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Network Interaction In the Thermoacidophile *Alicyclobacillus acidocaldarius*

In Response to Different Complex Carbon Sources

By

Brady D. Lee

A dissertation

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Committee Approval

To the Graduate Faculty:

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Dedication

I dedicate my dissertation work to my family who supported me through all of my course work and writing. A special feeling of gratitude to my wife, Hope, our children, Joey and Cassandra, who were all patient and supportive, and sacrificed a lot of time while I spent many hours performing lab work and writing. I would also like to thank my parents, Joe and Joan Lee for their constant support through every aspect of my life. I could not have completed "long road" without all of their support. All five of you were my cheering section.

I would also like to dedicate the dissertation to my committee members, Pete Sheridan, Linda DeVeaux, and Bill Apel for all of their guidance and hours of time spent reviewing the various chapters of my dissertation and the final document. Thank you for your valuable input and comments that helped me improve my writing skills as I worked through the discussion of my research.

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ABSTRACT

Lignocellulose is composed of a hexose-containing component, cellulose; a primarily pentose-containing component, hemicellulose; and an aromatic-containing component, lignin. Due to the massive amount of this lignocellulose in the environment, many microorganisms have developed the ability to hydrolyze the various components into oligomer and monomer subunits that can then be metabolized for cellular processes or fermented to produce alcohols and organic acids. *Alicyclobacillus acidocaldarius* is a Gram positive thermo- and acid- tolerant bacterium capable of growth on carbohydrates from lignocellulose ranging from simple sugars to complex polysaccharides such as xylan. Molecular analysis of *A. acidocaldarius* strain DSM 446 was performed using high density oligonucleotide microarrays. When tested with pentose and hexose sugars, regulation occurred primarily in three categories of genes: 1) genes encoding regulators, primarily activators; 2) genes encoding enzymes involved in cell synthesis; and 3) genes encoding sugar transporters. Catabolite repression did not appear to be active in *A. acidocaldarius* during these experiments. When DSM 446 was growing on wheat arabinoxylan, shifts in gene transcription were induced by the addition of either xylose or glucose. In general, genes related to carbohydrate metabolism and transport were down-regulated during the experiments. In contrast to gene regulation in other Gram-positive bacteria, xylose induced down-regulation of more genes than glucose, and typically the magnitude of regulation was also greater. *In silico* analyses of the regulated glycoside hydrolase enzymes, along with the results from the microarray analyses, yielded a hypothesized mechanism for arabinoxylan metabolism by *A. acidocaldarius* strain DSM 446. These analyses showed that glycoside hydrolase enzymes expressed by this strain

may have broad substrate specificity and that overall hydrolysis is catalyzed by an extracellular xylanase, while subsequent steps are likely performed inside the growing cell. Following depolymerization of wheat arabinoxylan outside the cell, transport of mono-, di-, oligo- and polysaccharides was accomplished, primarily by multi-facilitator superfamily type and ATP binding cassette type transporters. In addition, similar to other thermophiles, *A. acidocaldarius* appears to use a number of oligopeptide/dipeptide transporters for transport of products of wheat arabinoxylan hydrolysis. A model for wheat arabinoxylan hydrolysis and transport will be presented.

CHAPTER I: Literature Review

This literature review is broken into two primary sections: 1) an overview of *Alicyclobacillus acidocaldarius* with a description of the metabolic and physical features that make this bacterium important from an extremophilic and industrial perspective; and 2) a review of important phenotypic characteristics that allow for metabolism of complex carbohydrates, such as cellulose and hemicellulose, with a focus on thermophilic bacteria. Characteristics that will be considered are glycoside hydrolases, carbohydrate transport, central metabolism related to pentose and hexose sugars, and, finally, global regulation of carbohydrate metabolism.

Alicyclobacillus acidocaldarius: An Introduction

General Characteristics

A. acidocaldarius is a thermoacidophilic Gram-positive *Firmicute* that grows over a temperature range from 45 to 70 °C, with an optimum of 60°C, and within a pH range between 2 and 6, optimally between pH 3 and 4 (1). Due to these characteristics, *A. acidocaldarius* occupies diverse habitats including geothermal sites, submarine hot springs and also as a contaminant in heat processed foods (e.g., fruit juices) (2). *A. acidocaldarius* is a non-motile, endospore forming strict aerobe. Cells often occur in chains of 5-6, as rods measuring 2-3 µm long by 0.7 to 1.0 µm wide. Physiologically, *A. acidocaldarius* gains carbon and energy from a variety of 5- and 6-carbon sugars, including L-arabinose, ribose, D-xylose, D-galactose, D-fructose, D-mannose, rhamnose, mannitol, and tagatose; the disaccharides D-turanose, melibiose, cellobiose, lactose, maltose, sucrose, and trehalose; as well as the more complex

polysaccharides cellulose, hemicellulose (xylan), starch and glycogen (3). The thermoacidophilic nature of *A. acidocaldarius*, along with the broad substrate range, makes this bacterium important from an industrial perspective, both as a product contaminant and as a potential biocatalyst for industrial synthesis. As such, the remainder of this review will discuss isolation of *Alicyclobacillus* from the environment and as a contaminant in fruit juice, followed by a discussion of *A. acidocaldarius'* unique cellular structure and physiology related to carbohydrate metabolism.

At face value, *A. acidocaldarius* appears phenotypically and functionally similar to other Bacilli. However, adaptations that differentiate members of this species from other *Firmicutes* include tolerance to elevated temperature and acidic pH and a broad substrate range, including the ability to grow using carbon from plant wall poly- and oligosaccharides (1). Unique cell wall constituents, as well as the ability to produce a number of hydrolytic enzymes, are attributes associated with growth in extreme environments, as well as the ability to gain carbon from normally recalcitrant sources (4-6). This review of the literature will focus on these unique physiological and structural traits rather than an extended review of every aspect of morphology and physiology of this bacterium.

Sources and Initial Characterization

Alicyclobacillus species have been isolated from hot springs, geothermal soils and as spoilage agents in fruit juices. *A. acidocaldarius* was originally isolated from Nymph Creek, an acidic creek in the Norris Geyser Basin Area of Yellowstone

National Park (YNP) (7). Fourteen thermoacidophilic bacteria were isolated from a variety of acidic and thermal environments around this geyser basin. Morphological, physiological and molecular characteristics of these isolates indicated a homogeneous group of aerobic, spore-forming rods with an average % guanine plus cytosine ratio of 62%. Physiological and molecular properties differentiated these bacteria from other species in the genus *Bacillus*; therefore this new species was classified as *Bacillus acidocaldarius*. *B. acidocaldarius* species I04-IA was deposited as the type strain and currently exists as *A. acidocaldarius* ATCC 27009 in the American Type Culture Collection and *A. acidocaldarius* DSM 446 in the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

A. acidocaldarius strains have also been isolated from hot spring environments located around the world. Bacterial species isolated from mud and water samples from an acidic hot spring in Tengchong, China showed 99.5% similarity to *A. acidocaldarius* species in Genbank, based on 16S rRNA sequences (8, 9). An aerobic, spore-forming bacillus, tolerant to acidic pH and high temperatures was isolated from hot springs near Naples, Italy (10). Morphology and phenotypic characteristics indicated close resemblance to the *B. acidocaldarius* isolated by Darland and Brock (10, 11). Acidophilic, thermophilic spore-forming bacteria were also isolated from water taken from the Tamagawa Hot Spring in Japan (12). Growth characteristics, % G+C ratio, and DNA melting characteristics led to classification of these bacteria as *B. acidocaldarius*. A thermoacidophilic Gram-positive bacterium (Tc-4-1) that grows optimally at pH 3, at 70 °C was isolated from a hot spring in Tengchong, Yunnan,

China (13). Analysis of the 16S rRNA gene exhibited 99% similarity to *A. acidocaldarius* DSM 452. One final *Alicyclobacillus* species of note is species A4 isolated from the outflow of a hot spring in Baoshan City, Yunnan Province, China (14). This species was isolated in pH 2.0 growth medium at 60 °C and has been used for production of numerous glycoside hydrolase enzymes (15-18).

In addition to hot springs, Alicyclobacilli have been isolated from numerous geothermal soils. Three subspecies of *A. acidocaldarius* (MR1, MR2 and MR4) were isolated from unexplored heated soils near Mount Rittman in Antarctica (19). MR1 has been deposited as *A. acidocaldarius* subspecies *rittmannii*. *A. acidocaldarius* species were also isolated from hot soils (64 °C) on the slopes of Mount Erebus located on Ross Island in Antarctica (20, 21).

A number of Alicyclobacilli have also been attributed to cause off-flavor and scent in fruit juices through the production of aromatic compounds. Guaiacol and halophenols produced by Alicyclobacilli were identified as the causative agents related to smell and flavor. (22, 23). Alicyclobacilli have been isolated from fruit juices, fruit juice processing plants and orchard soils (22, 24-27). One additional species was isolated from wastewater from a rice production plant (28). The thermoacidophilic nature of Alicyclobacillus, as well as the ability to form spores, is thought to improve the probability of survival in these environments, where pasteurization is used to sterilize fruit juices prior to bottling.

Reclassification

In 1992, comparative sequence analysis of the 16S rRNA gene of *B. acidocaldarius, B. acidoterrestris* and *B. cycloheptanicus* led to the proposal of the new genus, *Alicyclobacillus* (1). The two thermoacidophilic species *B. acidocaldarius* and *B. acidoterrestris* showed 16S rRNA similarity of 98.8%, indicating they belong in the same genus. *B. cycloheptanicus* showed lower similarity (>92%) with the other two species. Percent similarity of the 16S rRNA gene for all three species to the 16S rRNA gene of *B. subtilis* was near 85%. Along with differences in 16S rRNA sequence, differences in secondary structure of the 16S rRNA were also noted. While there were several differences in helical regions compared to other species of the genus *Bacillus*, the primary difference was noted in helix 6 of the 16S rRNA. For Both *B. cycloheptanicus* and *B. acidoterrestris*, there was a 12-14 nucleotide deletion event leading to an abbreviated structure in this region.

As new species of *Alicyclobacillus* have been identified, additional evidence from the hypervariable region at the 5' end of the 16S rRNA has been shown as another site differentiating this group from other Bacilli (29). Using alignment of 16S rDNA sequences from the type strains of *A. acidiphilus, A. acidocaldarius, A. acidoterrestris, A. cycloheptanicus, A. herbarius, A. hesperidum* and *Alicyclobacillus* genomic species 1, differences in the HV region corresponding to nucleotide positions 70-344 (*B. subtilis* numbering system) differentiate these species from other species of *Bacillus*. In addition, variability in this region also enables distinction between the various species of *Alicyclobacillus*. Using this variability, species of *Alicyclobacillus*

were differentiated into five clusters: Clusters I and II associated with *A. acidocaldarius*; Cluster III associated with *A. acidoterrestris*; Cluster IV associated with *A. hesperidum*; and Cluster V associated with *A. cycloheptanicus*.

In addition to differences in the 16S rRNA sequence and secondary structure, re-designation of *B. acidocaldarius* to *A. acidocaldarius* was also supported by the presence of unique cyclic fatty acids within the cell lipids (30-33). The primary membrane fatty acids of *A. acidocaldarius* include ω-cyclohexylundecanoic and ω-cyclohexyltridecanoic acids. Approximately 90% of the membrane fatty acids (ω-cycloheptylundecanoic acid, ω-cycloheptyltridecanoic acid and ω-cycloheptyl-α-hydroxyundecanoic acid) of *A. cycloheptanicus* are comprised of a seven carbon ring, further differentiating them from other *Alicyclobacillus* species (5, 33).

Distinguishing Structural Characteristics

Cell Surface Structures

A. acidocaldarius shares a basic cell surface composition with other Gram-positive bacteria: a cell wall made up of peptidoglycan (murein), and a cytoplasmic membrane, made up of phospholipids and protein. No in-depth analysis of the peptidoglycan composition of *A. acidocaldarius*, or Alicyclobacilli in general, has been performed, but due to the sensitivity of *A. acidoterrestris* cell walls to lysozyme the peptidoglycan is thought to possess a standard backbone polymer of β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramate (34, 35). Additional evidence supporting this hypothesis is demonstrated by the use of lysozyme in parallel with proteinase K to extract DNA and RNA from *A. acidocaldarius* ATCC 27009 (See later research

chapters in this dissertation). Alicyclobacilli can be distinguished from other Bacilli by cytoplasmic membrane composition. In fact, the ability of *A. acidocaldarius* to survive in inhospitable environments, such as acid hot springs, comes from the makeup of the cytoplasmic membrane. As stated in the previous section, the cytoplasmic membrane of *A. acidocaldarius* is made up of ω-cyclohexyl acids (the predominant fatty acid), hopanoids and sulfonolipids (1). *A. acidocaldarius* does possess branched-chain fatty acids characteristic of other Bacilli, but lipid profiles are dominated by ω-cyclohexane fatty acids (65-70%) (11). In addition to the unusual lipid profile, complex lipids of *A. acidocaldarius*, specifically the glycolipids, are very exotic (36). The hopanoid compounds 1-(O-β-N-acyl-glucosaminyl)-2,3,4-tetrahydroxypentane-29-hopane and 1,2,3,4-tetrahydroxypentane-29-hopane are found in approximately 15% of the lipids. The unusual architecture of the cytoplasmic membrane likely enables *A. acidocaldarius* to grow in conditions of elevated temperature and low pH (37). Results from model membranes containing ω-cyclic fatty acids suggest that high acyl chain density at the free fatty acid end of the molecule likely stabilizes the membrane and influences permeability, characteristics deemed necessary for adaptation to life in hot, acidic conditions (38). This hypothesis is supported by the observation that changes in growth temperature and pH also lead to shifts in the fatty acid profile of lipid extracts from *A. acidocaldarius* (32). Likewise, the occurrence of hopanoids in bacterial membranes is thought to demonstrate a condensing effect in membranes, resulting in less surface area per molecule, giving *A. acidocaldarius* an advantage in acid hot springs (39). In a manner similar to fatty

acids, hopanoid content in the cell membrane increased with elevated temperature, but only moderately with decreasing pH (40).

Hopanoid and ω-cyclohexyl fatty acids likely contribute to the survival of *A. acidocaldarius* in acid hot springs from a structural perspective but also from a bioenergetics and solute uptake perspective (41, 42). The cytoplasmic membrane of extremophilic bacteria often determines the composition of the cytoplasm, especially related to maintenance of pH. *A. acidocaldarius* thrives in acidic environments, but maintains a cytoplasmic pH between 5.85 and 6.31 (43). This range of pH is also paralleled by optimal activity pH of enzymes isolated from the cytoplasm of *A. acidocaldarius*. β-galactosidase, glyceraldehyde-3-phosphate dehydrogenase and squalene-hopene cyclase activity were optimum at pH near 6 (12, 44, 45).

Not only are the cell wall constituents of *A. acidocaldarius* unique, but mechanisms for synthesis of these building blocks is also distinctive. Biosynthesis of the cyclohexanecarboxylic acid starter unit of the ω-cyclic fatty acids is basically formed by diversion of shikimic acid, the starting unit for aromatic amino acids and other compounds in the cell, to lipid synthesis (46). Likewise, production of hopanoid compounds uses a unique terpene biosynthetic pathway that includes a novel squalene-hopene cyclase that forms the triterpene hopane subunits found in the cytoplasmic membrane.

In bacteria and plants, shikimic acid is a central metabolite between metabolism of carbohydrates and biosynthesis of aromatic compounds (46). A seven step pathway converts phosphoenolpyruvate and erythrose-4-phosphate to chorismate,

which is a precursor to aromatic amino acids and many aromatic metabolites. Synthesis of the ω-cyclohexyl fatty acids found in the cytoplasmic membrane of *A. acidocaldarius* also originates from shikimic acid (47). Cyclohexanecarboxylic acid, which is the starter unit for the ω-cyclohexyl fatty acids, is synthesized from shikimic acid through a series of dehydrations and double bond reductions, which interestingly do not create an aromatic intermediate during synthesis. Shikimic acid, acetic acid and phenylalanine feeding experiments performed in 1972 indicated that ^{14}C from shikimate and acetate accounted for most of the labeled carbon in methyl esters from the cells' lipids, while only a small proportion came from phenylalanine (30). Moore *et al*. (48) confirmed these results using ^{13}C and ^{2}H-labeled shikimic acid and possible intermediates in wild-type *A. acidocaldarius* and two blocked mutants. A more detailed analysis of ω-cyclic fatty acid biosynthesis in *A. cycloheptanicus* may provide additional insight into ω-cyclohexyl fatty acid formation in *A. acidocaldarius* (5). Feeding with ^{13}C and deuterium labeled metabolites indicated that the cyclohexyl ring may come from shikimic acid, while phenylacetic acid and acetate are used for chain elongation from a carboxylated cylohexane. While similar detailed experiments have not been performed using *A. acidocaldarius*, the phenylacyl ω-cylohexylundecanoate isolated from *A. acidocaldarius* was identical to the ω-cyclic fatty acid formed by *A. cycloheptanicus* (5). While experiments were not performed with labeled xylose, experiments by Moore *et al*. (1997), with uniformly labeled ^{13}C-glucose, showed that all of the carbon in the ω-cyclohexyl fatty acid came from glucose.

Hopanoids represent another interesting constituent of the *A. acidocaldarius* cytoplasmic membrane. Hopanoids are pentacyclic triterpenoid lipids produced by many prokaryotes as cell membrane components (49, 50). Up to 15% of *A. acidocaldarius* lipids consist of these hopanoid pentacyclic triterpine glycolipids with ω-cyclohexylundecanoate and ω-cyclohexyltridecanoate (37, 51, 52). Hopanoid biosynthesis in *A. acidocaldarius* is catalyzed by prenyltransferases that catalyze stepwise, head-to-tail additions of isopentenyl diphosphates to dimethylallyl diphosphate, resulting in geranyl diphosphate (C10), farnesyl diphosphate (C15) and geranylgeranyl diphosphate (C20). Two farnesyl diphosphate molecules can be condensed to squalene which is then cyclized to form hopene (45). Gene annotation from *A. acidocaldarius* indicates that the isopentyl diphosphate used during biosynthesis is produced through the mevalonate-independent pathway or the 5-methyl erythritol phosphate (MEP) pathway (53, 54). Squalene-hopene cyclase is the enzyme responsible for this complex, one-step enzymatic reaction resulting in formation of the five ring structures, 13 covalent bonds, and nine stereocenters of the pentacylic triterpene. The squalene-hopene cyclase from *A. acidocaldarius* was purified first in 1986, and since that time has served as the model triterpene cyclase for mechanistic studies (55-58).

Lignocellulose Metabolism as It Relates to *Alicyclobacillus acidocaldarius*

Lignocellulose is primarily made up of hemicellulose, cellulose and lignin (59). Bacterial growth and metabolism using hemicellulose and cellulose fractions of lignocellulose as a carbon source requires a complex physiological response that

includes: 1) production of glycoside hydrolase enzymes for depolymerization of the poly- and oligosaccharides; 2) a variety of carbohydrate/sugar transporters that facilitate transport of small oligosaccharides and sugar monomers into the cell; 3) a central metabolism that allows for metabolism of a range of hexose and pentose sugars; and 4) a set of global transcriptional regulators that prioritize expression of genes related glycoside hydrolase production, solute transport and central metabolism (60). In *A. acidocaldarius*, this process is further complicated because enzymes associated with all of these processes must be thermostable, and in some cases both thermo- and acid-stable, depending on cellular location. This portion of the literature review will briefly describe each of these four physiological attributes, as they pertain to *A. acidocaldarius*, so the focus will be on these systems in thermoacidophilic, Gram-positive bacteria.

Glycoside Hydrolase Enzymes

Lignocellulosic biomass is currently being studied as a feedstock for the production of fuels and other chemicals. Chemically, lignocellulose is a heterogeneous three dimensional matrix made of a carbohydrate component, consisting primarily of hemicellulose (20-30%) and cellulose (40-50%), and a lignin component. These three compounds are intertwined, providing a structure that is highly resistant to microbial degradation (60, 61). In fact, complete degradation of lignocellulose into monosaccharides requires the synergistic activity of a variety of glycoside hydrolase enzymes, a process most thoroughly studied in mesophilic microorganisms such as fungi and *Actinomycetes* (62). Likewise, numerous other

bacteria have also demonstrated the ability to produce glycoside hydrolase enzymes under aerobic and anaerobic conditions (63). Thermostable (stability at temperatures ≥ 50 °C) enzymes, which typically demonstrate higher rates of hydrolysis and more complete depolymerization, represent the next logical step in development of enzymes for lignocellulose breakdown (64).

The complete depolymerization of cell wall polysaccharides to sugar monomers requires substantially more enzymatic activities than simply endoglucanases and endoxylanases. Depolymerization of cellulose to glucose requires the synergistic action of three glycosyl hydrolase activities in three classes (65, 66). The complete depolymerization of hemicellulose to xylose, mannose, galactose, glucose, arabinose, and various uronic acids requires the synergistic action of at least 13 different glycosyl hydrolases (67). A list of cellulases and hemicellulases are summarized in Table 1.

Table 1: Glycosyl hydrolase activities required for complete depolymerization of cellulose and hemicellulose. The glycosyl hydrolase activities required to depolymerize the hemicellulose in a particular lignocellulosic substrate will vary based on the monomeric hemicellulose composition, which can vary widely.

Glycosyl Hydrolase Activities Required For Lignocellulose Polysaccharide Depolymerization

Cellulose (Lynd *et al* 2002)	**Hemicellulose (Shallom and Shoham 2003)**
endo-β-1,4-glucanase (EC 3.2.1.4)	endo-β-1,4-xylanase (EC 3.2.1.8)

1,4-β-glucan cellobiohydrolase (EC 3.2.1.91) β-glucosidase (EC 3.2.1.21)	exo-β-1,4-xylosidase or β-xylosidase (EC 3.2.1.37) α-L-arabinofuranosidase (EC 3.2.1.55) endo-α-1,5-arabinanase (EC 3.2.1.99) α-glucuronidase (EC 3.2.1.139) endo-β-1,4-mannanase (EC 3.2.1.78) exo-β-1,4-mannosidase (EC 3.2.1.25) α-galactosidase (EC 3.2.1.22) β-glucosidase (EC 3.2.1.21) endo-β-1,4-galactanase (EC 3.2.1.89) acetylxylan esterase (EC 3.1.1.72) acetylmannan esterase (EC 3.1.1.6) ferulic and p-cumaric acid esterases (EC 3.1.1.73)

A general schematic of cellulose showing the linear β-1,4-linked D-glucopyranose structure and enzymes involved in cellulose degradation are shown in Figure 1. Endoglucanase randomly hydrolyzes cellulose producing glucose, cellobiose and oligosaccharides. Exoglucanase or 1,4-β-glucan cellobiohydrolase produces cellobiose by acting on reducing and non-reducing ends. Finally, β-glucosidase hydrolyzes cellobiose to glucose. Bhalla *et al*. reviewed thermophilic and hyperthermophilic glycoside hydrolases in 2013 (64). Since that time, a number of other thermostable cellulose degrading enzymes have been

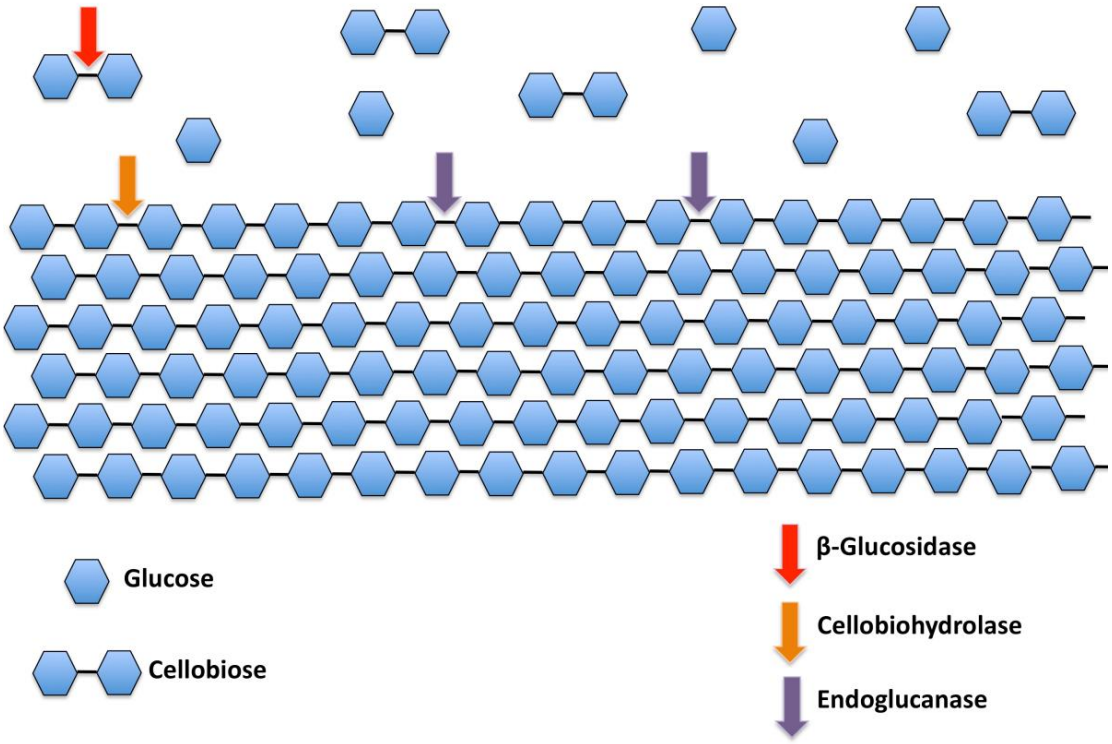

Figure 1. Schematic showing structure of cellulose and glycoside hydrolase enzymes associated with depolymerization. From: (68).

discovered and tested. Numerous (hyper)thermophilic bacteria produce thermostable, and in some instances thermo- and acid-stable, cellulose depolymerizing enzymes. Bacteria from the genera *Bacillus*, *Geobacillus*, *Caldibacillus*, *Acidothermus*, *Caldocallum*, *Clostridium*, *Alicyclobacillus*, *Thermotoga*, *Anaerocellum*, *Rhodothermus*, *Thermoanaerobacter*, and *Caldocellulosiruptor* have produced endoglucanase, exoglucanase and β-glucosidase enzymes that have demonstrated optimum activity over a pH range from 2.6 – 8.0 and temperatures from 55 – 106 °C. In addition, a number of Archaea have recently been shown to produce thermostable

enzymes for cellulose deconstruction. Included are species of *Pyrococcus* (69), *Sulfolobus* (70), and *Thermofilum* (71).

Hemicellulose has a complex chemical structure composed of different residues branched in three backbone structures (xylan, xyloglucan and mannan) that requires the synergistic action of 13 hydrolytic enzymes for complete depolymerization (See Table 1) (67, 72). In addition to endo- and exo-acting enzymes, complete hemicellulose depolymerization requires a number of accessory enzymes that cleave side chains of the xylan backbone (68). For xylan, specifically arabinoxylan, the main structure of the polysaccharide is β-1,4-linked D-xylose units, with likely α-linked L-arabinose, D-galactose, D-glucuronic acid, acetyl groups, ρ-coumaric and ferulic acids (73, 74). The xylan backbone is cleaved into smaller oligosaccharides by β-1,4- endoxylanase. Xylose monomers are then produced from these oligosaccharide by the catalytic activity of β-1,4-xylosidase. Release of arabinose from the polymer is accomplished by the activity of α-arabinofuranosidase. D-glucuronic acid is liberated from arabinoxylan through the activity of α-glucuronidase enzymes, while α-galactosidase releases D-galactose from the side chains. Finally the activity of the accessory enzymes, acetylxylan esterase, ρ-coumaryl esterase and ferulic acid esterase, catalyze the release of acetyl groups, ρ-coumaric acid and ferulic acid from arabinoxylan. Figure 2 shows the structure of arabinoxylan and associated bonds cleaved by the various glycoside hydrolases and accessory enzymes (68).

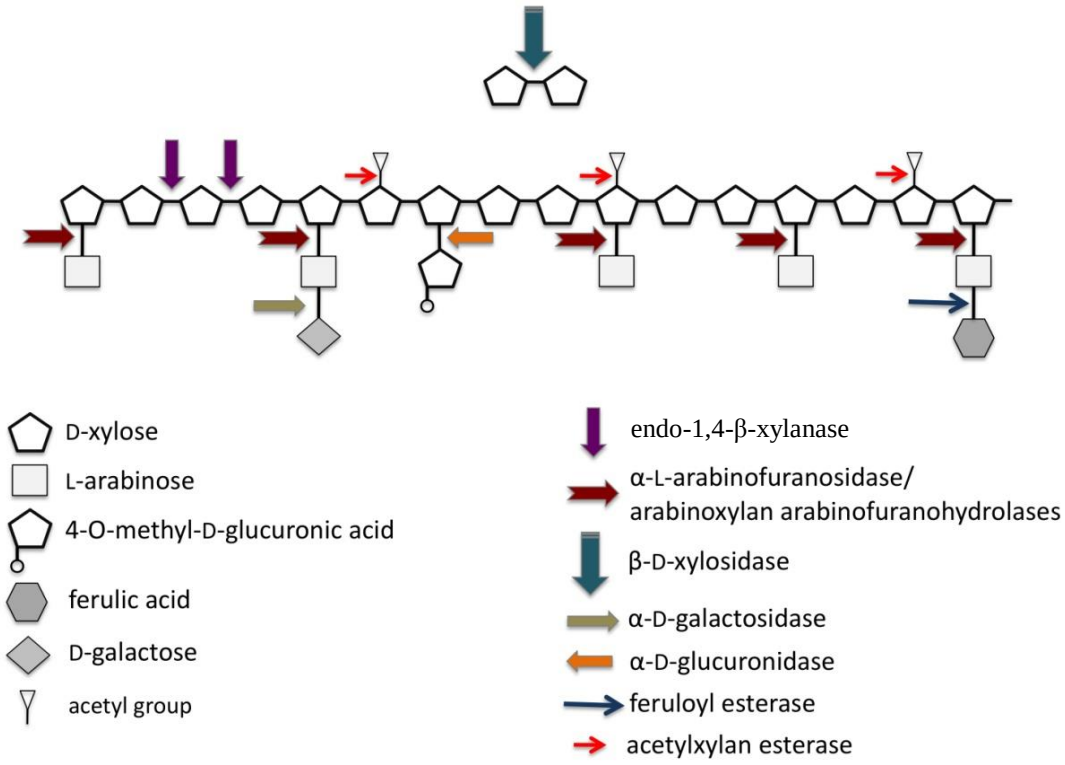

Figure 2. Schematic of arabinoxylan structure and glycoside hydrolase enzymes associated with depolymerization. Modified from: (68)

As with the suite of cellulase enzymes, thermo- and thermoacid-stable xylanolytic glycoside hydrolase enzymes are common in many (hyper)thermophilic bacteria. Enzymes involved in xylan degradation have demonstrated activity over a temperature range from 55 to 100 °C and over a pH range from 5.5 to 10.5 (64). As would be expected from the diverse group of bacteria expressing thermostable cellulase enzymes, similar species produce thermostable hemicellulose enzymes. Hemicellulases from bacteria in the genera *Bacillus*, *Geobacillus*, *Thermotoga*, *Acidothermus*, *Cellulomonas*, *Paenibacillus*, *Thermoanaerobacterium*, *Actinomadura*,

Alicyclobacillus, Anoxybacillus, Nesterenkonia, Caldicellulosiruptor, and *Enterobacter* have been studied. Two species of *Sulfolobus solfataricus* and one *Thermococcus* appear to be the only Archaea producing a thermostable xylan hydrolyzing enzyme (75-77).

The ability of *A. acidocaldarius* to grow on a variety of lignocellulosic biomass sources (e.g., wheat arabinoxylan, oat spelt xylan, birchwood xylan) indicates that this bacterium expresses and secretes many of the glycoside hydrolase enzymes required to release the sugar monomers present in cellulose and hemicellulose biomass by breaking the various bonds connecting them in the lignocellulosic matrix. As such, *A. acidocaldarius* represents a source of thermostable glycoside hydrolase enzymes for application as catalysts for the hydrolysis of the cellulose and hemicellulose components of lignocellulose. The potential for utilization of plant derived oligo- and polysaccharides by *A. acidocaldarius* is further demonstrated by the numerous glycoside hydrolase enzymes that have been found in the genome sequence or those that have been characterized. To date, 19 glycoside hydrolase genes have been found encoded in the *A. acidocaldarius* genome (78), and β-galactosidase, α-amylase, cellulase, neopullulanase, exo-pectinase, mannanase, β-glycosidase, and endoglucanase enzymes have been expressed and characterized (3, 9, 28, 79-93). An *A. acidocaldarius* species isolated from a hot spring in Tengchong, Yunnan, China was recently sequenced, and the genome was found to contain genes encoding xylanase, mannanase, β-glucosidase, β-galactosidase, and other glycoside hydrolase enzymes (13). Numerous glycoside hydrolase enzymes have been isolated and expressed from

Alicyclobacillus sp. A4, a bacterium enriched from the outflow of a hot spring in Baoshan City, Yunnan Province, China (14, 15, 17, 18, 94). Glycoside hydrolase enzymes include a novel xylanase, an α-amylase, a β-1,3 (4)-glucanase and an extremely acid tolerant β-1,4-glucanase. All of the enzymes listed demonstrate optimum activity at temperatures above 60 °C. Some of the glycoside hydrolases listed, presumably those secreted extracellularly, also demonstrate stability and activity at or below pH 2.

Genome information from *A. acidocaldarius* shows genes for 9, but not all 16, of the glycoside hydrolase enzymes required for complete depolymerization of cellulose and hemicellulose. In addition, there are 11 other glycoside hydrolase genes encoded in the *A. acidocaldarius* strain DSM 446 genome available through the Joint Genome Institutes Integrated Microbial Genome (JGI-IMG) webpage. Enzymes encoded by these genes are likely active toward other polysaccharides such as starch or polygalactose. The presence of 20 total enzymes related to polysaccharide depolymerization indicates that *A. acidocaldarius* has evolved to survive in environments where adapting to available nutrients is necessary (78). In hot springs or heated soils, lignocellulose-containing biomass or other polysaccharides are deposited in the growth environment and the availability of an assortment of glycoside hydrolase enzymes give *A. acidocaldarius* the ability to use carbohydrates present in these complex carbon sources.

Carbohydrate Transport

Activity of some of these acid- and thermostable hydrolases produced by *A. acidocaldarius* catalyze degradation of cellulose and hemicellulose, providing soluble oligomers and monomers that are small enough to be transported into the cell as sources of carbon and energy. Transport of carbohydrates, including mono-, di-, oligo-, and polysaccharides, into a bacterial cell is typically accomplished using three general families of transporters (Figure 3) (95). Specifically, these families are: primary transporters, such as ATP-binding cassette (ABC) type transporter

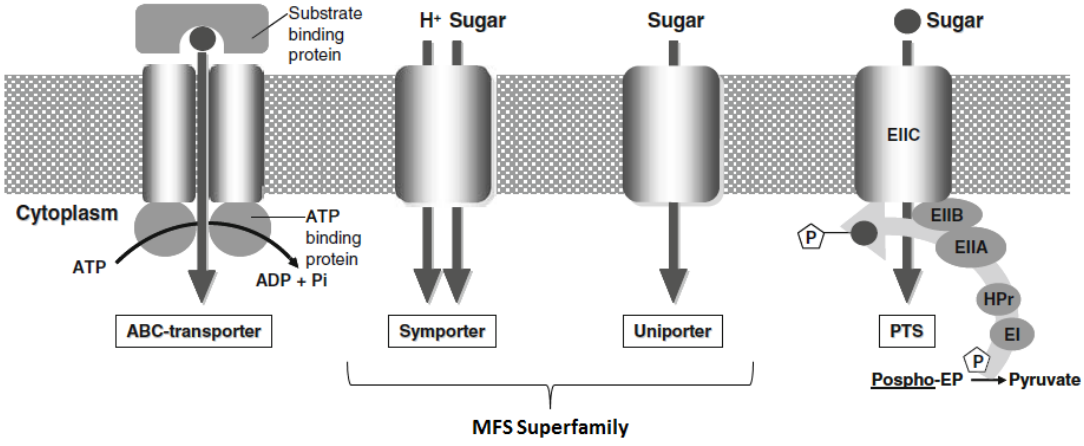

Figure 3. Three major families of carbohydrate transporters found in bacteria. Examples of primary transporters (ABC-transporter), secondary transporters (MFS Superfamily), and bacteria specific transporters (PTS).

superfamily transporters, secondary transporters consisting primarily of major facilitator superfamily (MFS) transporters, and finally, phosphoenolpyruvate (PEP):carbohydrate phosphotransferase systems (PTS). Primary transporters, such as ABC-transporters, are active transporters that transport solutes across the cell

membrane at the expense of a molecule of ATP (96). Relative to carbohydrate transport, proton gradients generated in the extracellular medium cause the flow of protons down a chemical and charge concentration gradient, facilitating uptake of sugars or other carbohydrates, which are examples of secondary transport. These types of transport systems are important because the bacterial cell wall represents a barrier between the cytoplasm and the external environment; therefore, the cytoplasmic membrane represents the primary permeability barrier for the passage of most, if not all, nutrients, including carbohydrates, into the cell.

ATP-Binding Cassette (ABC) Transporters

ABC-type transporters, are considered primary transport systems that require chemical energy to function (97). Carbohydrate transport using ABC-type transporters is catalyzed by the hydrolysis of ATP to drive the process against concentration gradients (98-100). Structurally, ABC-type transporters involved in carbohydrate transport, as well as other solutes, are composed of two transmembrane domains (TMD) providing a transport pathway and two cytoplasmic nucleotide binding domains (NBDs) where ATP hydrolysis occurs to drive the transport process (101). ABC transporters used for solute import require substrate binding proteins (SBPs) on the surface of the cytoplasmic membrane that interact with the external medium. SBPs are typically transcribed with the TMD and NBD domains, and become anchored to the membrane in close proximity to the transporter, allowing interaction with the translocation pore within the TMD (102). More recently, it has been discovered that some ABC-transporters have two or four SBPs, increasing affinity and uptake of

solutes (103). Multiple SBPs in association with the uptake pore may also broaden substrate range for a transporter. The high affinity and specificity of SBPs allow bacteria to scavenge low concentrations of nutrients efficiently (104). NBDs contain highly conserved domains, but TMDs can vary widely depending on the nature of the substrate transported (105, 106). The NBD domains harbor the ABC motifs where ATP hydrolysis takes place (107).

Depending on the type of carbohydrate transported, ABC transporters can be further subdivided into carbohydrate uptake transporters 1 and 2 (CUT1, CUT2) (108). Functionally CUT1 and CUT2 transporters differ by substrate; a variety of di- and oligosaccharides are transported by the CUT1 family, while transporters in the CUT2 family transport only monosaccharides (109, 110). Functional differences related to saccharide specificity is controlled by the SBP, but there are structural differences that exist between the NBDs and TMDs in CUT1 and CUT2 family transporters (111-113). Four gene products self-assemble to form CUT1 family transporters: one for the SBP, two integral membrane proteins (TMDs) which typically span the membrane six times, and the ATPase subunit (NBD). Two ATPase subunits assemble into a functional enzyme complex with one copy of each TMD. In contrast, only three gene products are assembled in the formation of CUT2 family transporters. While substrate specificity will require different SBP for CUT2 transporters, the ABC domain (NBD) is encoded by one gene that is twice the length of average NBD.

Mechanisms of ABC-type transporters have been studied in Gram-negative and Gram-positive bacteria as well as Archaea (97, 101, 107, 108, 114-120). Probably the

best characterized transporter from all three groups is the transporter for the import of maltose (121-126). The generalized mechanism can be seen in Figure 4. Prior to substrate binding by the SBP, the TMD and NBD are situated in an inward-facing

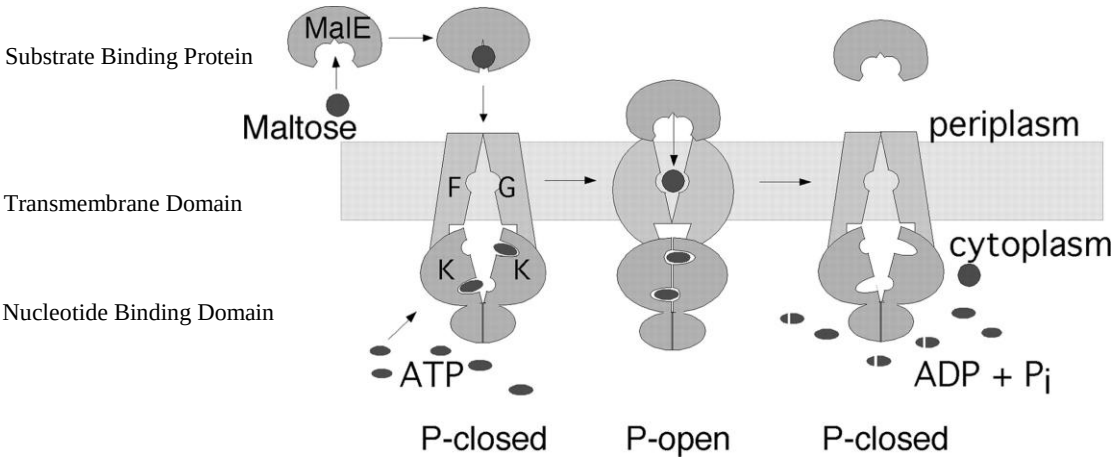

Figure 4. Example of *E. coli* ABC-type transporter for maltose. Schematic of ABC-type transporter, showing various conformations during substrate binding, ATP loading, ATP hydrolysis and transport of substrate from periplasm to cytoplasm side of membrane. From: (116, 127)

conformation, so that the translocation pore is open to the cytoplasm and closed to the external environment. Upon binding of substrate to the SBP, the SBP interacts with the external surface of the TMD, forming a pre-translocation conformation where the NBDs are brought closer together, allowing binding of ATP and concerted closure of the NBD, reorientation of the TMD and opening of the SBP. Substrate is then transferred to the binding site of the TMD during formation of the outward facing conformation. In the outward-facing conformation, the bound ATP is positioned at the catalytic site for hydrolysis. Upon ATP hydrolysis, a high-energy form (ADP bound)

of the inward-facing conformation is established releasing the substrate to the cytoplasm. At this stage in the process, the transporter is reset through release of ADP and the SBP.

Major Facilitator Superfamily Transporters

Major facilitator superfamily (MFS) transporters are the largest know superfamily of secondary transporters found across all domains of life (128). Initially broken into five clusters, MFS transporters were first categorized in 1993 to include: 1) drug resistance proteins; 2) sugar facilitators; 3) facilitators of metabolic pathway intermediates, 4) phosphate ester-phosphate antiporters: and 5) groups of oligosaccharide proton symporters (129). There are three primary types of MFS type transporters, each distinguished by the potential driving force of the transport reaction (130). Transporters driven by substrate gradients are considered uniporters and transport a solute from high concentration to low concentration. Symporters, on the other hand, transport two or more solutes in the same direction using the electrochemical gradient of one of the solutes as the driving force for transport. The third kind is antiporters, in which solutes are transported in opposite directions across the cell membrane presumably using an electrochemical gradient. Further division of MFS transporters into 17 families based on sequence similarity was done in 1998 (131), with further expansion to 28 families recognized in 2000 (98). As of 2012, this superfamily consists of 74 individual families, each associated with transport of a different solute (128). The Transporter Classification Database currently contains 76 individual families of MFS transporters (132). According to the literature, while

uniporters predominate for sugar transport in eukaryotes, symporters seem to be the most prevalent MFS transporter for carbohydrate uptake in bacteria (95).

Structurally, the majority of MFS transporters have 12 transmembrane helices, but some may contain more (133, 134). These helices are known as the MFS fold, which are further divided into N- and C-domains, organized into two 3 by 3 inverted repeats of these transmembrane helices. The N- and C-domains provide a transport path for the substrate which is facilitated by a substrate binding site located halfway through the membrane.

During the transport process, MFS transporters alternate through a cycle of conformational changes that allow binding of a substrate on the external surface of the membrane and subsequent release of the substrate on the cytoplasmic side of the membrane (135). The alternating access model hypothesis has been used to describe the series of conformational changes required to describe substrate transport by MFS transporters (136). Along with substrate binding and transport, binding and release of the co-transported cation (typically H^+, Na^+ or K^+) must be considered. Application of biophysical techniques, such as X-ray and EM crystallography, resonance energy transfer and double-electron spin resonance have allowed for generation of 2D and 3D structures (137, 138). Unfortunately, only a few conformational structures have been generated for each type of transporter, so all structures throughout the entire cycle must be inferred using existing data from other transporters. For this reason molecular dynamic simulations have also been employed to elucidate transport mechanisms (139). Since MFS transporters are so diverse, information available for the bacterial

sugar transporters *LacY*, *XylE*, *GlcP* and *FucP* will be discussed (137, 139-149). These transporters, which belong to the oligosaccharide/H^+ and sugar/H^+ symporter subfamilies of the MFS superfamily, use protons as the co-transport ion for lactose, xylose, glucose and fucose, respectively.

While there are likely subtle differences between each of these transporters for different sugars and the associated ion co-transported, comparing conformations identified, a general cycle is proposed. A generalized schematic depicting the alternating access model is shown in Figure 5. The process is initiated by an outward open (toward periplasm or external growth environment) transporter conformation, where a co-transported ion and the sugar substrate are bound to their associated

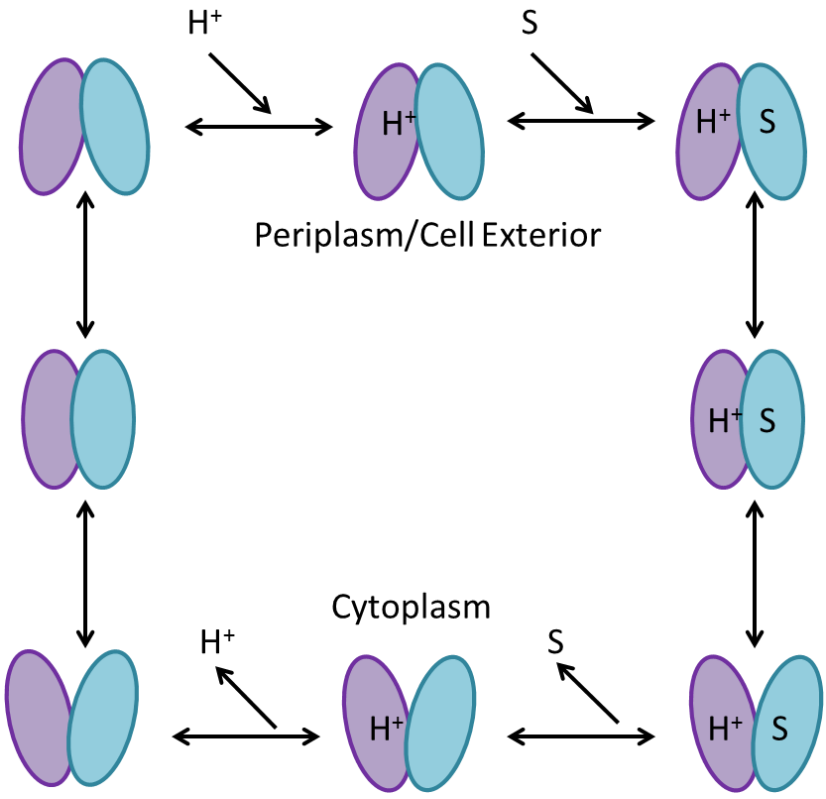

Figure 5. Schematic showing steps of proton binding, substrate binding and shifts in transporter conformation for a H^+/solute (S) transporter.

binding sites, leading to an occluded conformation of the transporter. This occluded conformation appears to be the transition from outward open to inward open (toward the cytoplasm) conformation, where the substrate and co-transported ion can be released into the cytoplasm. Once the substrate and co-transported ion are released, the transporter returns to the outward open conformation, completing the cycle. While the alternating access model has received a lot of support, two examples of these protein conformations exist. The first is called the rocker-switch, in which the N- and C-domains open in the center in an action similar to a clothes pin (138). The second is an airlock model, in which sequential gates open first to the external environment,

similar to "outward open", and then a fully occluded (i.e., both gates closed), followed by opening of cytoplasmic gates, to release the solute to the cytoplasm (137).

Another type of secondary transporters are those within the sodium solute symporter (SSS) family. Like previously described transporters, SSS transporters are also found in prokaryotic and eukaryotic cells (150). A structure having 13 transmembrane domains appears to be the most common structural fold, but others with 12, 14 and 15 exist (151). While these transporters use a similar transport mechanism to MFS transporters, no sequence or motif similarity have been detected between the two families (98). Unlike MFS transporters, SSS family proteins are only used for uptake, using a sodium symport mechanism. In a manner similar to proton coupled sugar transport, a Na^+-electrochemical gradient provides energy for transport to occur (152). Spectroscopic methods indicate that solute/Na^+ binding leads to conformational changes that facilitate transport. Using proteoliposome supported MelB from *E. coli*, solid supported membrane electrophysiology was used to monitor conformational changes during melibiose transport across the membrane (153). These studies led to a 6-state kinetic model exhibiting conformations similar to those outlined for the MFS transporters described above. MelB can facilitate import and export of melibiose, and SSM-based electrophysiology showed Na^+ binding to the cytoplasm-opened conformation, followed by melibiose binding. Transition through an occluded conformation to a periplasm-opened conformation where melibiose was release followed quickly by Na^+ release and generation of the empty transporter to start the cycle again was seen. Structurally induced conformational changes in MelB catalyzed

by Na^+ binding analyzed using substrate-induced FTIR difference spectroscopy also indicated binding was required for efficient coupling to substrate binding (154). Similar results were found for a sodium/ galactose transporter from *Vibrio parahaemolyticus* (141). Crystal structures from the wild-type and mutant of binding site residues were analyzed and results refined with *in silico* modeling to generate the alternating access model for Na^+ and galactose binding and release on alternating sides of the membrane. Along with Na^+ ions, SSS transporters can also couple solute co-transport with H^+ and Li^+ ions (155).

Phosphoenolpyruvate (PEP): Carbohydrate Phosphotransferase Systems (PTS)

The final type of transport, or permease, system that will be discussed, the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), is involved in transfer and phosphorylation of the sugar as it is transferred through the membrane (156). Unlike MFS and ABC-type transporters which are found in eukaryotes and prokaryotes, PTS transporters are only found in bacteria (95). While many bacteria have complete PTS transport systems, many do not, including a number of *Mycobacterium* species, cyanobacteria, methylotrophs and thermophiles such as *Thermotoga maritima* and *Thermus thermophilus* (157). In Archaea, PTS homologs have only been detected in the haloarchaea, with the only complete PTS system characterized in *Haloferax volcanii* (158, 159). The PTS is active in Gram-positive and Gram-negative bacteria, and consists of membrane and cytoplasmic components (Figure 5). In addition to carbohydrate transport, the PTS system is part of a global

transcriptional regulatory system (160). This regulatory system, called carbon catabolite repression (CCR), will be discussed in detail later in this review.

PTS transporters are subdivided into four superfamilies, which can be further distributed into seven permease families, depending on the sugar substrate transported (157, 161). Superfamilies include the Glc-Fru-Lac superfamily, the Asc-Gat superfamily, the Man superfamily and the Dha superfamily. Differentiation of the four superfamilies is based on phylogeny of the Enzyme II (EII) components of the PTS system (160). Likewise, the carbohydrate specificity of each PTS system is differentiated by the EIIs, specifically the EIIA and EIIB domains (162). EIIA domains of the four superfamilies have no sequence similarity, while EIIB domains vary in sequence with the exception of the active site loop. EIIC and EIID represent the transmembrane part of the transporter (157). EIIC is the permease and sugar specific receptor (163). EIID appears to be specifically involved in mannose transport. An example of a PTS transporter for glucose can be seen in Figure 6.

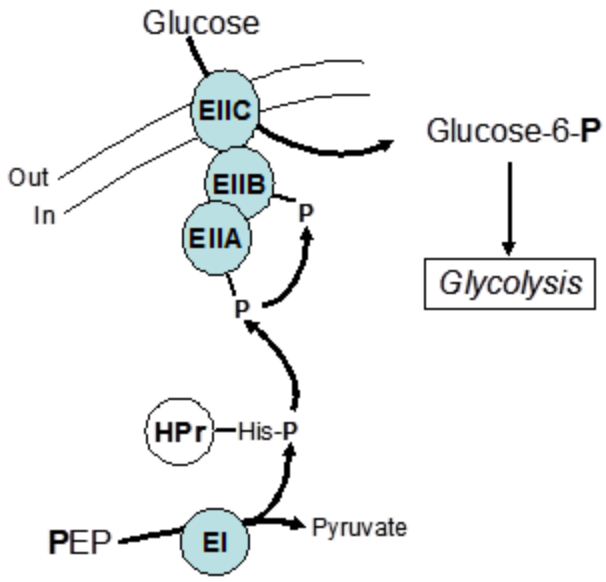

Figure 6. Schematic of cytoplasmic and cell wall components of a PTS transporter for glucose. Modified from (164)

Carbohydrate transport using the PTS is initiated by two cytoplasmic components that are common to all sugars: Enzyme I (EI) and the histidine phosphocarrier protein (HPr) (162). These two components are considered the energy coupling component of the PTS system (165). Carbohydrate transport is initiated when two molecules of the high energy metabolite PEP are produced via glycolysis; one is used to drive the initial transport and phosphorylation of carbohydrates assimilated by the cell via the PTS system. HPr is phosphorylated by the activity of EI, which is autophosphorylated using a phosphoryl group from PEP. The phosphoryl group from HPr is then transferred to the EII complexes. Specifically, in the presence of Mg^{2+}, EI is autophosphorylated on a conserved histidine (His-189 in *E. coli*) in the N-terminus of the enzyme, while PEP binding occurs in the C-terminus (160). HPr is

also phosphorylated on a histidine residue (His-15), located in the N-terminus of the protein. EIIA then accepts the phosphoryl group from HPr, and subsequently donates it to EIIB. The transport process is completed when the phosphoryl group is transferred from EIIB to the transmembrane EIIC which catalyzes the coupled translocation and phosphorylation of the sugar (163, 166).

Central Metabolism

Lignocellulosic biomass is currently being studied as a feedstock for the production of fuels and chemicals. The carbohydrate component of lignocellulose, hemicellulose and cellulose, is comprised of chains of pentose and hexose monosaccharides that when hydrolyzed provide sugar monomers that are then fermented to fuels such as ethanol. In order to increase product yield from pretreated lignocellulose, microorganisms must be able to use both the pentose (e.g., xylose and arabinose) and the hexose (glucose) sugars. Many fermentative microorganisms cannot use xylose and must be genetically modified for xylose utilization to increase yield (167, 168). Likewise, carbon catabolite repression (CCR) is active in many bacteria that can metabolize both pentose and hexose monosaccharides, causing preferential use of the hexoses, primarily glucose, which leads to sequential rather than simultaneous sugar utilization (169). While the general mechanism of CCR is different when comparing Gram-positive and Gram-negative bacteria, in each case, gene regulation occurs so that a preferred carbon source such as glucose is utilized compared to secondary carbon sources such as xylose or arabinose (160, 170-172). In many microorganisms, CCR is associated with the

phosphoenolpyruvate:phosphotransferase system (PTS), where the incoming sugar is phosphorylated during transport through the outer membrane using a phosphoryl group from phosphoenolpyruvate, which can lead to catabolite repression or inducer exclusion (173). Genes and operons encoding glycoside hydrolase enzymes for breakdown of hemicellulose and cellulose, as well as transporters of oligosaccharides are also regulated by CCR (174-185). When a preferred carbon source, such as glucose, is present, expression of these enzymes is typically down-regulated in an effort to conserve cellular resources. CCR will be described in more detail in the next section.

While carbon metabolism in many microorganisms appears to be regulated by CCR, some Bacteria, Archaea, and yeast have been shown to simultaneously use mixtures of pentose and hexose monosaccharides (169). While CCR has been demonstrated to be active in many lactic acid bacteria, there are species that have been shown to possess the ability to simultaneously use pentose and hexose monosaccharides. *Lactobacillus buchneri* strain NRRL B-30929 simultaneously fermented xylose and glucose into lactate, acetate (169, 186, 187) and ethanol (186). This bacterium demonstrated the ability to ferment various pentose and hexose monosaccharides, as well as pentose- and hexose- containing disaccharides and oligosaccharides. In addition, *L. buchneri* NRRL B-30929 used the mixed sugars in corn stover and wheat straw hydrolysates for the production of ethanol (188). *L. brevis* is another bacterium which appears to have relaxed control of monosaccharide use and can therefore use pentose and hexose monosaccharides simultaneously (187).

Like *L. buchneri*, *L. brevis* and *L. plantrum* have been shown to simultaneously utilize sugars in hydrolysates from sour cabbage, corn cob and rice straw (189, 190). Simultaneous utilization of pentose and hexose monosaccharides in *L. brevis* has been attributed to use of non-PTS sugar transporters, such as H^+ symporters and sugar uptake by facilitated diffusion (169).

Ruminal strains of *Butyrivibrio fibrosolvens* were also able to grow via the concurrent use of xylose and glucose. Interestingly, not all strains of *B. fibrosolvens* were able to co-utilize the pentose and hexose monosaccharides (191). CCR was also absent in *Clostridium thermohydrosulfuricum* strain Rt8.B1, allowing for simultaneous utilization of glucose and xylose (192). Research results indicated biphasic utilization of glucose implicating high- and low-affinity transport, while xylose uptake appeared to occur through a facilitated diffusion mechanism. Likewise, the solventogenic bacterium *C. beijerinckii* SA-1 demonstrated the ability to co-ferment glucose and xylose when grown in single- and two-stage chemostats (193). The carbohydrate transport mechanism used during this process was not determined. *C. beijerinckii* P260 was able to co-utilize glucose, xylose, arabinose, galactose, and mannose present in wheat straw hydrolysate for production of acetone, butanol and ethanol (194-196). Co-utilization of pentose and hexose monosaccharides from barley straw, corn stover and switchgrass hydrolysate was also demonstrated by *C. beijerinckii* P260 (197, 198). Simultaneous utilization was demonstrated when pentose and hexose monosaccharides were present in equimolar amounts, as well as at concentrations differing by almost an order of magnitude.

Simultaneous utilization of pentose and hexose has also been demonstrated in a number of thermophilic non-cellulolytic and cellulolytic anaerobes. *Thermoanaerobacter* X514, a non-cellulolytic anaerobe, was able to simultaneously ferment xylose and glucose to ethanol (199-201). Carbon utilization network analysis indicated that glucose, xylose, fructose and cellobiose catabolism are all distinct networks in X514. In this network, fructose, glucose and cellobiose transport is facilitated by PTS transporters, while xylose is transported via an ATP-binding cassette (ABC) transporter. While pentose metabolism is regulated in other Gram-positive anaerobes, such as *Clostridia*, similar regulation was not noted in X514. Georgieva *et al*. (202, 203) observed simultaneous xylose and glucose utilization during growth and ethanol production by *Thermoanaerobacter* BG1L1 when grown on wet-exploded wheat straw hydrolysate and dilute acid hydrolysate from corn stover. This species was genetically modified for improved ethanol production, but no modification to the sugar transport and utilization genes was performed.

Caldicellulosiruptor saccharolyticus, *Thermotoga neapolitana* and other *Thermotoga* species are examples of cellulolytic bacteria able to co-utilize pentose and hexose sugars produced from hydrolysis of plant biomass (204). Isolates from both genera have been studied primarily for hydrogen production. The extreme thermophile *C. saccharolyticus* has demonstrated the ability to grow using arabinose, fructose, galactose, glucose, mannose and xylose, as individual monosaccharides and in mixtures where monosaccharides present were utilized simultaneously (205). While *C. saccharolyticus* was able to simultaneously consume all monosaccharides in the

mixtures, the monosaccharides were not used to the same extent, which indicated that sugar transporters were not substrate-specific and showed different affinities for the different sugars. The genome of *C. saccharolyticus* possesses 177 ABC transporter genes, most of which encode transporters related to uptake of monomeric and dimeric sugars (206). Interestingly, only one PTS transporter, for fructose, was found in the genome. *C. saccharolyticus* was shown to use pentose and hexose monosaccharides from *Miscanthus*, wheat straw, sweet sorghum plant and juice, treated and un-treated maize leaves, sugarcane bagasse and *Silphium* leaves for hydrogen production (204, 207). Various species of *Thermotoga* have also been shown to grown on the hydrolysate from lignocellulose, and like *C. saccharolyticus* have demonstrated co-utilization of pentose and hexose monosaccharides (204). *T. neapolitana* was able to use glucose, xylose and arabinose, all of which were consumed in parallel supporting growth and hydrogen production (208). *T. neapolitana*, along with other *Thermotoga* species, was also able to use pentose and hexose monosaccharides from *Miscanthus*, wet oxidized wheat straw, and with untreated and dilute acid or ammonia treated rice straw (208, 209). Genome studies, transcriptional analysis and physiological studies of a variety of *Thermotoga* species have shown the abilities to metabolize a variety of mono-, oligo- and polysaccharides (210-212). As with bacterial species discussed previously, co-utilization of pentose and hexose monosaccharides was attributed to the variety of ABC-type carbohydrate transporters, as well as the diversity of sugar kinases present in the cells.

Finally, simultaneous utilization of pentose and hexose sugars has been demonstrated in the hyperthermophilic archaeon, *Sulfolobus acidocaldarius*, and two yeast species, *Trichosporon cutaneum* and *Candida shehatae* (213-216). *S. acidocaldarius* was able to simultaneously utilize xylose and glucose, as well as a mixture of glucose, arabinose and galactose. Genome studies of *Sulfolobus* species has demonstrated the absence of PTS, and that sugar transport is mediated by ABC-type transporters, which may be the reason glucose-induced catabolite repression was not noted (117, 217, 218). *C. shehatae* was able to ferment xylose and glucose to ethanol; while ethanol production by yeast is common, many require genetic modification for efficient xylose metabolism (214, 219). Mixed substrate utilization by *C. shehatae* was noted over a range of dilution rates, when growing on xylose and glucose supplied at a 1:1 ratio (215). The ability of *C. shehatae* to grow on hydrolysates from lignocellulosic material was not determined. *T. cutaneum*, an oleaginous yeast, assimilated glucose and xylose simultaneously, and accumulated intracellular lipid when grown on a 2:1 mixture of glucose to xylose (216). Lipid production was also noted when *T. cutaneum* was grown on corn-stover hydrolysate, but the lipid content of the cells was decreased. While no specific studies have been performed to look at the mechanisms and rates of sugar assimilation in *C. shehatae* and *T. cutaneum*, genome studies indicate that sugar transport is typically accomplished via facilitated diffusion or proton symport (220).

Global Regulation of Carbon Metabolism in Gram-positive Bacteria

Along with acting as the primary signal for phosphorylation and transport of carbohydrates, HPr appears to be integral to the CCR mechanism functioning in Gram-positive bacteria (Figure 7). HPr is phosphorylated at two specific residues: His-15 which is phosphorylated by EI, which is the form utilized during carbohydrate transport, and Ser-46, which is phosphorylated by the regulatory enzyme HPr kinase in an ATP-driven reaction (221). High concentrations of glycolytic intermediates, such as fructose-1,6-bisphosphate, trigger the activity of HPr kinase which leads to HPr-Ser46-P, which is no longer involved in sugar transport (222). An additional HPr-like protein, the catabolite repression HPr (crh) protein, has been shown to be involved in repression but not in the PTS transport function (164, 223). This protein contains the regulatory site serine but not the active site histidine.

Interestingly, both phosphorylated forms of HPr are central to CCR; HPr-Ser46-P is involved in catabolite control protein A (CcpA)-mediated repression, while HPr-His15-P appears to be responsible for inducer exclusion and induction prevention (224). Inducer exclusion is an indirect form of CCR which occurs when

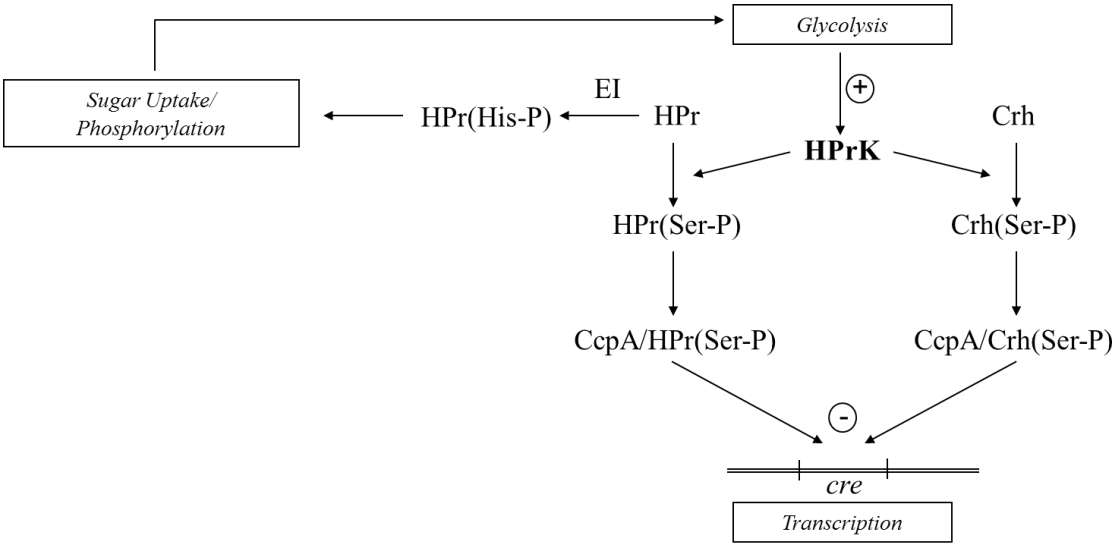

Figure 7. Schematic showing general mechanism of carbon catabolite repression in Gram-positive bacteria.

phosphorylated HPr binds to the EIIABC of transporters used for less favorable carbohydrates, inhibiting transfer of the phosphate moiety to the competing carbohydrate, keeping the carbohydrate from being transferred to the cytoplasm. Induction of HPr kinase by the presence of glycolytic intermediates leads to formation of the HPr-Ser46-P protein facilitating binding to CcpA; this complex then binds to an imperfect palindromic sequence, a catabolite response element (*cre*), located in the promoter or protein-coding regions of the target gene, leading to negative regulation of the target gene or operon (225).

Functionally, examples of CcpA-mediated repression can be seen for operons coding for pentose utilization by numerous Gram-positive bacteria, when grown on a preferred hexose substrate such as glucose. Typically, Gram-positive bacteria from the *Bacillus/Clostridium* group can use various carbohydrates as carbon and energy

sources (226). Using extracellular glycoside hydrolases, bacilli degrade lignocellulose from plant cell walls, producing oligo-, di- and monosaccharides. These potential carbon sources are transported into the cell, phosphorylated using the system described above and catabolized via glycolysis or the pentose phosphate pathway. The catabolized monosaccharides can be divided into hexoses, such as glucose, and several pentoses, namely arabinose, xylose and ribose. Enzymes necessary to catabolize a specific monosaccharide are usually synthesized only when their substrate is present and the preferred carbon and energy source is absent.

Interestingly, CcpA also regulates activity of genes as an activator; in other words, carbon catabolite activation (CCA) (222, 227, 228). Genes central to the glycolytic pathway are induced by the presence of glucose through the activity of CcpA. The glycolytic *gapA* operon, which encodes glyceraldehyde-3-phosphate dehydrogenase, is induced by glucose and other sugars (229). Luesink *et al*. (230) demonstrated that in the lactic acid bacteria *Lactobacillus lactis*, CcpA in the presence of PTS transported sugars activated the transcription of the *lac* operon carrying the genes for phosphofructokinase (*pfk*) and pyruvate kinase (*pyk*). Interestingly, in *L. casei*, these genes are activated by PTS sugars but repressed by CcpA (231).

Operons controlling utilization of pentose sugars are also controlled by transcriptional factors which bind to operator sites upstream from the operon. When the inducer is present, usually the monosaccharide for which the enzymes encoded are catalytically active, operon repression is alleviated and the genes are transcribed. Alternately, when glucose is present, the CcpA-mediated repression of gene

transcription is activated as described above. In the second case, even if the inducer monosaccharide is present, transcription is still repressed due to CCR, making the CcpA-mediated response the master regulator. Similar CCR mechanisms seem to be present in numerous strains of *B. subtilis* and *B. megaterium, Paenibacillus* and *Lactobacillus pentosus* and *L. casei* (182, 184, 232, 233).

Summary of Literature

A. acidocaldarius represents a unique microorganism relative to phenotypic characteristics related to bioprocessing of plant biomass or lignocellulose. *A. acidocaldarius* is able to tolerate extremes in temperature and pressure allowing compatibility with current lignocellulose pretreatment technologies (234). The presence of unique cell wall features including hopanoids and ω-cyloheptyl fatty acids, allow for life at extremes of pH and temperature. Numerous glycoside hydrolase enzymes for polysaccharide depolymerization have been found in the *A. acidocaldarius* genome and many have been characterized. Likewise, a number of primary and secondary carbohydrate transporters have been found in the *A. acidocaldarius* genome. Interestingly, very few PTS transporters are found in the *A. acidocaldarius* genome. A diversity of transporters would likely allow for uptake of a wider diversity of mono-, di-, oligo- and polysaccharides.

From a regulatory standpoint, the *A. acidocaldarius* genome appears to encode a number of Gram-positive CCR components, indicating preferential use of carbon sources in the environment. Understanding CCR in *A. acidocaldarius* and associated

carbohydrate use is the objective of the research performed and discussed in chapters to follow.

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Chapter II: Concurrent Metabolism of Pentose and Hexose Sugars by the Polyextremophile *Alicyclobacillus acidocaldarius*

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ABSTRACT

Alicyclobacillus acidocaldarius is a Gram-positive thermoacidophilic bacterium capable of growth on mono-, di-, oligo- and polysaccharides present in plant biomass. Many bacteria preferentially metabolize monosaccharides because expression of genes encoding proteins related to metabolism are regulated through a global regulatory mechanism called carbon catabolite repression (CCR). While allowing the bacterium to focus cellular resources on a monosaccharide that provides the most efficient carbon use for cellular processes, such as anabolism and energy production, CCR also causes sequential rather than simultaneous utilization when more than one monosaccharide is

present in the growth environment. The *A. acidocaldarius* genome encodes all components of Gram-positive CCR, but all transporters used for pentose and hexose monosaccharides found in biomass are multifacilitator superfamily or ATP-binding cassette transporters, types not common to CCR. Because of these interesting functional and regulatory characteristics, global transcriptome analysis of *A. acidocaldarius* growing on xylose or fructose was performed on steady-state cultures in chemostats, followed by attempted induction of CCR using either glucose or arabinose. Sugar and cell density analyses showed that *A. acidocaldarius* was able to grow while simultaneously metabolizing xylose and glucose, xylose and arabinose, and fructose and glucose, indicating that normal Gram-positive CCR did not appear to control carbon metabolism. Global transcriptome analysis showed that, with few exceptions, transcription of most genes was down-regulated when *A. acidocaldarius* transitioned from steady-state growth on one monosaccharide to non-steady state-state growth on two monosaccharides. Likewise, when the culture transitioned from non-steady-state growth with two monosaccharides to steady-state growth on the monosaccharide mixture, the same genes with a similar magnitude of up-regulation occurred. Regulation occurred primarily in three categories of genes: 1) genes encoding regulators, primarily activators; 2) genes encoding enzymes involved in cell synthesis; and 3) genes encoding sugar transporters. In essence, when the second monosaccharide was added, *A. acidocaldarius* appeared to suspend anabolic processes. Following reestablishment of steady-state, metabolism returned to a condition similar to steady-state on the original monosaccharide.

INTRODUCTION

Alicyclobacillus acidocaldarius is a thermoacidophilic, Gram-positive, spore forming bacterium that grows optimally in strictly aerobic conditions at 60°C and at pH between 3 and 4. Isolated from Nymph Creek in Yellowstone National Park (1), it was originally designated as *Bacillus acidocaldarius.* Because of the prevalence of ω-cyclic fatty acids in the cell wall and an abbreviated helix 6 of the 16S rRNA, the genus was reclassified as *Alicyclobacillus* in 1992 (2). Due to acid and thermal tolerance, *A. acidocaldarius* has been found in diverse habitats including geothermal sites, submarine hot springs, orchard soils, and also as a contaminant in heat processed foods (e.g., fruit juices) (3-8). Physiologically, *A. acidocaldarius* has demonstrated the ability to utilize a variety of 5- and 6-carbon sugars as both carbon and energy source, including, L-arabinose, ribose, D-xylose, D-galactose, D-fructose, D-mannose, rhamnose, mannitol, and tagatose, the disaccharides D-turanose, melibiose, cellobiose, lactose, maltose, sucrose, and trehalose, as well as the more complex polysaccharides cellulose, hemicellulose (xylan), starch and glycogen (9). The potential for utilization of plant derived oligo- and polysaccharides by *A. acidocaldarius* is further demonstrated by the numerous glycoside hydrolase enzymes that have been found in the genome sequence, some of which have been characterized. To date, 20 glycoside hydrolase genes have been found encoded in the *A. acidocaldarius* genome (10), and β-galactosidase, α-amylase, cellulase, neopullulanase, exo-pectinase, mannanase, β-glycosidase, and endoglucanase enzymes have been expressed and characterized (6, 9, 11-26).

Lignocellulosic biomass is currently being developed as a feedstock for the production of fuels and chemicals. The carbohydrate component of lignocellulose--hemicellulose and cellulose--is composed of chains of pentose and hexose monosaccharides that provide sugar monomers, which are then fermented to fuels such as ethanol. In order to increase product yield from pretreated lignocellulose, microorganisms must be able to use both the pentose (e.g., xylose and arabinose) and the hexose (e.g., glucose). Many fermentation microorganisms cannot use xylose and must be genetically modified for xylose utilization to increase yield (27, 28). Likewise, carbon catabolite repression (CCR) is active in many bacteria that can metabolize both pentose and hexose monosaccharides, causing preferential use of the hexoses, primarily glucose, which leads to sequential rather than simultaneous sugar utilization (29). While the general mechanism of CCR is different when comparing Gram-positive and Gram-negative bacteria, in each case, gene regulation occurs so that a preferred carbon source such as glucose is utilized before secondary carbon sources such as xylose or arabinose (30-33). In many microorganisms, CCR is associated with the phosphoenolpyruvate (PEP): phosphotransferase system (PTS), where the incoming sugar is phosphorylated during transport using a phosphoryl group from PEP, which can lead to catabolite repression or inducer exclusion (34). Genes and operons encoding glycoside hydrolase enzymes for breakdown of hemicellulose and cellulose, as well as transporters of oligosaccharides, are also regulated by CCR (35-46). When a preferred carbon source, such as glucose, is present, expression of these enzymes is typically down-regulated in an effort to conserve cellular resources.

While carbon metabolism in many microorganisms appears to be regulated by CCR, there are a number of Bacteria, Archaea, and some yeast that have been shown to simultaneously use mixtures of pentose and hexose monosaccharides (Table 1) (29). The Bacteria include a number of species of lactic acid bacteria that have been shown to possess the ability to simultaneously use pentose and hexose monosaccharides, supplied either as individual sugars or as the product of hydrolysis of lignocellulose biomass (47-49). Simultaneous utilization of pentose and hexose monosaccharides in *L. brevis* and other Lactobacilli has been attributed to use of non-PTS sugar transporters, such as H^+ symporters and sugar uptake by facilitated diffusion (29).

A variety of bacteria isolated from animal digestive tracts have also shown the ability to simultaneously metabolize pentose and hexose sugars. Species from the genera *Butyrivibrio* and *Clostridium* are bacteria commonly found in rumen material. Ruminal strains of *Butyrivibrio fibrosolvens* were able to grow via the concurrent use of xylose and glucose. While transport mechanism were not determined during these experiments, other *B. fibrosolvens* strains demonstrate pentose uptake using ABC transporters (50). Interestingly, not all strains of *B. fibrosolvens* were able to co-utilize the pentose and hexose monosaccharides (51). Active CCR also appears to be absent in a number of Clostridia (Table 1) (52-57). *Clostridium thermohydrosulfuricum* strain Rt8.B1 showed the absence of catabolite repression, allowing for simultaneous utilization of glucose and xylose. Research results indicated biphasic utilization of glucose implicating high- and low-affinity transport, while xylose uptake appeared to occur through a facilitated diffusion mechanism (52). While sugar transport hasn't

been characterized in many of these bacteria, for those that have, PTS transporters don't appear to be responsible for transport of both pentose and hexose sugars, which explains why CCR may not be active in these species.

Simultaneous utilization of pentose and hexose has also been demonstrated in thermophilic non-cellulolytic hydrogen producing and cellulolytic anaerobes. Species in the genera *Thermoanaerobacter*, *Caldicellulosiruptor*, and *Thermotoga* are primary examples of thermophiles in this category of metabolic potential (58-66). In the carbon utilization network of *Thermoanaerobacter* strain X514, fructose, glucose and cellobiose transport is facilitated by PTS transporters, while xylose is transported via an ABC transporter. Regulation of pentose metabolism in other Gram-positive anaerobes has been seen in other *Thermoanaerobacter* species, but was not noted in X514. In cellulolytic thermophiles, such as *Caldicellulosiruptor saccharolyticus* and different *Thermotoga* species, carbohydrate transport is also catalyzed by non-PTS type transporters. The genome of *Cc. saccharolyticus* possesses 177 ABC transporter genes, most of which encode transporters related to uptake of monomeric and dimeric sugars (67). Interestingly, only one PTS transporter, for fructose, was found in the *Cc. saccharolyticus* genome. Various *Thermotoga* species were also able to use pentose and hexose monosaccharides from hydrolyzed lignocellulose materials (65, 68). As with bacterial species discussed previously, co-utilization of pentose and hexose monosaccharides was attributed to the variety of ABC-type carbohydrate transporters, as well as the diversity of sugar kinases present in the cells (69-71).

Finally, simultaneous utilization of pentose and hexose sugars has been demonstrated in the hyperthermophilic archaeon, *Sulfolobus acidocaldarius*, and two yeast species, *Trichosporon cutaneum* and *Candida shehatae* (72-75). *S. acidocaldarius* was able to simultaneously utilize xylose and glucose, as well as a mixture of glucose, arabinose and galactose. Genome studies of *Sulfolobus* species has demonstrated the absence of PTS, and that sugar transport is mediated by ABC-type transporters, which may be the reason glucose-induced catabolite repression was not noted (76-78). While no specific studies have examined the mechanisms and rates of sugar assimilation in the yeast, *C. shehatae* and *T. cutaneum*, genome studies indicate that sugar transport is typically accomplished via facilitated diffusion or proton symport (79).

A. acidocaldarius is an industrially important bacterium that produces a variety of extracellular and intracellular glycoside hydrolase enzymes, and can also grow on a variety of pentose and hexose sugars (2). Annotation of the *A. acidocaldarius* genome shows the presence of PTS, proton symport and many ABC-type carbohydrate transporters (10). In addition, the genome has all components of the Gram-positive CCR, including catabolite control protein A (CcpA), histidine protein (HPr), HPr kinase/phosphorylase, Crh, and cis-acting catabolite responsive elements (*cre*). The purpose of this study was to characterize carbon metabolism and gene regulation in *A. acidocaldarius* during growth on monosaccharides. Chemostat studies and global transcriptome analysis using high-density microarrays were used to accomplish this

goal. This research represents the first global transcriptome analysis of *A. acidocaldarius*.

MATERIALS AND METHODS

Inoculum Development.

A. acidocaldarius ATCC 27009 was purchased from the American Type Culture Collection (ATCC) and used for all experiments. To ensure that the same generation was used in all experiments, stock cultures collected from cultures two transfers from the ATCC stock were maintained in 5% DMSO and stored at -80 °C. Chemostat inoculum was prepared by inoculating 1 ml of the frozen stock into 25 ml of Modified 402 Medium, which contained the following (g/L): $(NH_4)_2SO_4$ (1.3), Fe(III) EDTA (0.047), $CaCl_2 \cdot 2H_2O$ (0.07), $MgSO_4 \cdot 7H_2O$ (0.25), KH_2PO_4 (3.0), and glucose (4.0). In addition, 1 mL of a mineral (Solutions A and B) and vitamin stock (Solution C) were added. Solution A (g/L): $MgCl_2$ (25), $CaCl_2 \cdot 2H_2O$ (6.6), H_3BO_3 (0.58), $FeCl_3 \cdot 6H_2O$ (5), $Co(NO_3)_2 \cdot 6H_2O$ (0.05), $NiCl_2 \cdot 6H_2O$ (0.02). Solution B (g/L): $MnSO_4 \cdot H_2O$ (2.0), $ZnSO_4 \cdot 7H_2O$ (0.5), $CuSO_4 \cdot 5H_2O$ (0.15), $Na_2MoO_4 \cdot 2H_2O$ (0.025). Solution C (g/L): pyridoxine hydrochloride (0.08), folic acid (0.012), thiamine hydrochloride (0.13), riboflavin (0.042), nicotinamide (0.084), *p*-aminobenzoate (0.088), biotin (0.01), cyanocobalamin (0.0004), D-pantothenic acid, calcium salt (0.086), *myo*-inositol (0.021), choline bromide (0.053), orotic acid, sodium salt (0.021), spermidine (0.1). Base medium was autoclaved (121 °C, 20 psi) for 30 min prior to use; KH_2PO_4 solution was adjusted to pH 4.0, autoclaved

separately and added once bulk media had cooled. Solutions A, B and C were filter-sterilized (0.22 μm) to maintain sterility during storage. During media preparation, solutions A and B were autoclaved with the bulk media, whereas Solution C was added to the base medium once it had cooled. A 25 mL *A. acidocaldarius* culture was grown overnight and then used to inoculate 250 mL of Modified 402 Medium containing 4 g/L of the sugar that was used for the subsequent chemostat experiment. This culture was then used to inoculate the chemostat.

Chemostat Studies.

Carbon utilization studies to determine whether CCR governed carbon metabolism in *A. acidocaldarius* was performed in a BioFlo 3000 chemostat system (New Brunswick Scientific, Enfield, CT). Medium was added to the reactor, oxygen and DO probes were inserted into appropriate ports, and the entire reactor was autoclaved at 121 °C, 20 psi, for 1 hour. The pH probe was calibrated using a two-point calibration with pH 2 and 7 buffers, prior to autoclaving. Prior to heating the reactor for operation, the pH of the medium was measured using an external pH meter and the pH on the BioFlo control unit was adjusted accordingly. The DO probe was allowed to polarize for six hours and then the dynamic range of the probe was set first by purging nitrogen gas through the medium (0% oxygen) and then air (100% oxygen). A working volume of 2.0 L of Modified 402 Medium was used for experiments, and cultures were grown at a dilution rate of 0.5/hr. Experiments were run at a temperature of 60 °C and the pH of the growth medium was automatically controlled to 4.0 by adding 1 N NaOH. To ensure that the cultures were not oxygen-

limited, dissolved oxygen was controlled to 10% using a cascade system based on changes in agitation along with the addition of pure oxygen to the inlet air stream. Experiments were performed in which xylose or fructose was used as initial carbon sources. In the case of xylose, CCR-induced conditions were established by the addition of glucose or arabinose, while for fructose, CCR induction was attempted using glucose. Each sugar was supplied at a concentration of 2.0 g/L, so when two sugars were present the total sugar concentration was 4 g/L.

Using the inoculum described above, the reactor was inoculated and run through an initial batch phase during which *A. acidocaldarius* was grown to mid- to late log phase using the initial sugars indicated above. During this time period, cell density was monitored using OD_{600}. At this point, a pump feeding the reactor with autoclaved Modified 402 Medium with the appropriate sugar monomer was started to achieve the 0.5/hr dilution rate. An effluent pump set at a slightly higher flow rate was used to remove spent medium from the reactor. To ensure that the culture was at steady-state prior to addition of the CCR-inducing sugar, the bioreactor was run for a time equivalent to five reactor volumes. Samples were taken at each residence time (i.e., every two hours) for sugars, cell density and RNA extraction. A "baseline" sample (t_0) to which other samples were compared was taken just prior to adding the inducing sugar. The induction phase of the experiment consisted of injection of a solution containing the inducing sugar that provided a final concentration of 2 g/L of the sugar in the chemostat medium, and then initiation of flow of autoclaved Modified 402 Medium containing the initial sugar and the inducing sugar. Upon injection and

initiation of flow for the medium containing both monosaccharides, samples were taken for sugars, cell density, and RNA extraction. This sample was considered the t_1 sample. The chemostat was run for an additional five reactor volumes, and as with the initial phase on a single monosaccharide, samples were taken every two hours. Once five reactor volumes had passed through the chemostat, a final sample (t_2) was taken for sugars, cell density and RNA extraction. Three biological replicates of each application of initial sugar for steady state growth and inducing sugar was accomplished using three separate chemostat runs, set up and operated in an identical manner.

Analyses

Bulk samples were taken from a sampling port in the chemostat using sterile syringes. Samples were then aliquoted for the required amount for each analysis.

Cell Density:

A Thermo Scientific Evolution 600 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the optical density of the culture at a wavelength of 600 nm. The spectrophotometer was controlled using VisionPro Software. Due to the high cell density achieved once the culture entered logarithmic growth, a 1/10 dilution using sterile, sugar-free medium was performed prior to analysis.

Carbohydrate Analysis:

An Agilent G1600AX 3D Capillary Electrophoresis (CE) system (Agilent, Santa Clara, CA) equipped with a diode array detector was used to analyze sugar

concentration in the chemostat supernatant. The CE was controlled using Agilent's ChemStation Software. Neutral carbohydrate (i.e., glucose, xylose, arabinose and fructose) concentrations were determined using the method of Rovio *et al*. with some modification (80, 81). Uncoated fused silica capillary columns with a total length of 60 cm and an ID of 50 µm were used for analysis. Carbohydrate separation was performed by holding the samples and the capillary at a temperature of 15 °C. Samples were injected using a pressure of 0.5 psi for 4 seconds, followed by a plug of electrolyte (0.5 psi for 5 seconds). Carbohydrates were separated using a voltage of +16 kV. The electrolyte used for the analysis consisted of 130 mM NaOH and 36 mM $Na_2HPO_4 \cdot 2H_2O$ having a pH value of 12.6. All solutions were made using Optima LC/MS Water (Fisher Scientific, Waltham, MA). Prior to use, the electrolyte was filtered through a 0.45 µm filter to remove particulates, and sonicated for 20 minutes at room temperature to degas. Monitoring of the carbohydrates was accomplished at a wavelength of 270 nm with a bandwidth of 10 nm. Duplicate measurements were made for each sample.

Prior to analysis, new capillaries were pre-conditioned using sequential 20 minute rinses with 0.1 M NaOH, water and electrolyte solution. Between individual samples, the capillary was rinsed with 10% acetic acid, Optima water, and electrolyte by applying a pressure of 15 psi for 5 minutes for each solution. Acetic acid was used to help in stabilizing current during sequential analyses.

Samples (5 mL) were acidified with concentrated H_2SO_4 (250 µL), mixed using a vortexer and then frozen until analyzed. Samples were diluted in Optima water

and then filtered through a 0.2 µm syringe filter prior to analysis. Concentrations of monosaccharides in the samples were determined by comparing absorbance to the absorbance of known standards for each sugar. Stock solutions (10 g/L) were made for each monosaccharide and were stored at 4 °C when not in use. Monosaccharide stocks were diluted in Optima water to generate a three point calibration of 50, 100, and 500 mg/L for analysis.

Isolation of Total RNA:

Samples for RNA extraction were taken from the chemostat and immediately mixed with RNA Protect Bacteria Reagent (Qiagen, Valencia, CA) at a 1:2 ratio. This mixture was incubated at room temperature for 5 minutes, centrifuged, the supernatant was discarded and the cell pellet was flash frozen in liquid nitrogen and then stored at -80 °C until the RNA was extracted. Total RNA was extracted from the *A. acidocaldarius* cells using an RNeasy Midi Kit (Qiagen, Valencia, CA) with slight modification of the manufacturer's protocol. *A. acidocaldarius* cells were thawed and lysis was accomplished by adding 200 µL of Tris-EDTA buffer containing 15 mg/mL lysozyme and 0.1 mg/mL proteinase K. Samples were vortexed for 10 seconds and then incubated at room temperature for 15 minutes with shaking. Buffer RLT (4 mL) containing β-mercaptoethanol was then added to each sample, the mixture was homogenized using a syringe with a 20G needle, and then incubated at room temperature for 10 minutes. Following incubation, 3.5 mL of 80% ethanol was added

and the resulting solution was mixed vigorously. The lysate was then passed through a Midi spin filter via centrifugation at 5,000 x g for five minutes to capture the RNA. RNA on the spin filter was washed sequentially with two 2.5 mL aliquots of Buffer RPE, followed by centrifugation at 5,000 x g for five minutes. RNA was eluted from the glass filter using 200 µL of DNase/RNase-free water: water was applied to the filter, incubated at room temperature for five minutes, and then centrifuged at 5,000 x g for three minutes. To increase RNA yield, the flow-through was re-applied to the spin filter and centrifuged. Residual DNA in the samples was removed by treatment with Ambion TURBO DNA-free kit (Life Technologies, Grand Island, NY). RNA was purified to remove compounds that might interfere with cDNA synthesis and concentrated using ethanol precipitation. The pellet was dried using an Eppendorf Vacufuge Concentrator 5301 (Brinkmann Instruments, Westbury, NY), and then resuspended in 20 µL of DNase/RNase-Free water. To inhibit RNA degradation during storage, 1 µl of Ambion Superase-In RNase Inhibitor (Life Technologies, Grand Island, NY) was added. RNA concentration and purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was determined using an RNA Nano Chip Kit run on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Synthesis of cDNA:

Double-stranded cDNA was synthesized from total RNA using the Invitrogen Superscript Double-Stranded cDNA Synthesis Kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Ten µg of total RNA was mixed with

random hexamer primers in DNase/RNase-Free Water, heated for 10 minutes at 70 °C and then quenched in an ice-water slurry for five minutes. While on ice, First Strand Buffer, dithiothreitol, and dNTPs, all supplied with the kit, were added and then brought to the reaction temperature of 42 °C, prior to addition of SuperScript II from the kit. Following addition of the reverse transcriptase, the reaction mixture was incubated at 42 °C for one hour. After this incubation the tubes were placed on ice and reaction components for second strand synthesis were added. The first strand reaction mixture was mixed with Second Strand Buffer, dNTPs, DNA ligase, DNA polymerase and RNase H (all supplied with the kit); the reaction was mixed and then incubated at 16 °C for two hours. An additional five minute incubation was performed after T4 DNA polymerase was added to the reaction. Residual RNA was degraded by adding RNase A and incubating the reaction mixture at 37 °C for 10 minutes. Proteins were removed by treating with phenol:chloroform:isoamyl alcohol, with phase separation being accomplished using Phase Lock Tubes (5 Prime, Inc., Gaithersburg, MD). cDNA in the aqueous phase was then precipitated and concentrated by ethanol precipitation. The pellet was dried using an Eppendorf Vacufuge Concentrator 5301 (Brinkmann Instruments, Westbury, NY), and then resuspended in 20 µL of DNase/RNase-Free water and allowed to solubilize overnight. cDNA concentration in each reaction was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A DNA 7500 Chip Kit run on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) was used to verify that most of the cDNA was $\geq$ 400 bp.

Microarray Experiments and Data Analysis:

Microarrays were designed and synthesized by NimbleGen using their 4 x 72K Custom Gene Expression Array format. Complete genome sequence information for *A. acidocaldarius* ATCC 27009 (DSM 446) was provided to NimbleGen. Seven probes, each 60 nt in length, were designed for each of the 3,554 identified open reading frames (ORFs) in the genome. Each probe was synthesized on the microarray in triplicate. Control probes were also included to ensure that there was no intra-quadrant contamination during the hybridization process.

Triplicate RNA samples isolated from *A. acidocaldarius* cells grown under the various conditions were used for microarray analysis. One-color cDNA labeling using Cy3, hybridization to the *A. acidocaldarius* microarrays, array imaging and initial analysis of the array data was performed by NimbleGen. Data was normalized using NimbleScan software which normalizes probe response using quantile normalization and gene calls generation using Robust Multichip Averaging (RMA) (82, 83). Log_2-transformed RMA data files were imported into ArrayStar 4 software (DNASTAR, Inc., Madison, WI) and the mean expression levels of three replicate arrays for each condition were considered. For comparison between gene expression during growth on the initial sugar, immediately upon addition of the CCR-inducing sugar, and growth on initial and inducing sugar, statistical significance was determined with a Bonferonni corrected moderate t-test and only genes that had a threefold or greater change in gene expression at 95% confidence were considered significant.

Bioinformatic Analysis:

Comparative analysis of the *A. acidocaldarius* genome was performed using the Integrated Microbial Genomes feature within the Joint Genome Institute (84). Homology determinations for *A. acidocaldarius* proteins were accomplished using the Basic Local Alignment Search Tool (BLAST) for protein sequences using the 'blastp' algorithm (85, 86). The non-redundant protein sequence database was used for searches and uncultured and environmental sample sequences were excluded from the search. Alignments demonstrating variation between protein sequences for the Hpr protein were generated using GeneDoc (87). Phylogenetic trees comparing protein sequences were generated using the Molecular Evolutionary Genetics Analysis (MEGA6) software program (88). Protein sequences were aligned using the MUSCLE program within MEGA6 (89, 90). Phylogenetic reconstruction was accomplished using the Maximum Likelihood statistical method and distances between sequences were determined using 1000 bootstrap replicates.

RESULTS AND DISCUSSION

The purpose of the experiments performed was to understand sugar metabolism and regulatory mechanisms controlling metabolism in the Type strain of *A. acidocaldarius* (ATCC 27009/DSM 446). Mixtures of monosaccharides were used monitor changes in sugar metabolism, growth, and gene transcription in *A. acidocaldarius* when tested in continuous-flow chemostat studies. Information from the annotated genome of *A. acidocaldarius* indicates the presence of regulatory components common to Gram-positive CCR, which is a common global regulator of

gene transcription when bacteria grow on a preferred carbon source, such as glucose, even when other sugars are present (10). To determine whether CCR does control sugar metabolism, growth and gene transcription in *A. acidocaldarius*, cells were grown to a metabolic steady-state on one sugar and then a second sugar was added to the growth medium. Monosaccharide pairs tested were: xylose/glucose, xylose/arabinose, and fructose/glucose, where the first sugar listed represents the monosaccharide used to establish the initial steady-state of growth, and the second monosaccharide was used for potential induction of CCR. Xylose, glucose and arabinose were selected because they represent the monosaccharides commonly found in the cellulose and hemicellulose fractions of plant biomass. Fructose was tested because, according to the genome, transport of this monosaccharide is facilitated by a PTS transporter, the type of transporter typically associated with CCR. Samples for analysis of cell growth and monosaccharide utilization were taken at multiple time points over the duration of the experiment, while samples for total RNA extraction were taken at three time points during the experiment. Gene transcription was analyzed using single-dye, high-density microarrays, and results were compared at the three sample points. The three time points considered were: steady-state on one sugar (initial phase); non-steady-state on two sugars (transition); and steady-state on two sugars (sugar1/sugar2)

Comparison of Sugar Metabolism

Glucose and Xylose Metabolism

An initial experiment was performed to monitor the effect of glucose inclusion on sugar metabolism, growth and gene transcription of *A. acidocaldarius* that was growing on xylose. Xylose and glucose concentrations and growth as determined by OD_{600} are shown in Figure 1A. During the initial phase, at a dilution rate of 0.5/hr, *A. acidocaldarius* was using approximately half of the xylose supplied. During the initial growth phase on xylose, *A. acidocaldarius* cell density was stable at an OD_{600} of approximately 1.5. Cell density for only the final residence time before the transition phase where the second sugar was added is plotted. Following addition of enough glucose to bring the calculated concentration in the chemostat to 2.0 g/L, the measured glucose concentration in the media was just below 1.7 g/L. Following the initial spike of glucose and initiation of the chemostat influent containing xylose and glucose, the *A. acidocaldarius* culture was receiving a total of 4 g/L of total carbon for growth. During this transition phase, a gradual increase in xylose concentration coincided with high glucose concentrations in the chemostat. At face value, this initial observation may have been an indication of CCR, but *A. acidocaldarius* cells quickly began using both the xylose and glucose for growth which was demonstrated by a gradual increase in cell density over time compared to the initial steady-state (See OD_{600} in Figure 1A). Since glucose was being supplied continuously in the chemostat influent, if CCR were occurring, then xylose metabolism would be expected to drop off for the remainder of the experiment, and little to no additional growth should have been noted. Xylose and

glucose concentrations in the chemostat dropped to gradually to near zero, and the cell density nearly tripled after the glucose was added indicating that the *A. acidocaldarius* cells were growing on both monosaccharides, and CCR was not likely active.

Arabinose and Xylose Metabolism

A second set of experiments was performed in which two pentose sugars were tested. Metabolism of xylose and arabinose and associated effects on growth are shown in Figure 1B. Sugar use between experimental replicates was very different between chemostat runs. *A. acidocaldarius* was completely using the xylose in the influent medium during the initial phase when grown on xylose only. In contrast to the previous experiment with xylose and glucose, the culture for this set of experiments was completely using the xylose, meaning it was growing under carbon-limited conditions. Upon addition of the calculated 2.0 g/L arabinose, there was a spike in arabinose concentration to an average just over 2.5 g/L. As the experiment proceeded, the mean xylose concentration in the medium began to rise to an average maximum just over 1.0 g/L. The measured arabinose concentration in the chemostat medium decreased as the xylose concentration increased and then both monosaccharides were metabolized nearly equally after the first residence time (2 hours). By the end of the third residence time, neither monosaccharide was detectable in samples of growth medium taken from the chemostat. When both monosaccharides were present in the chemostat influent, cell density nearly doubled (Figure 1B). As with the xylose/glucose monosaccharide pair, no apparent CCR was demonstrated as *A. acidocaldarius* was able to use both xylose and arabinose simultaneously. This

result is less surprising, since xylose and arabinose are both pentose sugars, and are likely metabolized using similar cellular machinery. In addition, neither sugar has been shown to elicit CCR in other bacteria.

Fructose and Glucose Metabolism

A third and final set of chemostat experiments was performed in which growth and monosaccharide use were compared when *A. acidocaldarius* was exposed to two hexose sugars. *A. acidocaldarius* was not able to completely use fructose when supplied at an inlet concentration of 2.0 g/L (Figure 1C). During the initial phase when grown only on fructose, approximately 60% of the incoming fructose was used by *A. acidocaldarius*. This result suggests that *A. acidocaldarius* was not carbon-limited during growth on fructose, with the chemostat conditions tested. Interestingly, cell densities achieved were still equivalent to cell densities when grown on xylose as the sole carbon source. When the steady-state culture was spiked with glucose, medium concentrations of nearly 2.0 g/L of glucose were noted; however, there was only a slight increase in the fructose concentration in the growth medium. As the culture proceeded into the second steady-state on both monosaccharides, fructose concentrations returned to levels near those seen during the initial phase and glucose concentrations decreased to near zero. These results may indicate that *A. acidocaldarius* prefers glucose over fructose. Following addition of the second monosaccharide, cell density nearly doubled, indicating that *A. acidocaldarius* was able to use fructose and glucose in parallel, and that CCR was probably not elicited by the addition of glucose.

Global Transcription Analysis

RNA extracted from *A. acidocaldarius* was reverse-transcribed to cDNA and used for microarray analysis in an effort to understand global regulation of transcription during growth on individual and mixtures of pentose and hexose monosaccharides. *A. acidocaldarius* was grown in chemostats to steady-state on one sugar, followed by addition of a second sugar. Gene regulation during the initial phase, when growth was attributed to the first sugar, was then compared to the transition phase with two sugars, and then to steady-state growth on two sugars. Three sets of experiments were performed: xylose/glucose, xylose/arabinose and fructose/glucose; results from each experiment are presented. Overall results for regulated genes are shown in Figure 2.

Numbers of genes that were regulated during the experiments were 56, 64 and 71of the 3,554 *A. acidocaldarius* ORFs tested for xylose/glucose, xylose/arabinose, and fructose/glucose, respectively. These results demonstrate differences in genes regulated under the different conditions tested. During the xylose/glucose and fructose/glucose experiments, regulated genes that demonstrated greater than 3-fold with a confidence level of 95% or higher when comparing growth on the first sugar to growth after the transition when the second sugar was spiked into the growth medium, were selected for analysis. A few genes are discussed for which the change is less than 3-fold, but the confidence interval is still above 95%, but only in cases where the gene annotation links it to a set of genes where the fold-change was greater than three. When *A. acidocaldarius* was tested with xylose and arabinose, for unknown reasons,

confidence intervals for the microarray analysis dropped substantially. Consequently, genes that were highly regulated (4-fold or higher) will be discussed as significant, and will represent confidence intervals of 80% or higher. This cut-off was chosen because at this high level of change for gene transcription still remains significant even at 80% confidence. Factors related to carbon-limitation during the xylose/arabinose chemostat experiments may have led to the low *p*-values demonstrated during this experiment.

Up-regulated genes were either below the fold- change cut-off or the 95% confidence interval set for the research. During the transition from non-steady-state on xylose and glucose to steady-state on the same two sugars, gene transcription trends reversed and most genes were up-regulated for all three conditions. Of particular interest to this research, none of the genes related to CCR were regulated. Only two genes were up-regulated within the established criteria for the experiment during the fructose/glucose experiment.

When comparing COG categories of functions for genes, overall, gene transcription analysis showed that genes related to lipid transport and metabolism were the most regulated (Figure 2). Relatively high numbers of genes associated with amino acid transport and metabolism, carbohydrate transport and metabolism, and energy production and conversion were also regulated. Substantially higher degrees of down-regulation (>19-fold) of transcription of a number of genes was noted when *A. acidocaldarius* transitioned from growth using fructose to growth on fructose and glucose, than was demonstrated for the other conditions tested. More energy production and conversion genes, as well as genes related to transcription, were

regulated during the fructose/glucose experiment than for the other two conditions tested.

Systems Level Analysis of Gene Transcription

Gene transcription analysis of the Gram-positive thermoacidophilic *A. acidocaldarius* growing on individual pentose and hexose sugars, and mixtures of pentose and/or hexose sugars was performed. Sugar mixtures tested were xylose/glucose, xylose/arabinose and fructose/glucose. *A. acidocaldarius* was grown to steady-state on the first sugar listed and then RNA was extracted and compared to RNA extracted when growing at non-steady-state and steady-state on both sugars. The goal of these experiments was to better understand monosaccharide metabolism by *A. acidocaldarius* and regulatory mechanisms controlling this metabolism. Results from microarray experiments for different cellular systems are discussed in more detail below.

Transcription Regulators

Transcriptional regulators are usually *trans*-acting elements that become active when the bacterial cell receives one or more environmental or metabolic signals that the growth environment has changed and that cellular resources may need to be redistributed for optimal growth. A number of transcriptional regulators were affected by transition of *A. acidocaldarius* from growth on one sugar to transitory and steady-state growth on two sugars. Figure 3 shows regulators of gene transcription that were controlled during testing of *A. acidocaldarius* on different individual monosaccharide and monosaccharide mixtures.

The most highly down-regulated ORF for regulation of gene transcription was Aaci_0876 that encodes a helix-turn-helix, AraC (HTH-AraC) family regulator. This gene was down-regulated at levels greater than 11-fold during testing with xylose/glucose and fructose/glucose, but only 9-fold when *A. acidocaldarius* was grown on xylose/arabinose. HTH-AraC family regulators are common in *Firmicutes* and represent two-component response regulators (91). These types of regulators have been found to regulate three primary processes in bacteria: 1) carbon metabolism; 2) stress response and adaptation; and 3) expression of virulence factors. Nearly 45% of AraC regulators have been shown to regulate carbon catabolism and global metabolism (92). In other *Firmicutes*, such as *Paenibacillus* sp. Strain JDR-2, the HTH-AraC regulator appears to be in the same regulatory element as a histidine kinase, which is most likely involved in signal transduction related to glycoside hydrolase enzymes and related carbon metabolism (93). When compared to other AraC-type regulators cataloged in the NCBI database, Aaci_0876 is nearly identical to the genes in two other *A. acidocaldarius* genome sequences in the database (Accession ZP03495134.1 and YP_00551834.1), with the next closest matches being to AraC transcriptional regulators found in numerous species of *Paenibacillus*. Many of the *Paenibacillus* genomes contain a histidine kinase just upstream from genes encoding the AraC-type regulator. This, however, is not the case with *A. acidocaldarius*; the gene upstream of Aaci_0876 encodes a hypothetical protein, which from gene ontology appears to be a UvrD/Rep helicase family protein, and it was not co-regulated with Aaci_0876. Likewise, none of the 25 histidine kinase related signal

transduction genes found in the *A. acidocaldarius* genome were co-regulated with Aaci_0876.

Aaci_0444, which is annotated as encoding a PAS modulated Fis family Sigma-54-dependent transcriptional activator, was also down-regulated during all three experiments. During transition from growth on the first monosaccharide to the transition phase when the second monosaccharide was added, Aaci_0444 was down-regulated 7.3-fold with xylose/glucose, 6.7-fold with xylose/arabinose, and 9.5-fold when grown on fructose/glucose. Further analysis of Aaci_0444 indicates that this is most likely a bacterial enhancer binding protein (bEBP) that activates transcription using the Sigma-54 RNA polymerase holoenzyme (94). *In silico* analysis of the protein encoded by this gene indicates that it contains the three primary domains of bEBPs: 1) a signal sensing domain (N-terminus); 2) an HTH DNA binding domain (C-terminus); and 3) a Sigma-54 interaction domain (95). The Sigma-54 interaction domain also contains a conserved AAA^+ domain which is responsible for ATP hydrolysis required for activation of transcription. Interestingly, another PAS modulated Fis family Sigma-54-dependent transcriptional activator (Aaci_0324), located in the same genome neighborhood as *rpoN* (Aaci_0326), which encodes the Sigma-54 subunit of RNA polymerase, was not up- or down-regulated. *In silico* analysis of 842 prokaryotic genomes in the GOLD database showed Sigma-54 and bEBPs homologs in 62% of the genomes (96). Analysis of promoters containing Sigma-54 and bEBP recognition/binding sequences indicated COG functional categories of genes that encode proteins related to energy production and conversion,

cell wall/membrane/envelope biogenesis, cell motility, post-translational modification, signal transduction and intracellular trafficking/secretion were found more often than other categories of genes. These findings, along with the demonstrated down-regulation of lipid synthesis genes in *A. acidocaldarius* discussed below, indicate that the Sigma-54 RNA polymerase holoenzyme and Aaci_0444 may be responsible for transcription of genes encoding enzymes for cell wall construction and other carbon metabolism. Aaci_0443, which is annotated as a major facilitator superfamily (MFS) general substrate transporter, appeared to be co-regulated with this bEBP during the xylose experiments, and will be discussed in additional detail below.

Aaci_0139 was annotated as a GAF-modulated transcriptional regulator of the LuxR Family, and was downregulated 5.6-fold with xylose/glucose, 4.2-fold with xylose/arabinose and 5.9-fold with fructose/glucose. These regulators typically are involved in some type of signal transduction and contain a sensory domain, as well as an HTH DNA-binding domain. Down-regulation of this gene in parallel with Aaci_0444, the bEBP that interacted with Sigma-54, makes sense since GAF-modulated transcriptional regulators often impact Sigma-54-dependent transcription (97, 98).

Three other regulators that were down-regulated only on fructose/glucose were Aaci_1214 (Transcriptional regulator, LacI family), Aaci_2890 (Transcriptional regulator, AraC family) and Aaci_2901 (Transcriptional regulator, GnrR family with LacI sensor). These genes were also down-regulated when *A. acidocaldarius* was

grown on xylose/glucose, with p-values below 0.05, but the change in expression was 2.3, 2.6-fold and 1.9-fold, respectively.

As indicated previously, none of the genes associated with CCR were regulated within the criteria established for the experiments, during any of the three conditions tested. While most genes common to CCR are present in the genome of *A. acidocaldarius*, CCR did not appear to be a global regulator of carbon metabolism as in other Gram-positive bacteria, such as the CCR prototype *B. subtilis* (99, 100). Regulation of genes related to CCR is not a prerequisite for catabolite repression since expression of these genes may be constitutive, but when analyzed with the physiological data from the experiments, CCR does not appear to be active. The primary physiological indicator that sugar metabolism in *A. acidocaldarius* is not regulated in a manner consistent with CCR is the fact that growth occurred when a second sugar expected to elicit CCR was added to the steady-state culture of *A. acidocaldarius*. Carbon metabolism in *A. acidocaldarius* appears to be most similar to *L brevis*: CCR components are present, but due to the prevalence of MFS-family and ABC-type transporters for sugar uptake, *L. brevis* can grow on two sugars simultaneously and CCR does not appear to be active (29, 101). This is important because CCR in most Gram-positive bacteria seems to be associated with PTS-type sugar transport systems. Transport of glucose, xylose and arabinose is facilitated by non-PTS-type transporters, while fructose may be transported by a PTS-type transporter. Even though this is the case, CCR did not appear to be active when glucose was added to the *A. acidocaldarius* culture growing at steady-state on fructose.

The protein sequence of one of the three genes that have been annotated as phosphotransferase, phosphocarrier protein HPr (Aaci_0224), has the catalytic and regulatory domains associated with HPr proteins from other Gram-positive bacteria (Figure 4). This gene is adjacent to Aaci_0225, which is annotated as a phosphoenolpyruvate-protein phosphotransferase, also associated with CCR in Gram-positive bacteria. , the catalytic histidine (His-15) and the regulatory serine (Ser-46) in the protein encoded by Aaci_0224 of *A. acidocaldarius* are shifted downstream two amino acids relative to their position in other Gram-positive bacterial HPr homologs. The His-15 is shown in the conserved HARPA region, while the regulatory Ser-46 is shown conserved KSI domain. While the effect of this shift on phosphorylation and activity of Aaci_0224 is not known, it may alter or even inhibit phosphorylation. This hypothesis is supported by the fact that point mutations in HPr proteins in *B. subtilis* have shown relaxed CCR (102, 103). If HPr is not phosphorylated on the regulatory serine (Ser-46), then proper interaction with CcpA may not occur, which would lead to less specific binding of *cres*, thereby inhibiting CCR.

Aaci_0224 is part of what appears to be a gene cassette related to metabolism of mannitol as well as containing a number of regulatory elements (Figure 5A). Aaci_0220 encodes a PTS system, mannitol-specific enzyme II (EIIC) subunit. The transcriptional anti-terminator, BglG, which is proposed as the mannitol operon activator, is encoded by Aaci_0221. Aaci_0222 encodes a sugar phosphotransferase that may be the enzyme II (EIIA) subunit for the mannitol system. Mannitol dehydrogenase is encoded by Aaci_0223. As stated previously, Aaci_0225 encodes

the phosphoenolpyruvate-protein phosphotransferase which encodes enzyme I (EI) of the phosphotransferase system. Figure 5B shows the gene cassette from *B. subtilis* strain 168 for which CCR from glucose is active. There are two regulators (YkvZ and GlcT), the glucose EIIABC subunit (PtsG), the HPr encoding gene (PtsH), and EI component (PtsI). If gene proximity and co-expression is important, then an alternate hypothesis for the lack of CCR in *A. acidocaldarius* may be that CCR is not triggered by glucose but may be triggered by mannitol, which was not tested. No in-depth analysis of similar phenomena in other bacteria has been performed for comparison.

Transporters

A variety of solute transporters were regulated in *A. acidocaldarius* during transition from growth on one monosaccharide to growth on two monosaccharides (Figure 6). While generally maintaining the pattern of dominant down-regulation of gene transcription, three genes or gene cassettes that encode apparent transporters were up-regulated: Aaci_2204, which is a sodium solute superfamily transporter (SSS); Aaci_2842 through Aaci_2844, which appear to encode an ABC-type transporter for sulfate; and Aaci_2879, which has been annotated as a general substrate transporter.

One of the more interesting results during transition from growth on xylose to growth on xylose/arabinose was the down-regulation (average of nearly 9-fold) of three genes (Aaci_0259, Aaci_0260 and Aaci_0261) which encode the different subunits of an ABC-transporter for D-xylose. This transporter has been designated a carbohydrate uptake transporter 2 (CUT2) family, which are specific for transport of

monosaccharides (104). The change in expression levels of these genes in the xylose/glucose and fructose/glucose experiments were below the cut-offs established for these experiments. These results suggest that these genes were down-regulated by the presence of arabinose, but not glucose, and may be constituently expressed when pentose sugars are present. These genes appear to be organized in an operon similar to *xylFGH* of many bacteria, including *Thermoanaerobacter ethanolicus* (105). Aaci_0259 has been annotated as the gene encoding a periplasmic xylose binding protein (XylF) that bears highest homology to other species of *Alicyclobacillus* (60 – 77%), and greater than 55% homology to XylF proteins of *Acidiphilium* and *Acidocella* species. The encoded ATP-binding protein (XylG;Aaci_0260) which, as would be expected, shows homology to XylG proteins in other Alicyclobacilli and to species of *Chloroflexi* and *Actinobacteria*. Aaci_0261, encoding the inner membrane translocator/permease (XylH), shows homology to XylH proteins in other *Alicyclobacillus* species, as well as, *Chloroflexi* and *Proteobacteria*.

Five of the transporters that were down-regulated during all three experiments have been annotated as major facilitator superfamily, MFS_1, transporters: Aaci_0335, Aaci_0443, Aaci_2515, Aaci_2622, and Aaci_2906. In all three conditions tested, a variety of transporter types responded to the second sugar added to the *A. acidocaldarius* culture growing on one sugar; however, there were differences when growing on xylose, regardless of inducing sugar added, and fructose/glucose. A gene encoding an MFS transporter (Aaci_0335) was down-regulated 4.8-fold with xylose/glucose, 5.4-fold with xylose/arabinose and 6.9-fold for fructose/glucose.

SEED and Pfam classifications of this transporter indicated it is most likely a sugar transporter for pentose sugars. Interestingly, besides the other *A. acidocaldarius* genomes in the NCBI database, this MFS transporter shows the most homology to MFS-type transporters found in nearly 40 crenarchaeotes, even showing less similarity to similar transporters in other *Alicyclobacillus* species. The phylogenetic tree shown in Figure 7 demonstrates the similarity of the MFS transporter encoded by Aaci_0335 in *A. acidocaldarius* to similar MFS transporters in a large number of Archaea.

A second gene annotated as an MFS transporter, Aaci_0443, was down-regulated during the xylose experiments and has been classified as a sugar transporter in Prosite (PS00216), but no specific metabolite was given. KEGG Orthology (KO:K08369) indicates that this transporter most likely functions through a proton symport mechanism. This gene locus was down-regulated ~7-fold in *A. acidocaldarius* when tested on xylose/glucose and xylose/arabinose, but not during the fructose/glucose experiment. While no metabolite has been specified for this transporter, these results indicate that it may be involved with xylose, or more generally, pentose transport. As was the case with Aaci_0335, this protein exhibits homology to a number of Archaeal MFs transporters, as well as Actinobacteria.

An ORF that has been annotated as a hypothetical protein (Aaci_2515) was down-regulated under all conditions tested. Highest levels of down-regulation when comparing the three test conditions were noted during growth on fructose/glucose (6.6-fold), compared to below 5-fold when grown on xylose/glucose or xylose/arabinose. Comparison to other proteins in the InterPro database indicates that this ORF may

encode an MFS Family transporter, of the general substrate type (IPR016196). When compared to other genes in the non-redundant protein database in NCBI, this gene shows the highest homology (95% identity) to ORF TC41_2812 from *A. acidocaldarius* Tc-4-1, which has been annotated as a D-galactonate transporter of the MFS type. The same classification has been given for AaLAA1DRAFT for *A. acidocaldarius* LAA1. Aaci_2515 also shows 76% identity to a gene that has been annotated as an MFS transporter in *A. hesperidum* URH17-3-68. This 148 amino acid protein also shows greater than 68% identity to MFS proteins in a number of *Geobacillus* species. While the specific function of this protein in *A. acidocaldarius* is not known, the gene is located downstream of Aaci_2516, which was annotated as a xylose isomerase domain protein TIM barrel. Conserved domain analysis of this enzyme shows that it may be involved in transport and metabolism of phosphate sugars. Aaci_2516 was also down-regulated, but only to a level of 3.3-fold. Aaci_2157, which was annotated as an alcohol dehydrogenase GroES domain protein, was also down-regulated 3.2-fold during the fructose/glucose experiment.

A fourth MFS transporter (Aaci_2622) was also differentially down-regulated when *A. acidocaldarius* was grown on xylose/glucose or xylose/arabinose, compared to fructose/glucose. This gene was down-regulated 3.2-fold on xylose/glucose, 4.6-fold on xylose/arabinose, but did not change for the fructose/glucose experiment. While no specific substrate has been given for this transporter, the protein sequence shows greater than 40% similarity to MFS transporters in a number of *Burkholderia* and *Pseudomonas* species. Some have been annotated as sugar phosphate permeases.

Comparison of Aaci_2622 with other COGs suggests that this 421 amino acid protein is a sugar phosphate permease (COG2271). Comparison of the protein encoded by this gene with similar proteins, along with data from the microarray experiments, indicates that this protein may be involved in xylose transport, or pentose transport, in general.

Aaci_2906 was down-regulated 4-fold when tested with fructose/glucose, but less than 3-fold when tested with xylose/glucose, and has been annotated as an MFS transporter. As with other MFS transporters discussed, Aaci_2906 is a general substrate transporter for which no substrate was given. This gene is in the same gene neighborhood as genes encoding two other transporters: an ABC transporter (Aaci_2902 through Aaci_2904), which was also down-regulated and will be discussed below, and Aaci_2900, which has been annotated as a sugar transporter, but was not differentially expressed. Interestingly, Aaci_2906 is transcribed in the opposite direction from Aaci_2902 to Aaci_2905. While not all genes in this particular gene neighborhood were regulated, many genes appear important to processing of pentose monosaccharides, or pentose-containing oligosaccharides. In addition to the aforementioned transporters, Aaci-2899 encodes an L-ribulokinase, Aaci_2897 encodes an L-arabinose isomerase, and Aaci_2894 encodes the glycoside hydrolase, α-N-arabinofuranosidase.

Finally, gene locus Aaci_2879 has been annotated as a general substrate transporter, which is most likely an MFS transporter. This ORF also represents one of only a few genes demonstrating up-regulation during all three experimental conditions.

Transcription of this gene was up-regulated during growth on xylose/glucose, xylose/arabinose and fructose/glucose, at levels of 2.5-, 2.7- and 3.8-fold, respectively. The predicted 465 amino acid protein encoded by this gene shows greater than 55% identity with MFS transporters found in a number of *Sulfobacillus acidophilus*. Conserved domain analysis indicates that this transporter is best represented by the model TIGR00887, which is for a proton symporter for phosphate. Using COG Functional Category classification, this protein has been annotated as a sugar phosphate permease.

Four down-regulated gene loci, Aaci_0403, Aaci_2510, Aaci_2889, and Aaci_2474, have been annotated as Family 5 extracellular solute binding proteins. Aaci_2474 has been annotated as the OppA component of an oligopeptide transporter and will be discussed in more detail below. Domain analysis of these proteins demonstrates the possibility of binding for multiple solutes, including nickel, peptide and carbohydrate transport, as part of the ABC transport process. KEGG Orthology (KO:K02035) and InterPro (IPR000914) indicate these genes encode a protein that may be the periplasmic substrate binding protein for peptide or nickel ABC transporter. Conserved domain analysis indicates that these substrate binding proteins from *A. acidocaldarius* contain domains similar to the cellulose-binding domain of a protein from the hyperthermophile *Thermotoga maritima*. This periplasmic-binding fold protein superfamily not only functions in transport of peptides, but also metabolites such as amino acids and carbohydrates.

Aaci_0403 encodes a 565 amino acid protein that has been annotated as a Family 5 extracellular solute-binding protein, and was downregulated following growth on xylose when the culture was spiked with glucose or arabinose, but not when the culture growing on fructose was spiked with glucose. Down-regulation was 3.3-fold upon addition of the second sugar during the xylose/glucose experiment, and 11.5-fold when tested with xylose/arabinose. These results indicate that the signal for regulation of this solute-binding protein may be controlled by arabinose, or pentose sugars in general. Fructose and glucose, both hexoses, appeared to have less of an effect on gene transcription. Conserved domain analysis shows that model cd08509 for PBP2_TmCBP_oligosaccharides_like binding proteins best fits the protein encoded by this gene. The fact that this gene was differentially regulated with different sugars may be an indication that it is involved in carbohydrate transport. Sugars such as arabinose were found to be transported by dipeptide transporters in *Sulfolobus solfataricus* (78), further supporting this hypothesis.

A second gene (Aaci_2510) encoding a Family 5 extracellular solute-binding protein was down-regulated during all three conditions tested. The gene was down-regulated nearly 7-fold when *A. acidocaldarius* was spiked with glucose after growing at steady-state on either xylose or fructose, but only 4.4-fold when growing on xylose/arabinose. KEGG Orthology (KO2035) indicates that this protein may be the extracellular solute binding protein associated with an ABC transporter involved in peptide or nickel transport. Comparison to conserved domains for oligopeptide

transporters indicates that Aaci_2510 may function in a manner similar to OppA (Aaci_2474) which will be discussed in more detail below.

The final Family 5 extracellular solute-binding protein-encoding gene that will be discussed as a stand-alone gene is Aaci_2889. This gene was down-regulated 4.6-fold during the xylose/glucose and 6.3-fold during the xylose/arabinose experiment, but not during the fructose/glucose experiment. This indicates that this transporter was being transcribed when growing at steady-state on xylose and when the second sugar was added, transcription was significantly down-regulated, regardless of whether the second sugar was a pentose or hexose. As with Aaci_0403 and Aaci_2510, KEGG Orthology (KO2035) indicates this protein is most likely a peptide/nickel transport system substrate-binding protein. Gene orthology, along with the fact that these genes were strongly down-regulated when the second sugar was added, indicate the possible function in monosaccharide transport.

Another set of genes (Aaci_2471, Aaci_2472, Aaci_2473 and Aaci_2474) that was down-regulated > 5-fold when *A. acidocaldarius* was grown on xylose and then spiked with either glucose or arabinose has been annotated as the OppA, OppB, OppC and OppD components of an ATP-binding cassette (ABC) oligopeptide transporter. This set of genes was not regulated when grown on fructose/glucose. The OppF component (Aaci_2470), which is part of this operon, was also down-regulated to a level of 2.5-fold with a *p*-value of 0.02, when tested with xylose/glucose. As discussed above, three other genes (Aaci_0403, Aaci_2510 and Aaci_2889) that have also been annotated as Family 5, extracellular solute binding proteins for peptides or

nickel were down-regulated during testing. Since Aaci_0403 and Aaci_2889 were down-regulated under similar conditions, they may be involved in a network with Aaci_2471 through Aaci_2474. While *opp* genes are typically arranged in operons, as is the case with *A. acidocaldarius*, additional OppA components are often present in other locations of the genome and may be co-regulated with the genes in the operons (106, 107). *A. acidocaldarius* appears to have at least five genes dispersed across the genome that encode proteins that carry out similar functions to OppA. The increased number of these solute binding proteins is thought to increase the efficiency of oligopeptide binding (106). Another *opp* operon present in the *A. acidocaldarius* genome (Aaci_2258-Aaci_2263) did not appear to be regulated during any of the three experiments. In general, oligopeptide transporters play a central role in internalization of peptides for nutrition and recycling of cell wall peptides. If transport of peptides was the sole function of the transporter encoded by this group of genes, then regulation during growth on fructose/glucose would have been expected.

While the function of the oligopeptide transporters in *A. acidocaldarius* is not known at this time, additional insight can be obtained by Opp/Dpp transporters found in *Thermotoga maritima* (108, 109). Transcription of components for two distinct Opp/Dpp family transporters was high in the presence of xylose and xylan polysaccharides. The primary difference between *T. maritima* and *A. acidocaldarius* is that the Opp/Dpp transporter in *T. maritima* is located in an apparent xylan utilization cluster with a Family 10 xylanase transversely transcribed from the transporter, while in *A. acidocaldarius* it is not (Figure 8A). Comparing gene ortholog

neighborhoods based on COG functional categories, the *A. acidocaldarius* group of genes is more similar to Opp/Dpp in *Thermotoga* that is adjacent to two endoglucanase enzymes (Figure 8B). The OppA-family binding protein (MtpA) was more highly regulated when *T. maritima* was grown on xylose, but other transporter components were more regulated when grown on mannans. Figure 8C shows the primary difference between these clusters of genes when comparing *T. maritima* to *A. acidocaldarius;* a glycoside hydrolase gene (Aaci_2475) is located adjacent to the transporter genes, but is also oriented in the same direction as the transporter genes.

Aaci_1215 encodes a 446 amino acid protein, also characterized as a Family 1 extracellular solute-binding protein (SBP), which differ from the Family 5 SBPs discussed above. KEGG Orthology (KO:K10188) and comparison with other COGs (COG1653) for bacterial proteins indicate this protein is most likely the periplasmic component of a sugar transporter. The Integrated Microbial Genomes (IMG) tool within the Joint Genome webpage has given an IMG term annotation of a carbohydrate ABC transporter substrate-binding protein, of the carbohydrate uptake transporter-1 (CUT1) family (TC 3.A.1.1.-). This protein was most down-regulated when tested with fructose/glucose (4.8-fold) and 3.5-fold when tested with xylose/glucose, but not with xylose/arabinose. This Family 1 SBP appeared to be co-regulated with other components of the ABC transporter (Aaci_1216, an inner membrane transport component; and Aaci_1217, also an inner membrane transport component). These two genes were down-regulated within the statistical cut-off for the experiments but were below the fold change cut-offs established for the experiment. As was indicated when

discussing global transcription analysis, most genes were up-regulated to an equal extent when *A. acidocaldarius* transitioned from growing at non-steady-state on two sugars to steady-state on both monosaccharides. This, however, was not the case during testing with the fructose/glucose sugar pair. Up-regulation was 6.4-fold for Aaci_1215, 5.8-fold for Aaci_1216 and 4.8-fold for Aaci_1217, indicating that increased transcription was occurring when both hexose monosaccharides were present. These results indicate that these genes were down-regulated by the presence of glucose, or hexoses in general, since the response was stronger with fructose and glucose.

Gene loci Aaci_1853 and Aaci_1253, both encoding hypothetical proteins mapped to integral membrane proteins of the GPR1/FUN34/*yaaH* family, were also down-regulated. Aaci_1253 demonstrated higher levels of down-regulation than Aaci_1853. Aaci_1253 was most down-regulated during the fructose/glucose experiment, while Aaci_1853 was least down-regulated when *A. acidocaldarius* was grown on fructose/glucose. Description of the gene from InterPro (IPR000791) classifies GPR1/FUN34/*yaaH* as a hydrophobic protein with six transmembrane regions, which indicates involvement in transport. Various studies in fungi and bacteria have implicated GPR1/FUN34/*yaaH* in the transport of volatile fatty acids, such as acetate, from inside the cell to the external medium (110, 111). In *Bacillus subtilis*, *yaaH* has been shown to encode genes related to sporulation (112). Analysis of Aaci_1853 in the NCBI database shows that this gene demonstrates the highest level of homology to genes in organic acid producing and metabolizing bacteria such

as *Acetobacter*, *Thermoacetogenium*, *Gluconobacter* and others. In all instances, the gene has been annotated as an inner membrane transporter for acetate. Within the JGI database, Aaci_1253 has also been annotated as an inner membrane transporter, specifically in the YaaH Family, transporters involved in organic acid transport. The gene shows high homology (>87%) to other *Alicyclobacillus* genes in the NCBI database, and is closely related to GPR1/FUN34/*yaaH* inner membrane transporters in Archaea (*Sulfolobus*) and β-Proteobacteria (*Burkholderia*). Acetyl-coenzyme A synthetase (Aaci_1254) is the upstream nearest neighbor of Aaci_1253. This strengthens the argument that Aaci_1253 may be involved in acetate transport, since Acetyl-coenzyme A synthetase catalyzes acetogenesis from pyruvate, and while not down-regulated to the level of Aaci_1253, Aaci_1254 was down-regulated 6.2-fold at the same sampling point. In addition, when growing at high rates on monosaccharides such as glucose and xylose, *A. acidocaldarius* has been shown to produce organic acids such as acetate, lactate and propionate (data not shown). This type of carbon overflow is common in other *Firmicutes* (113, 114).

Aaci_2204, which has been annotated as a solute:sodium symporter (SSS) family transporter (TC:2.A.21), was up-regulated 9.1-fold when *A. acidocaldarius* was spiked with arabinose following steady-state growth on xylose. During the experiments when glucose was added as the second monosaccharide after xylose or fructose, up-regulation was just below 4-fold. SSS family transporters typically function to transport Na^+ with sugars, amino acids, inorganic ions or vitamins. Transport by this transporter must not be specific, since up-regulation was seen

whether glucose or arabinose was added as the second monosaccharide. The elevated transcription when arabinose was added indicates that this transporter may be more responsive to pentose sugars, specifically arabinose. High concentrations of Na^+ are not present in the growth medium, but were added when NaOH was pumped into the chemostat to help control pH, so Na^+ would be available for co-transport. For all three conditions tested, Aaci_2203, which encodes a hypothetical protein with no known function, was expressed at similar levels to Aaci_2204, indicating possible co-transcription.

A permease involved in the transport of L-lactate (Aaci_2529) was down-regulated 4.9-fold during transition from steady-state on xylose to non-steady-state growth on xylose and glucose but only 3.8-fold with xylose/arabinose and 3.7-fold with fructose/glucose. While metabolites were not measured during this experiment, previous studies (data not shown) indicate the production of organic acids, including lactate, by *A. acidocaldarius* when grown on xylose and glucose. The presence of lactate in the growth medium would support the presence and activity of transporters for L-lactate. In addition, the *A. acidocaldarius* genome contains genes that suggest three mechanisms for lactate formation. In one pathway, L-lactate dehydrogenase (Aaci_0520) catalyzes the reduction of pyruvate to lactate. In a second mechanism, lactate 2-monooxygenase (Aaci_0905) catalyzes formation of L-lactate from acetyl-CoA through acetate. Finally, *A. acidocaldarius* appears to use the methyglyoxal pathway as a mechanism for carbon overflow; this pathway also yields lactate. While Aaci_0520 was not regulated within the confidence intervals established for this

experiment, lactate 2-monooxygenase (Aaci_0905) was down-regulated 2-fold with a *p*-value of 0.03. Lactate 2-monooxygenase is also in the same genome neighborhood as Aaci_0902 and Aaci_0904, which encode the alpha-subunit of the pyruvate dehydrogenase E1 component, and a long-chain-fatty-acid-CoA ligase, respectively. These two genes were also down-regulated between 3- and 5-fold, respectively. This is important because these genes encode proteins from the same KEGG pathway as Aaci_0905, and are involved in pyruvate metabolism.

Another apparent operon that appears to encode the components of an ABC-type transporter for sugars was also down-regulated. The operon consists of a GntR Family transcriptional regulator (Aaci_2901), a periplasmic binding protein/LacI transcriptional regulator (Aaci_2902), an inner membrane translocator (Aaci_2903), and an ABC transporter related ATP-binding protein (Aaci_2904). Regulation was only seen when glucose was added as the second monosaccharide. Average down-regulation of all genes in this apparent operon were 2.4-fold for xylose/glucose and 4.6 fold during the fructose/glucose experiment, indicating the gene was being transcribed at a higher level when *A. acidocaldarius* was growing at steady-state on fructose. The periplasmic binding protein gene (Aaci_2902), that was down-regulated as part of this group of genes, encodes a protein that has been classified as a carbohydrate uptake transporter 2 (CUT2). CUT2 transporters are binding-protein-dependent transporters that only transport monosaccharides (104). A gene structure for ABC-type sugar transporters can be found in various anaerobes, most notably, numerous species of *Thermoanaerobacter* and *Caldicellulosiruptor* species.

Genes encoding what appears to be an ABC-transporter for sulfate (Aaci_2842 through Aaci_2844) were also up-regulated, but only during the xylose/glucose and xylose/arabinose experiments. Aaci_2842 and Aaci_2843 encode the inner membrane permease component, which is similar to the protein CysW. Aaci_2844 encodes the sulfate transporting ATPase, which is similar to CysA. Interestingly, Aaci_2841, which encodes the periplasmic sulfate-binding protein, was not up-regulated

Cell Wall, Fatty Acid and Amino Acid Metabolism

Genes related to metabolism of cellular constituents that were regulated during the three experiments are compared in Table 2. Genes within this category represent the largest set regulated during the testing. Many are present in apparent operons, while others are individual loci spread around the genome.

One apparent operon, Aaci_0135 through Aaci_0139, was down-regulated; on average, genes Aaci_0135, Aaci_0136 and Aaci_0137 were down-regulated the most when glucose was added as the second sugar, with down-regulation the highest (nearly 9-fold) with fructose/glucose. Aaci_0138 was not regulated during the xylose/arabinose and fructose/glucose experiments. Three of the five genes (Aaci_0135, Aaci_0137, and Aaci_0138) have been annotated as encoding hypothetical proteins of unknown function. Aaci_0136 was down-regulated 6.2-fold, 4.2-fold and 11-fold, when tested with xylose/glucose, xylose/arabinose and fructose/glucose, respectively. Aaci_0136 was annotated as a PE-PGRS family protein, because of the Pro-Glu (PE) in the N-terminal domain followed by a Gly-Ala-rich sequence (PE-PGRS). These proteins have been studied the most in

Mycobacteria, where the PE domain is thought to be responsible for translocation and localization in the cell wall (115). Some PE-PGRS family genes have also been classified as lipases/esterases that are up-regulated during starvation as the cell utilizes stored triacylglycerol (116). The C-terminal domain of this protein contains a Ser-rich sequence (…ASSSSSSSSGSSTSNGT) of unknown function, but may also be involved in protein translocation. This group of genes also contains Aaci_0139, the GAF- modulated transcriptional regulator discussed above.

Genes encoding proteins related to phenylacetate metabolism, Aaci_0339 (4-hydroxyphenylacetate 3-monooxygenase, oxygenase subunit), Aaci_0340 (3,4-dihydroxyphenylacetate 2,3-dioxygenase) and Aaci_0341 (2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase), were also down-regulated. In general, Aaci_0340 and Aaci_0341 were more down-regulated (~6-fold) during the fructose/glucose experiment than in the experiments with xylose. Levels of transcriptional regulation of Aaci_0339 were similar for all three conditions considered. These genes may be co-regulated with the genes discussed in the previous paragraph and may be involved with further metabolism of phenylacetate for ω-cyclic fatty acid production. Other genes for phenylacetate metabolism, Aaci_0810 (Phenylacetate-CoA oxygenase) and Aaci_0811 (Phenylacetate--CoA ligase), were also down-regulated. Although regulation of transcription occurred under all conditions tested, transcription of both genes appeared to be more down-regulated when *A. acidocaldarius* was tested on fructose/glucose. Genes encoding three other subunits for phenylacetate-CoA

oxygenase (Aaci_0807 through Aaci_0809) were not regulated within the bounds of this experiment.

A gene that has been annotated to encode a hypothetical protein (Aaci_0427) was down-regulated to similar levels under all three conditions tested. Comparison of this protein with other proteins within the NCBI Conserved Domain database indicates that this protein may be a medium chain dehydrogenases/reductase (MDR)/zinc-dependent alcohol dehydrogenase-like family protein. The protein contains an putative NAD(P) binding site, as well as structural and catalytic zinc sites.

Two genes that encode proteins that may be involved in fatty acid synthesis were down-regulated when *A. acidocaldarius* was grown on xylose and spiked with either glucose or arabinose. Aaci_0441, which is annotated as an iron-containing alcohol dehydrogenase, was down-regulated 3.9-fold, while Aaci_0442, a methylmalonate-semialdehyde dehydrogenase, was down-regulated nearly 6-fold. While the specific function of these two enzymes is not known, they are located in the same genome neighborhood as an MFS sugar transporter (Aaci_0443) and the bEBP (Aaci_0444), which were both down-regulated during all three experiments.

Aaci_1055 is annotated as an Alpha/beta hydrolase fold-3 domain protein and was down-regulated less than 3-fold when shifting from steady-state growth on xylose to non-steady-state growth on xylose and glucose, and nearly 4-fold when tested at the same transition with fructose and glucose. It was likely down-regulated less than 2-fold when arabinose was added to the xylose culture, but the p-value was also above the stated cut-off. Comparing with other COGs, the protein encoded by this gene

contains domains that indicate it is likely a lipase/esterase, involved in cell growth and metabolism.

A number of ORFs which appear to be included in operons related to fatty acid metabolism were also down-regulated when the *A. acidocaldarius* culture was spiked with glucose after growing at steady state on xylose. This gene group consisted of: acyl-CoA dehydrogenase (Aaci_1452), 3-hydroxyisobutyrate dehydrogenase (Aaci_1453), malonate/methylmalonate-semialdehyde dehydrogenase (acylating) (Aaci_1454), enoyl-CoA hydratase/isomerase (Aaci_1455), acyl-coenzyme A synthetases/AMP-(fatty) acid ligases (Aaci_1456), acetyl-CoA acetyltransferase (Aaci_1457), and a CDP-diacylglycerol/glycerol-3-phosphate 3-phosphatidyltransferase gene (Aaci_1470), farther downstream. Down-regulated genes for other enzymes related to fatty acid metabolism from a different genome locus were: the A and B subunits of 3-oxoacid CoA-transferase (Aaci_2060/Aaci_2059), acetyl-CoA acetyltransferase (Aaci_2058), Acetyl-CoA synthetase (Aaci_2057) and a gene farther downstream for an AMP-dependent synthetase/ligase (Aaci_2035). While most genes within this operon were highly down-regulated, the most significant results are those obtained when *A. acidocaldarius* was growing on fructose at steady-state and then spiked with glucose. Two genes were down-regulated greater than 20-fold, two above 10-fold, two above 5-fold and the last gene in the group near 4-fold. Differences in expression of the different genes in each group indicate that there may be secondary promoters for some genes in the operon allowing differential expression of individual genes. These results indicate,

once again, that when the second monosaccharide was added to the *A. acidocaldarius* culture, genes related to synthesis of lipids that are most likely used for cell wall assembly were down-regulated.

Another significant result from this experiment was the level of up-regulation of transcription of these same genes, when the *A. acidocaldarius* culture transitioned from non-steady-state on fructose and glucose to steady-state on fructose and glucose. As previously mentioned, most genes were up-regulated to the same level as was seen for down-regulation during the shift from steady-state on one monosaccharide to non-steady-state on two monosaccharides. The first two genes, Aaci_1452 and Aaci_1453, were up-regulated greater than 30-fold, Aaci_1453 nearly 40-fold. Aaci_1454 and Aaci_1455 were up-regulated greater than 20-fold, and Aaci_1456 and Aaci_1457 were up-regulated at greater than 10-fold. Aaci_1458 was also up-regulated, but less than 5-fold.

A gene that has been annotated as an aldehyde dehydrogenase (Aaci_2504) was down-regulated greater than 9-fold when glucose was spiked into the *A. acidocaldarius* culture growing at steady-state on xylose or fructose. Transcriptional down-regulation of this gene was 5-fold during the xylose/arabinose experiment. The KEGG Orthology listed in the JGI attribute page for this gene has given the protein the function of a phenylacetaldehyde dehydrogenase. This enzyme represents the final step in phenylalanine catabolism to phenylacetic acid. A major component of the cell wall of *A. acidocaldarius* is the ω-cyclohexyl fatty acid, ω-cyclohexylundecanoic acid (117). Shikimic acid, acetic acid and phenylalanine feeding experiments performed in

1972 indicated that ^{14}C from shikimate and acetate accounted for most of the labeled carbon in methyl esters from the cells lipids, while only a small proportion came from phenylalanine (118). A more detailed analysis of ω-cyclic fatty acid biosynthesis in *A. cycloheptanicus* may provide additional insight into ω-cyclohexyl fatty acid formation in *A. acidocaldarius* (119). Feeding with ^{13}C- and deuterium-labeled metabolites indicated that the cyclohexyl ring comes from shikimic acid, while phenylacetic acid and acetate are used for chain elongation from a carboxylated cylohexane. While similar detailed experiments have not been performed using *A. acidocaldarius*, the phenylacyl ω-cylohexylundecanoate isolated from *A. acidocaldarius* was identical to the ω-cyclic fatty acid formed by *A. cycloheptanicus* (119). While experiments were not performed with labeled xylose, experiments by Moore *et al*. (1997) with uniformly labeled ^{13}C-glucose showed that all of the carbon in the ω-cyclohexyl fatty acid came from glucose. These studies and regulation of the annotated acetaldehyde dehydrogenase (Aaci_2504) may indicate the involvement of the enzyme product in cell wall formation by *A. acidocaldarius*.

Central Metabolism and Cellular Respiration

Two genes involved in central metabolism, Aaci_1608 (pyruvate, phosphate dikinase) and Aaci_1609 (fructose-1,6-bisphosphatase), were down-regulated 5.1- and 7.5-fold, respectively, when *A acidocaldarius* was tested with fructose/glucose. These two genes are transcribed in opposite directions from the same genetic locus. These were the only glycolytic genes regulated in *A. acidocaldarius* within the established criteria, under any of the sugar pairs tested. Down-regulation of fructose-1,6-

bisphosphatase makes sense because following phosphorylation of fructose during transport through the PTS transporter, this would be the next committed step in fructose metabolism.

One of the more interesting findings was that during growth on fructose, *A. acidocaldarius* appears to use a different pyruvate dehydrogenase complex than when grown on xylose. When transitioning from steady-state growth on fructose to non-steady-state growth on fructose and glucose, gene loci Aaci_0825 (pyruvate dehydrogenase E1 component alpha subunit), Aaci_0826 (pyruvate dehydrogenase E1 component beta subunit), and Aaci_0827 (catalytic domain of components of various dehydrogenase complexes) were down-regulated 4.7-, 4.5- and 3.2-fold, respectively. These results indicate that these genes may be transcribed as an operon. Aaci_0827 is most likely the pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase subunit. In contrast, during transition from steady-state growth on xylose to non-steady-state growth with xylose and either glucose or arabinose, Aaci_0902 (alpha subunit) and Aaci_0903 (beta subunit) were down-regulated. There was no dihydrolipoamide acetyltransferase subunit gene associated with this gene locus. *A. acidocaldarius* possess a third locus with genes for pyruvate dehydrogenase-- Aaci_0454 through Aaci_0457-- which encodes all three components of the pyruvate dehydrogenase complex; however this gene locus was not regulated within the bounds of the three experiments. Genes from this genome locus may be constitutively expressed, thereby, not regulated during the experiment.

Proteins related to cellular respiration were also down-regulated when *A. acidocaldarius* transitioned from steady-state growth on fructose to non-steady-state growth on fructose and glucose. Five genes that encode the four polypeptides of cytochrome C oxidase (Aaci_0511-Aaci_0514) and a gene annotated as encoding a hypothetical protein involved in cytochrome oxidase assembly (Aaci_0510) were down-regulated at different levels. Aaci_0510 is transcribed in the opposite direction from the other four genes, which appear to be arranged in an operon in a manner similar to other *Firmicutes*. Aaci_0504 has been annotated as an HesB/YadR/YfhF-family protein which was down-regulated only after steady-state growth on fructose. Domain analysis through Pfam (pfam01521) indicates that it may be a protein involved in iron-sulfur cluster biosynthesis. Iron-sulfur cluster proteins are prevalent in the electron transport chains of most organisms.

Conclusions

A. acidocaldarius ATCC 27009 is a Gram-positive, aerobic, thermoacidophile capable of using a wide range of mono-, di-, oligo- and polysaccharides. While the genome sequence of *A. acidocaldarius* contains genes encoding all components of CCR typical for Gram-positive bacteria, no in-depth analysis of CCR has been performed for this bacterium. Microarray, monosaccharide utilization and growth data from continuous-flow chemostat studies, in which *A. acidocaldarius* was grown to steady-state on an individual monosaccharide and then a second monosaccharide was added to elicit CCR, indicated that *A. acidocaldarius* was able to use xylose/glucose, xylose/arabinose and fructose/glucose simultaneously and suggested that CCR was not

the primary regulator of carbon metabolism during the conditions tested. Xylose, arabinose and glucose at concentrations of 2 g/L were completely metabolized, while fructose was only partially metabolized. In each experiment, the addition of the second sugar yielded twice as much carbon, which resulted in nearly doubling of the cell density of the culture as determined by optical density.

A common theme between all three experiments was that many of the same genes were regulated regardless of the sugar being use for growth, or the sugar added. Interestingly, with the exception of two transporters, most genes were down-regulated during transition from steady-state growth on the first monosaccharides to non-steady state with two monosaccharides. During transition from non-steady-state growth on two sugars to steady-state growth on two sugars, the same genes were up-regulated to nearly the same extent; however there were a few cases where up-regulation was several fold higher than the original down-regulation. Gene categories that were down-regulated included transcriptional regulators (mostly transcriptional activators), a variety of transporters, and enzymes related to metabolism of cellular components, such as cell walls lipids and amino acids, as well as carbon overflow. Some central metabolism genes were also down-regulated during growth on fructose and glucose, including genes for respiration. In general, these results indicate that through a yet-to-be-determined signal transduction mechanism, transport, growth and carbon overflow were temporarily halted when the inducing sugar was added, and then the transcriptional state of the cell returned to a similar level when growing on both sugars.

A. acidocaldarius, unlike many Gram-positive bacteria, is able to simultaneously use pentose and hexose sugars, indicating that this bacterium would be useful for breaking down plant biomass which contains both types of sugars. Parallel utilization of sugars appears to be a function of the types of transporters encoded in the genome, and a possibly defective HPr component of the CCR system, which may inhibit binding of CcpA to promoter regions which is the key step in CCR.

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Table 1. Microorganisms capable of simultaneous metabolism of pentose and hexose sugars found in lignocellulosic biomass.

Table 2. Microarray results for transcriptional regulation of *A. acidocaldarius* annotated genes for cell wall, amino acid and fatty acid synthesis, during continuous flow chemostat studies, while growing on xylose/glucose, xylose/arabinose, or fructose/glucose.

Figure 1. Graphs showing sugar use and growth (OD_{600}) for *A. acidocaldarius* growing in continuous flow chemostats during studies to determine the effect of different monosaccharides on gene transcription. (A) Growth on xylose and glucose, (B) Growth on xylose and arabinose, and (C) Growth on fructose and glucose. Error bars represent 95% confidence intervals

Figure 2. Comparison of functional gene categories regulated during growth of *A. acidocaldarius* on mixtures of xylose/glucose, xylose/arabinose or fructose/glucose.

Figure 3. Transcriptional regulators affected by addition of a second sugar to a chemostat culture of *A. acidocaldarius* growing at steady-state on either xylose or fructose.

Figure 4. Alignment of the *A. acidocaldarius* DSM 446 phosphocarrier protein HPr encoded by Aaci_0224 with HPr from other Gram-positive bacteria. AacDSM446 = *A. acidocaldarius* strain DSM446, AacTc-4-1 = *A. acidocaldarius* strain Tc-4-1, AheU17368 = *A. hesperidum* strain URH17-3-68, TabtenMB4 = *Thermoanaerobacter tengcongensis* strain MB4, TabMS = consensus for numerous *Thermoanaerobacter*

species, PdendC454 = *Paenibacillus dendritiformis* strain C454, PaenMS = consensus for numerous *Paenibacillus* species, Pdarw = *Paenibacillus darwiniensis*, Bacillales = consensus for numerous *Bacillales* species, Bsubt168 = *B. subtilis* strain 168.

Figure 5. Schematic of gene strings from genome loci containing phosphocarrier protein HPr from *A. acidocaldarius* strain DSM 446 (A) and *B. subtilis* strain 168 (B).

Figure 6. Regulation of genes encoding transporters catalyzed by addition of a second sugar to a chemostat culture of *A. acidocaldarius* growing at steady-state on either xylose or fructose.

Figure 7. Phylogenetic tree showing similarity of MFS transporter encoded by Aaci_0335 to MFS transporters in Archaea.

Figure 8. Schematic of genome loci encoding ABC-type peptide transporters (Opp) in *T. maritima* strain MSB8 (A and B) and *A. acidocaldarius* strain DSM 446 (C) that are highly regulated during growth on saccharides. (A) shows Opp locus form *T. maritima* that is adjacent to genes related to xylose and xylan metabolism, while (B) shows an Opp locus for mannose metabolism. (C) shows an Opp locus in *A. acidocaldarius* in proximity to a glycoside hydrolase of unknown function.

Table 1.

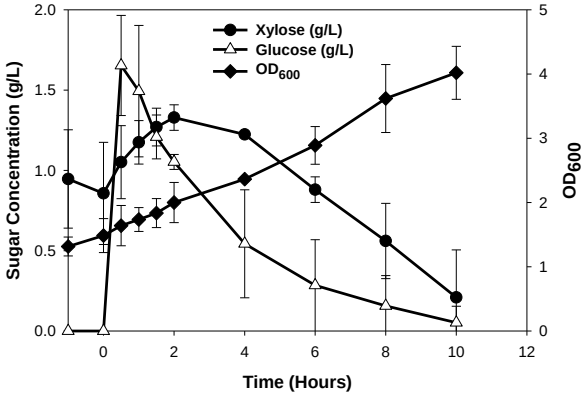
Microorganisms	Saccharides Metabolized	Transporter	Reference
Lactic acid bacteria			
Lactobacillus buchneri	xylose/glucose, corn stover hydrolysate, wheat straw hydrolysate	ND	Liu et al. 2008, 2009
Lactobacillus brevis strains NRRL 1834 and NRRL1836	xylose/glucose, rice straw hydrolysate,	MFS (H^+ symporters)	Kim et al. 2010a, 2010b
Lactobacillus brevis strain S3F4	corn cob hydrolysate, corn stover hydrolysate	ND	Guo et al. 2010
Lactobacillus plantrum Strain XS1T3-4	corn cob hydrolysate, corn stover hydrolysate	ND	Guo et al. 2010
Ruminal bacteria			
Butyrivibrio fibrosolvens strains ATCC 19171, 86	glucose/xylose	ND	Marounek and Kopecny 1994
Clostridium thermohydrosulfuricum strain Rt8.B1	glucose/xylose	Xylose permease, high and low affinity glucose transporters	Cook et al. 1993
Clostridium beijerinckii strain SA-1	xylose/glucose	ND	Heluane et al. 2011
Clostridium beijerinckii strain P260	Wheat straw, barley straw, corn stover and switch grass hydrolysates	ND	Qureshi et al. 2008a, 2008b, 2010a, 2010b
Thermophilic bacteria			
Thermoanaerobacter strain X514	xylose/glucose	Glucose – PTS transporter, xylose – ABC transporter	Feng et al. 2009, Hemme et al. 2011, Lin et al. 2011
Thermoanaerobacter strain BG1L1	Wheat straw and corn stover hydrolysate	ND	Gerogieva and Ahring 2007, Georgieva et al. 2008
Caldicellulosiruptor saccharolyticus strain DSM 8903	*Miscanthus* hydrolysate, wheat straw, sweet sorghum, maize, sugarcane bagasse	ABC transporters	deVrije et al. 2009, Willquist et al. 2010, Ivanova et al. 2009
Thermotoga neapolitana strain DSM 4359	glucose/xylose/arabinose, *Miscanthus* hydrolysate, wheat straw, rice straw	ND*	Eriksen et al. 2011, Nguyen et al. 2010
Archaea			
Sulfolobus acidocaldarius	xylose/glucose, glucose/arabinose/galactose	ABC transporter	Joshua et al. 2011, Elferink et al. 2001, Chen et al. 2005
Eukaryotes			
Trichosporon cutaneum	glucose/xylose, corn stover hydrolysate	Facilitated diffusion or H^+ symport	Hu et al. 2011
Candida shehatae	glucose/xylose	ND	Kastner and Roberts 1990, Kastner et al. 1998

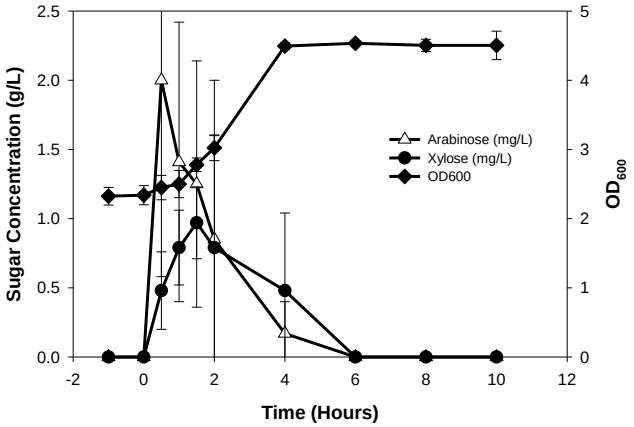
ND – Not Determined, ND* - Not determined but likely ABC transporter

Table 2.

Gene Locus Tag	Product Name	Xylose/ Glucose	Xylose/ Arabinose	Fructose/ Glucose
Aaci_0135	Hypothetical protein	5.4 down	4.2 down	10.4 down (p = 0.16)
Aaci_0136	PE-PGRS family protein	6.2 down	4.2 down	11 down (p = 0.13)
Aaci_0137	Hypothetical protein	4.6 down	3.1 down	4.9 down (p = 0.23)
Aaci_0150	ABC-1 domain protein	BC	BC	6.3 down
Aaci_0151	Hypothetical protein	BC	BC	5.2 down
Aaci_0152	Aldehyde dehydrogenase	BC	BC	4.3 down
Aaci_0153	Iron-containing alcohol dehydrogenase	BC	BC	4.2 down
Aaci_0159	AMP-dependent synthetase and ligase	4.9 down	3.6 down	5.2 down
Aaci_0160	MaoC domain-containing protein dehydratase	4.2 down	3.3 down	4.6 down
Aaci_0161	MaoC domain-containing protein dehydratase	3.3 down	BC	3.5 down
Aaci_0339	4-hydroxyphenylacetate 3-monooxygenase, oxygenase subunit	2.8 down (p = 0.02)	3.1 down	3.3 down
Aaci_0340	3,4-dihydroxyphenylacetate 2,3-dioxygenase	3.8 down	4.2 down	6.0 down
Aaci_0341	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase	4.2 down	3.6 down	6.4 down
Aaci_0427	Hypothetical protein	4.8 down	4.0 down	5.3 down
Aaci_0441	Iron-containing alcohol dehydrogenase	3.9 down	3.9 down	BC
Aaci_0442	Methylmalonate-semialdehyde dehydrogenase	5.8 down	5.8 down	BC
Aaci_0810	Phenylacetate-CoA oxygenase, PaaJ subunit	2.3 down (p = 0.02)	3.3 down	4.2 down
Aaci_0811	Phenylacetate--CoA ligase	3.3 down	5.7 down	10.6 down
Aaci_0904	AMP-dependent synthetase and ligase	4.6 down	3.9 down	3.2 down
Aaci_1055	Alpha/beta hydrolase fold-3 domain protein	2.3 down (p = 0.02)	BC	3.6 down
Aaci_1254	Acetate/CoA ligase	6.2 down	6.0 down	7.5 down
Aaci-1452	Acyl-CoA dehydrogenase domain protein	9.8 down	11.4 down	27.3 down
Aaci-1453	3-hydroxyisobutyrate dehydrogenase	7.2 down	7.6 down	21.8 down
Aaci-1454	Methylmalonate-semialdehyde dehydrogenase	5.8 down	6.7 down	11.7 down
Aaci-1455	Enoyl-CoA hydratase/isomerase	5.1 down	7.6 down	12.6 down
Aaci-1456	AMP-dependent synthetase and ligase	3.9 down	5.1 down	5.3 down
Aaci-1457	Acetyl-CoA acetyltransferase	4.1 down	6.8 down	7.5 down
Aaci-1458	Short-chain dehydrogenase/reductase SDR	2.7 down	3.1 down	3.8 down
Aaci-2035	AMP-dependent synthetase and ligase	4.2 down	3.2 down	3.7 down
Aaci-2057	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	5.3 down	4.6 down	8.7 down
Aaci-2058	Acetyl-CoA acetyltransferase	4.7 down	4.0 down	4.4 down
Aaci-2059	3-oxoacid CoA-transferase, B subunit	7.1 down	6.1 down	7.6 down
Aaci-2060	3-oxoacid CoA-transferase, A subunit	7.6 down	6.8 down	14.1 down
Aaci-2421	Cobalamin (vitamin B12) biosynthesis CbiX protein	BC	BC	3.8 down
Aaci-2504	Aldehyde dehydrogenase	9.0 down	5.0 down	9.6 down

Figure 1.


Xylose (g/L)
Glucose (g/L)
OD600
Sugar Concentration (g/L)
OD600
Time (Hours)


Arabinose (mg/L)
Xylose (mg/L)
OD600
Sugar Concentration (g/L)
OD600
Time (Hours)

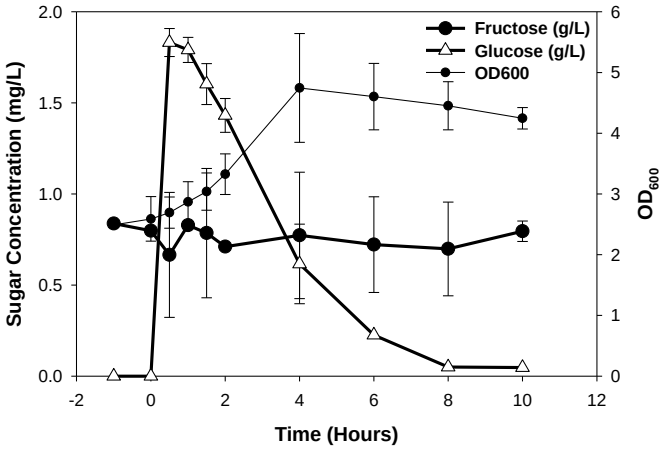

Fructose (g/L)
Glucose (g/L)
OD600
Sugar Concentration (mg/L)
OD600
Time (Hours)

Figure 2.

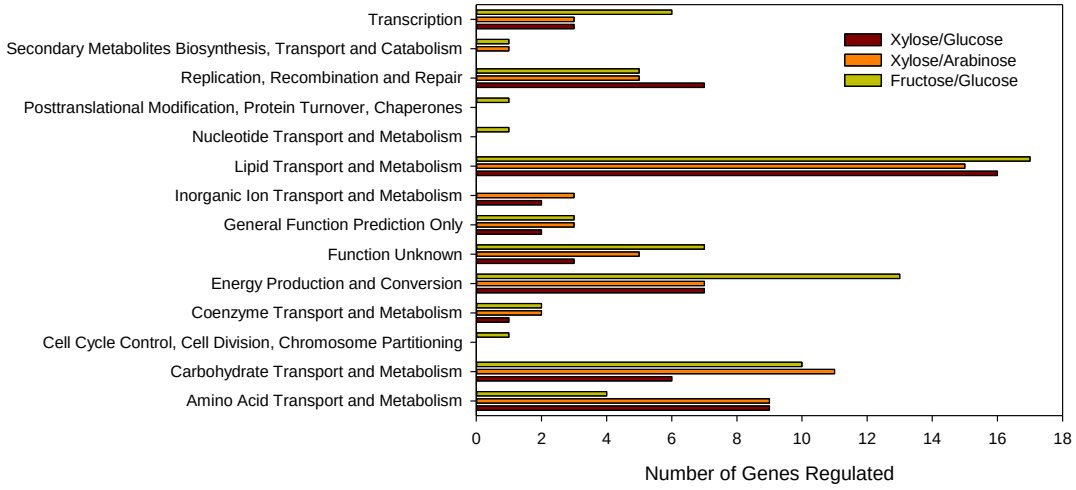

Transcription
Secondary Metabolites Biosynthesis, Transport and Catabolism
Replication, Recombination and Repair
Posttranslational Modification, Protein Turnover, Chaperones
Nucleotide Transport and Metabolism
Lipid Transport and Metabolism
Inorganic Ion Transport and Metabolism
General Function Prediction Only
Function Unknown
Energy Production and Conversion
Coenzyme Transport and Metabolism
Cell Cycle Control, Cell Division, Chromosome Partitioning
Carbohydrate Transport and Metabolism
Amino Acid Transport and Metabolism
Xylose/Glucose
Xylose/Arabinose
Fructose/Glucose
0
2
4
6
8
10
12
14
16
18
Number of Genes Regulated

Figure 3.

.

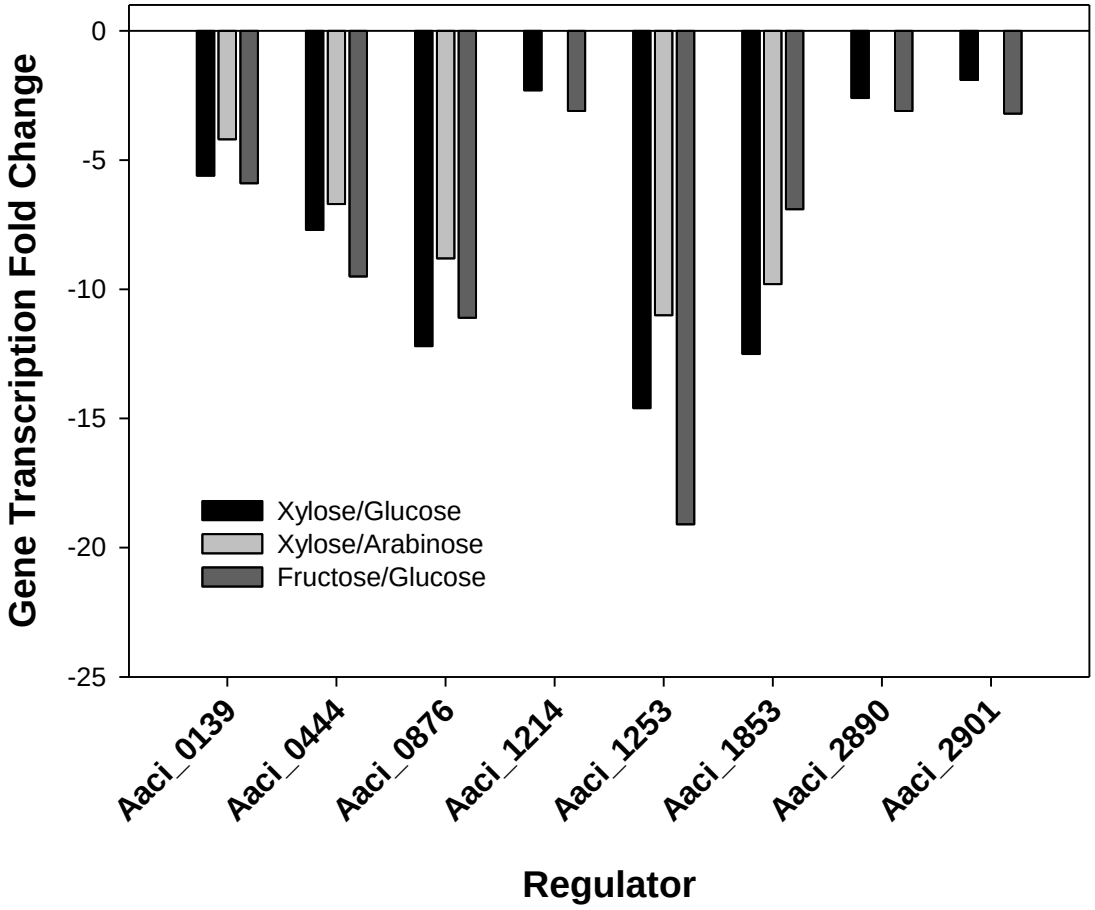

Gene Transcription Fold Change
0
-5
-10
-15
-20
-25
Xylose/Glucose
Xylose/Arabinose
Fructose/Glucose
Aaci_0139
Aaci_0444
Aaci_0876
Aaci_1214
Aaci_1253
Aaci_1853
Aaci_2890
Aaci_2901
Regulator

Figure 4.

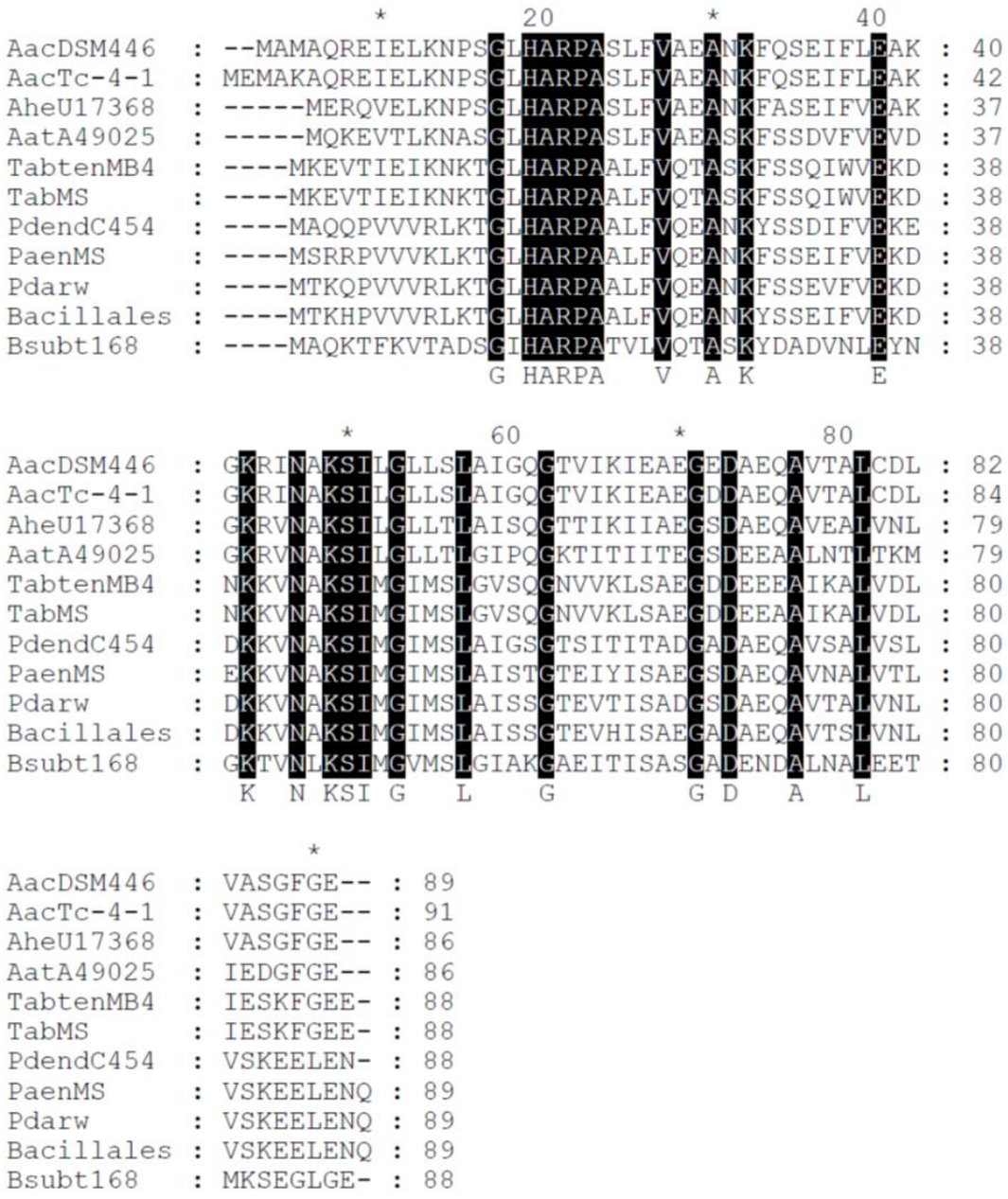

AacDSM446 : --MAMAQREIELKNPSGLHARPASLFVAEANKFQSEIFLEAK : 40
AacTc-4-1 : MEMAKAQREIELKNPSGLHARPASLFVAEANKFQSEIFLEAK : 42
AheU17368 : -----MERQVELKNPSGLHARPASLFVAEANKFASEIFVEAK : 37
AatA49025 : -----MQKEVTLKNASGLHARPASLFVAEASKFSSDVFVEVD : 37
TabtenMB4 : ----MKEVTIEIKNKTGLHARPAALFVQTASKFSSQIWVEKD : 38
TabMS : ----MKEVTIEIKNKTGLHARPAALFVQTASKFSSQIWVEKD : 38
PdendC454 : ----MAQQPVVVRLKTGLHARPAALFVQEANKYSSDIFVEKE : 38
PaenMS : ----MSRRPVVVKLKTGLHARPAALFVQEANKFSSEIFVEKD : 38
Pdarw : ----MTKQPVVVRLKTGLHARPAALFVQEANKFSSEVFVEKD : 38
Bacillales : ----MTKHPVVVRLKTGLHARPAALFVQEANKYSSEIFVEKD : 38
Bsubt168 : ----MAQKTFKVTADSGIHARPATVLVQTASKYDADVNLEYN : 38
G HARPA V A K E
AacDSM446 : GKRINAKSILGLLSLAIGQGTVIKIEAEGEDAEQAVTALCDL : 82
AacTc-4-1 : GKRINAKSILGLLSLAIGQGTVIKIEAEGDDAEQAVTALCDL : 84
AheU17368 : GKRVNAKSILGLLTLAISQGTTIKIIAEGSDAEQAVEALVNL : 79
AatA49025 : GKRVNAKSILGLLTLGIPQGKTITIITEGSDEEAALNTLTKM : 79
TabtenMB4 : NKKVNAKSIMGIMSLGVSQGNVVKLSAEGDDEEEAIKALVDL : 80
TabMS : NKKVNAKSIMGIMSLGVSQGNVVKLSAEGDDEEAAIKALVDL : 80
PdendC454 : DKKVNAKSIMGIMSLAIGSGTSITITADGADAEQAVSALVSL : 80
PaenMS : EKKVNAKSIMGIMSLAISTGTEIYISAEGSDAEQAVNALVTL : 80
Pdarw : DKKVNAKSIMGIMSLAISSGTEVTISADGSDAEQAVTALVNL : 80
Bacillales : DKKVNAKSIMGIMSLAISSGTEVHISAEGADAEQAVTSLVNL : 80
Bsubt168 : GKTVNLKSIMGVMSLGIAKGAEITISASGADENDALNALEET : 80
K N KSI G L G G D A L
AacDSM446 : VASGFGE-- : 89
AacTc-4-1 : VASGFGE-- : 91
AheU17368 : VASGFGE-- : 86
AatA49025 : IEDGFGE-- : 86
TabtenMB4 : IESKFGEE- : 88
TabMS : IESKFGEE- : 88
PdendC454 : VSKEELEN- : 88
PaenMS : VSKEELENQ : 89
Pdarw : VSKEELENQ : 89
Bacillales : VSKEELENQ : 89
Bsubt168 : MKSEGLGE- : 88

Figure 5.

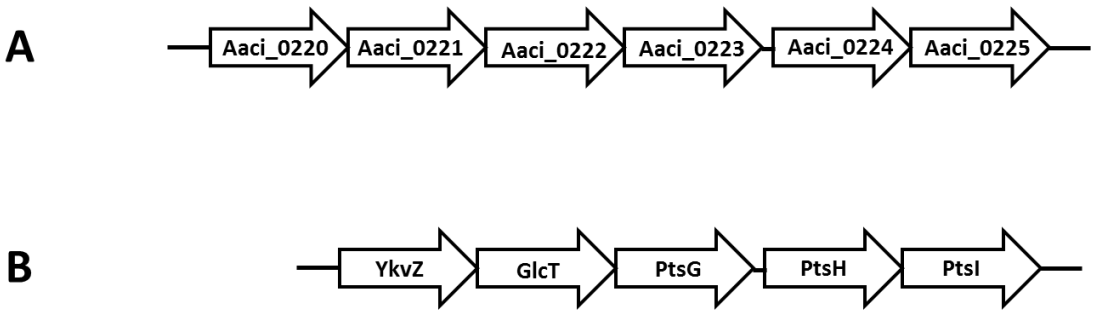

A
Aaci_0220
Aaci_0221
Aaci_0222
Aaci_0223
Aaci_0224
Aaci_0225
B
YkvZ
GlcT
PtsG
PtsH
PtsI

Figure 6.

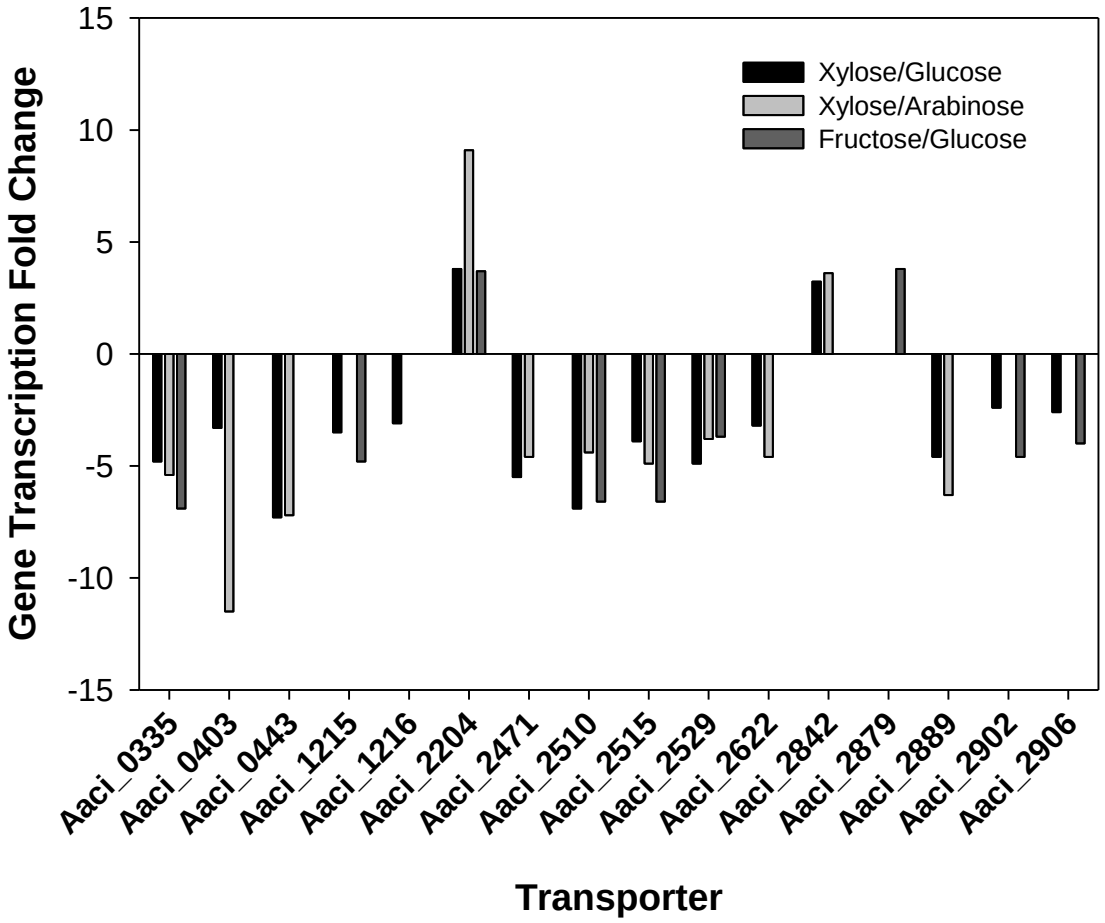
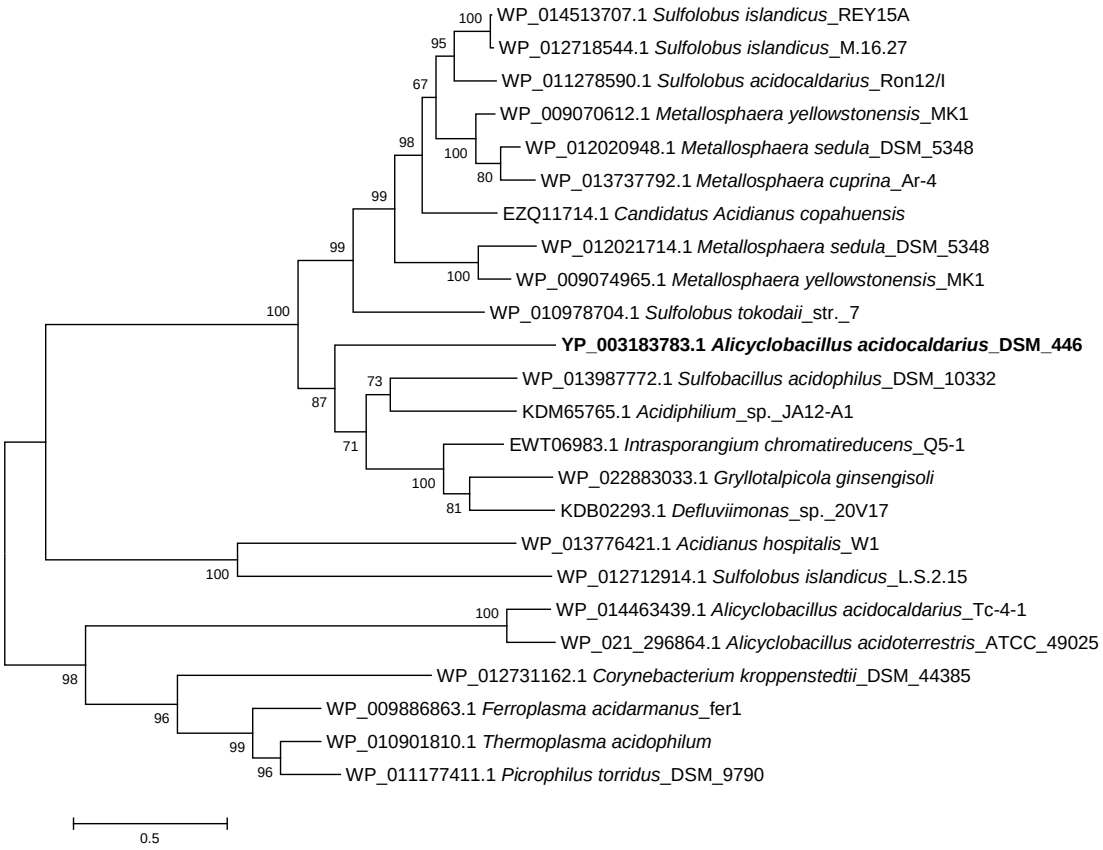

Gene Transcription Fold Change
15
10
5
0
-5
-10
-15
Xylose/Glucose
Xylose/Arabinose
Fructose/Glucose
Aaci_0335
Aaci_0403
Aaci_0443
Aaci_1215
Aaci_1216
Aaci_2204
Aaci_2471
Aaci_2510
Aaci_2515
Aaci_2529
Aaci_2622
Aaci_2842
Aaci_2879
Aaci_2889
Aaci_2902
Aaci_2906
Transporter

Figure 7.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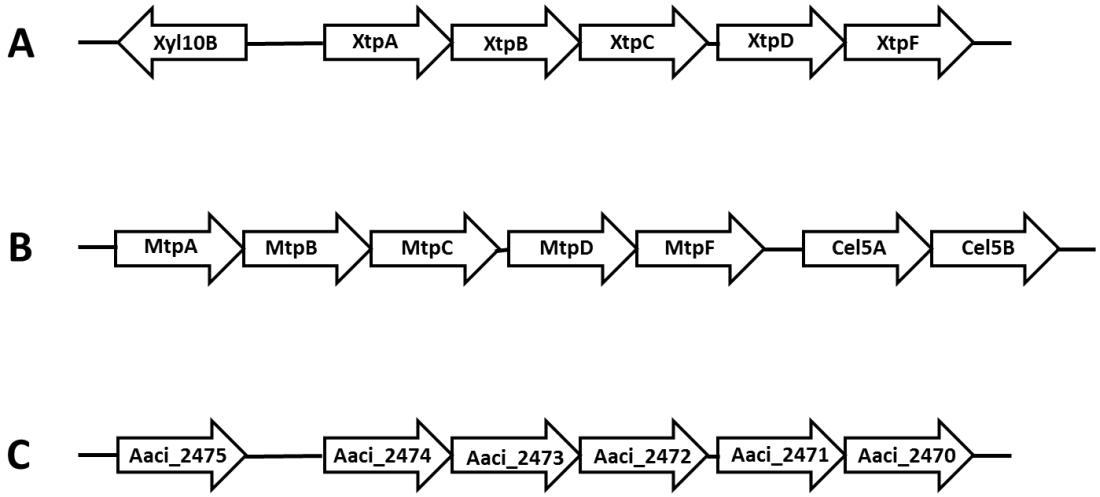
WP_012718544.1 Sulfolobus islandicus_M.16.27
WP_011278590.1 Sulfolobus acidocaldarius_Ron12/I
WP_009070612.1 Metallosphaera yellowstonensis_MK1
WP_012020948.1 Metallosphaera sedula_DSM_5348
WP_013737792.1 Metallosphaera cuprina_Ar-4
EZQ11714.1 Candidatus Acidianus copahuensis
WP_012021714.1 Metallosphaera sedula_DSM_5348
WP_009074965.1 Metallosphaera yellowstonensis_MK1
WP_010978704.1 Sulfolobus tokodaii_str._7
YP_003183783.1 Alicyclobacillus acidocaldarius_DSM_446
WP_013987772.1 Sulfobacillus acidophilus_DSM_10332
KDM65765.1 Acidiphilium_sp._JA12-A1
EWT06983.1 Intrasporangium chromatireducens_Q5-1
WP_022883033.1 Gryllotalpicola ginsengisoli
KDB02293.1 Defluviimonas_sp._20V17
WP_013776421.1 Acidianus hospitalis_W1
WP_012712914.1 Sulfolobus islandicus_L.S.2.15
WP_014463439.1 Alicyclobacillus acidocaldarius_Tc-4-1
WP_021_296864.1 Alicyclobacillus acidoterrestris_ATCC_49025
WP_012731162.1 Corynebacterium kroppenstedtii_DSM_44385
WP_009886863.1 Ferroplasma acidarmanus_fer1
WP_010901810.1 Thermoplasma acidophilum
WP_011177411.1 Picrophilus torridus_DSM_9790
100
95
67
98
100
80
99
99
100
100
73
87
71
100
81
100
100
98
96
99
96
0.5

Figure 8.


A
Xyl10B
XtpA
XtpB
XtpC
XtpD
XtpF
B
MtpA
MtpB
MtpC
MtpD
MtpF
Cel5A
Cel5B
C
Aaci_2475
Aaci_2474
Aaci_2473
Aaci_2472
Aaci_2471
Aaci_2470

CHAPTER III: Glycoside Hydrolase Gene Transcription by *Alicyclobacillus acidocaldarius* During Growth on Wheat Arabinoxylan and Monosaccharides: A Proposed Xylan Hydrolysis Mechanism

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ABSTRACT

Metabolism of carbon bound in wheat arabinoxylan (WAX) polysaccharides by bacteria requires a number of glycoside hydrolases active toward a number of different bonds between sugars and other molecules. *Alicyclobacillus acidocaldarius* is a Gram-positive thermoacidophile capable of growth on a variety of mono-, di-, oligo- and polysaccharides. In addition, roughly 19 proposed glycoside hydrolases have been annotated from genes found in the *A. acidocaldarius* strain DSM 446 genome. Molecular analysis of *A. acidocaldarius* strain DSM 446 when growing on WAX was performed using high density oligonucleotide microarrays. When a culture growing exponentially at the expense of arabinoxylan saccharides was challenged with glucose or xylose, most glycoside hydrolases were down-regulated. Interestingly, regulation was more intense when xylose was added to the culture than when glucose was added, a clear departure from classical carbon catabolite repression demonstrated by many

Gram-positive bacteria. *In silico* analyses of the regulated glycoside hydrolases, along with the results from the microarray analyses yielded a hypothesized mechanism for arabinoxylan metabolism by *A. acidocaldarius* strain DSM 446. These analyses showed that glycoside hydrolases expressed by this strain may have broad substrate specificity, and that initial hydrolysis is catalyzed by an extracellular xylanase, while subsequent steps are likely performed inside the growing cell.

INTRODUCTION

Alicyclobacillus acidocaldarius ATCC 27009 is a Gram-positive, spore forming, thermophilic acidophile that grows optimally in strictly aerobic conditions at 60°C and at pHs between 3 and 4. This bacterium was isolated from a hot spring in Nymph Creek area of Yellowstone National Park (1). Originally classified as *Bacillus acidocaldarius*, this bacterium was reclassified as *A. acidocaldarius* based on the prevalence of ω-cyclic fatty acids in the cell wall and an abbreviated helix 6 of the 16S rRNA (2). *A. acidocaldarius* has been isolated from diverse habitats including water and soil from geothermal sites, submarine hot springs, orchard soils, and also as a contaminant in heat processed foods (e.g., fruit juices) (3-8). *A. acidocaldarius* has demonstrated the ability to gain cellular carbon and energy from a wide variety of 5- and 6-carbon sugars including, L-arabinose, ribose, D-xylose, D-galactose, D-fructose, D-mannose, rhamnose, mannitol, and tagatose, the disaccharides D-turanose, melibiose, cellobiose, lactose, maltose, sucrose, and trehalose, as well as the more complex polysaccharides, cellulose, hemicellulose (xylan), starch and glycogen (9).

Lignocellulosic biomass is currently being studied as a feedstock for the production of fuels and chemicals (10, 11). Chemically, lignocellulose is a heterogeneous three dimensional matrix made of a carbohydrate component, consisting primarily of hemicellulose (20-30%) and cellulose (40-50%), and lignin component. These three components are intertwined, providing a structure that is highly resistant to microbial degradation. In fact, complete degradation of lignocellulose into monosaccharides requires the synergistic activity of a variety of glycoside hydrolases, a process most thoroughly studied in mesophilic microorganisms such as fungi and *Actinomycetes* (12). Likewise, numerous bacteria have also demonstrated the ability to produce glycoside hydrolases under aerobic and anaerobic conditions (13). Thermostable (stability at temperatures ≥ 50 °C) enzymes, which typically demonstrate higher rates of hydrolysis and more complete depolymerization, represent the next logical step in enzyme development of enzymes for lignocellulose breakdown, since lignocellulose pretreatment typically occurs using high process temperatures (14).

Hemicellulose accounts for nearly one-third of the renewable carbon found on earth (15, 16). Hemicellulose is composed predominantly of xylan, which is comprised of a conserved backbone of 1,4-linked β-D-xylose residues, decorated with side groups made up of arabinose, glucuronic acid, 4-O-methyl-glucuronic acid and possibly even galactose (17). Figure 1 shows the structure of arabinoxylan and shows the glycoside hydrolases necessary for depolymerization and production of monomers, which are then metabolized through the central metabolism of the microbial cell.

A. acidocaldarius represents a source of thermostable glycoside hydrolases for application as catalysts for the hydrolysis of the cellulose and hemicellulose components of lignocellulose. The potential for utilization of plant derived oligo- and polysaccharides by *A. acidocaldarius* is further demonstrated by the numerous glycoside hydrolases that have been found in the genome sequence or those that have been characterized. To date, 19 glycoside hydrolase genes have been identified in the *A. acidocaldarius* genome (18), and β-galactosidase, α-amylase, cellulase, neopullulanase, exo-pectinase, mannanase, β-glycosidase, and endoglucanase enzymes have been expressed and characterized (6, 9, 19-36). Table 1 shows enzymes required for the complete depolymerization of lignocellulose, and which of these activities have been found in genome of *A. acidocaldarius* or have been characterized using recombinant methods.

Carbon catabolite repression (CCR) is active in many bacteria that can metabolize both pentose and hexose monosaccharides and polysaccharides, causing preferential use of the hexoses, primarily glucose, which leads to sequential rather than simultaneous sugar utilization (37). In many bacteria, including mesophiles and thermophiles, biosynthesis of glycoside hydrolases requires cellular resources that are normally used for cell growth; therefore, transcription of genes that encode glycoside hydrolase is often under the control of CCR. While the general mechanism of CCR is different when comparing Gram-positive and Gram-negative bacteria, in each case, gene regulation occurs so that a preferred carbon source such as glucose is utilized compared to secondary carbon sources such as xylose, fructose, arabinose or

polysaccharides (38-41). Genes and operons encoding glycoside hydrolases for breakdown of hemicellulose and cellulose, as well as transporters of oligosaccharides are also regulated by CCR (42-53). When a preferred carbon source, such as glucose, is present, expression of these enzymes is typically down-regulated in an effort to conserve cellular resources. A well-studied example of the regulation of glycoside hydrolases is present in the hyperthermophile *Thermotoga maritima*. Gene transcription analysis using microarrays showed that genes encoding glycoside hydrolases were down-regulated when this bacterium was grown on glucose and other monosaccharides (46, 54-58).

The relevance of *A. acidocaldarius* for lignocellulose depolymerization is underscored by this bacterium's ability to use a wide range of monosaccharides for growth, as well as the capacity to produce a range of thermo- and acid-stable glycoside hydrolases. A thorough understanding of polysaccharide, monosaccharide and mixed saccharide metabolism by *A. acidocaldarius* will help in determining the utility of this bacterium for lignocellulose depolymerization. Part of this analysis will be to understand the effect of transcriptional regulation (i.e., CCR as one example) on expression of genes encoding glycoside hydrolases, which represents the first step in metabolism of lignocellulose. Genome analysis of *A. acidocaldarius* has revealed the presence of all components of Gram-positive CCR, as well as other regulators of genes encoding enzymes for monosaccharide and polysaccharide metabolism. The purpose of this study was to monitor gene transcription during growth on the polysaccharide, WAX, and to use the regulatory effects of monosaccharides on transcription of *A.*

acidocaldarius genes related to xylan utilization (i.e., glycoside hydrolases). Analyses were performed using batch chemostat studies and global transcriptome analysis using high density oligonucleotide microarray studies. This research represents the first transcriptome analysis of *A. acidocaldarius* while growing on plant polysaccharides, such as WAX.

MATERIALS AND METHODS

Inoculum Development.

A. acidocaldarius ATCC 27009 was purchased from the American Type Culture Collection (ATCC) and used for all experiments. To assure that the same generation was used in all experiments, stock cultures collected from cultures two transfers from the ATCC stock were maintained in 5% DMSO and stored at -80 °C. Chemostat inoculum was prepared by inoculating 1 ml of the frozen stock into 25 ml of Modified 402 Medium, which contained the following (g/L): $(NH_4)_2SO_4$ (1.3), Fe(III) EDTA (0.047), $CaCl_2 \cdot 2H_2O$ (0.07), $MgSO_4 \cdot 7H_2O$ (0.25), KH_2PO_4 (3.0), and xylose (4.0). In addition, 1 ml of a mineral (Solutions A and B) and vitamin stock (Solution C) were added. Solution A (g/L): $MgCl_2$ (25), $CaCl_2 \cdot 2H_2O$ (6.6), H_3BO_3 (0.58), $FeCl_3 \cdot 6H_2O$ (5), $Co(NO_3)_2 \cdot 6H_2O$ (0.05), $NiCl_2 \cdot 6H_2O$ (0.02). Solution B (g/L): $MnSO_4 \cdot H_2O$ (2.0), $ZnSO_4 \cdot 7H_2O$ (0.5), $CuSO_4 \cdot 5H_2O$ (0.15), $Na_2MoO_4 \cdot 2H_2O$ (0.025). Solution C (g/L): pyridoxine hydrochloride (0.08), folic acid (0.012), thiamine hydrochloride (0.13), riboflavin (0.042), nicotinamide (0.084), *p*-aminobenzoate (0.088), biotin (0.01), cyanocobalamin (0.0004), D-pantothenic acid, calcium salt (0.086), *myo*-inositol (0.021), choline bromide (0.053), orotic acid,

sodium salt (0.021), spermidine (0.1). Base medium was autoclaved (121 °C, 20 psi) for 30 min prior to use; KH_2PO_4 solution was adjusted to pH 4.0, autoclaved separately and added once the base medium had cooled. Solutions A, B and C were filter sterilized (0.22 μm) separately prior to addition to the base medium. Solutions A and B were autoclaved with the bulk media, whereas Solution C was filter sterilized and added to the base medium once it had cooled. The 25 ml *A. acidocaldarius* culture was grown overnight and then used to inoculate 250 ml of Modified 402 Medium containing 4 g/L xylose. This overnight culture grown at pH 4 at a temperature of 60 °C was then used to inoculate the chemostat.

Chemostat Studies.

Experiments to monitor gene transcription by *A. acidocaldarius* when growing on WAX and xylose or glucose were performed in a BioFlo 3000 chemostat system (New Brunswick Scientific, Enfield, CT). Medium was added to the reactor, oxygen and DO probes were inserted into appropriate ports, and the entire reactor was autoclaved at 121 °C, and a pressure of 20 psi for 1 hour. Solution C and WAX were added to the chemostat following autoclaving. The pH probe was calibrated using a two point calibration with pH 2 and 7 buffers, prior to autoclaving. Prior to heating the reactor for operation, the pH of the medium was measured using an external pH meter and the pH on the BioFlo control unit was adjusted accordingly. The chemostat was heated to 60 °C and the DO probe was allowed to polarize for six hours and then the dynamic range of the probe was set first by purging nitrogen gas through the medium (0% oxygen) and then air (100% oxygen). A working volume of 2.0 L of

Modified 402 Medium was used for experiments. The chemostat was run in batch mode and the temperature was held at a temperature of 60 °C and the pH of the growth medium was automatically controlled to 4.0 using the addition of 1 N NaOH. To ensure that the cultures were not oxygen limited, dissolved oxygen was controlled to 10% using a cascade system based on changes in agitation along with the addition of pure oxygen to the inlet air stream. WAX, which is relatively insoluble, was prepared by first wetting 2 g with 95% ethanol. Once in a slurry, 190 ml of sterile distilled water (pH 4.0) was added and the solution was heated for 30 minutes to evaporate residual ethanol. This solution was then added to the 2 L of Modified 402 Medium in the chemostat, giving a final WAX concentration of 1 g/L. *A. acidocaldarius* was grown to mid-exponential phase (OD_{600} of ~0.5) and then either glucose or xylose was spike into the reactor at concentrations of 2 g/L to induce regulation of gene transcription.

Once the *A. acidocaldarius* culture reached an OD_{600} of 0.5 while growing at the expense of sugar liberated from the hydrolysis of WAX, a sample was taken for RNA extraction, either glucose or xylose was added, and then a second sample was taken for RNA extraction. Three biological replicates for each condition were performed.

Analyses

Bulk samples were taken from a sampling port in the chemostat using sterile syringes. Samples were then aliquoted for the required amount for each analysis.

Isolation of Total RNA.

Samples for RNA extraction were taken from the chemostat and immediately mixed with RNA Protect Bacteria Reagent (Qiagen, Valencia, CA) at a 1:2 ratio. This mixture was incubated at room temperature for 5 minutes, centrifuged, the supernatant was discarded and the cell pellet was flash frozen in liquid nitrogen and then stored at -80 °C until the RNA was extracted. Total RNA was extracted from the *A. acidocaldarius* cells using an RNeasy Midi Kit (Qiagen, Valencia, CA) with slight modification of the manufacturer's protocol. *A. acidocaldarius* cells were thawed and lysis was accomplished by adding 200 µl of Tris-EDTA buffer containing 15 mg/ml lysozyme and 0.1 mg/ml proteinase K. Samples were vortexed for 10 seconds and then incubated at room temperature for 15 minutes with shaking. Buffer RLT (4 ml) containing β-mercaptoethanol was then added to each sample, the mixture was homogenized using a syringe with a 20G needle, and then incubated at room temperature for 10 minutes. Following incubation, 3.5 ml of 80% ethanol was added and the resulting solution was mixed vigorously. The lysate was then passed through a Midi spin filter via centrifugation at 5,000 x g for five minutes to capture the RNA. RNA on the spin filter was washed sequentially with two 2.5 ml aliquots of Buffer RPE, followed by centrifugation at 5,000 x g for five minutes. RNA was eluted from the glass filter using 200 µl of DNase/RNase-free water; water was applied to the filter, incubated at room temperature for five minutes, and then centrifuged at 5,000 x g for three minutes. To increase RNA yield, the flow through was re-applied to the spin filter and centrifuged. Residual DNA in the samples was removed by treatment with Ambion TURBO DNA-free kit (Life Technologies, Grand Island, NY). RNA

was purified to remove compounds that might interfere with cDNA synthesis and concentrated using ethanol precipitation. The pellet was dried using an Eppendorf Vacufuge Concentrator 5301 (Brinkmann Instruments, Westbury, NY), and then resuspended in 20 µl of DNase/RNase-Free water. To inhibit RNA degradation during storage, 1 µl of Ambion Superase-In RNase Inhibitor (Life Technologies, Grand Island, NY) was added. RNA concentration and purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was determined using an RNA Nano Chip Kit run on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

***Synthesis of cDNA*.**

Double stranded cDNA was synthesized from total RNA using the Invitrogen Superscript Double-Stranded cDNA Synthesis Kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Ten µg of total RNA was mixed with random hexamer primers in DNase/RNase-Free Water, heated for 10 minutes at 70 °C and then quenched in an ice-water slurry for five minutes. While on ice, First Strand Buffer, dithiothreitol, and dNTPs were added and then brought to the reaction temperature of 42 °C, prior to addition of SuperScript II. Following addition of the reverse transcriptase, the reaction mixture was incubated at 42 °C for one hour. After this incubation the tubes were placed on ice and reaction components for second strand synthesis were added. The first strand reaction mixture was mixed with Second Strand Buffer, dNTPs, DNA ligase, DNA polymerase and RNase H; the reaction was mixed and then incubated at 16 °C for two hours. An additional five minute incubation was

performed after T4 DNA polymerase was added to the reaction. Residual RNA was degraded by adding RNase A and incubating the reaction mixture at 37 °C for 10 minutes. Proteins were removed by treating with phenol:chloroform:isoamyl alcohol, with phase separation being accomplished using Phase Lock Tubes (5 Prime, Inc., Gaithersburg, MD). cDNA in the aqueous phase was then precipitated and concentrated by ethanol precipitation. The pellet was dried using an Eppendorf Vacufuge Concentrator 5301 (Brinkmann Instruments, Westbury, NY), and then resuspended in 20 µl of DNase/RNase-Free water and allowed to solubilize overnight. cDNA concentration in each reaction was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A DNA 7500 Chip Kit run on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) was used to verify that most of the cDNA was ≥ 400 bp.

Microarray Experiments and Data Analysis.

Microarrays were designed and synthesized by NimbleGen using their 4 x 72K Custom Gene Expression Array format. Complete genome sequence information for *A. acidocaldarius* ATCC 27009 (DSM 446) was provided to NimbleGen. Seven probes, each 60 nt in length, were designed for each of the 3,554 identified open reading frames (ORFs) in the genome. Each probe was synthesized on the microarray in triplicate. Control probes were also included to ensure that there was no intra-quadrant contamination during the hybridization process.

Biological triplicate RNA samples isolated from *A. acidocaldarius* cells grown under the various conditions used to determine the level of CCR demonstrated were used for

microarray analysis. One color cDNA labeling using Cy3, hybridization to the *A. acidocaldarius* microarrays, array imaging and initial analysis of the array data was performed by NimbleGen. Data was normalized using NimbleScan software which normalizes probe response using quantile normalization and gene calls generation using Robust Multichip Averaging (RMA) (59, 60). Log_2-transformed RMA data files were imported into ArrayStar 4 software (DNASTAR, Inc., Madison, WI) and the mean expression levels of three replicate arrays for each condition were considered. For comparison between gene expression during growth on WAX, immediately upon addition of the CCR inducing sugar, and growth on initial and inducing sugar, statistical significance was determined with a Bonferonni corrected moderate *t*-test and only genes that had a 2-fold or greater change in gene expression at 95% confidence ($p \leq 0.05$) were considered significant. In a few instances, genes may be discussed for which transcription was down-regulated less than 2-fold, but *p*-values were still below 0.05.

Comparative analysis of the *A. acidocaldarius* genome was performed using the Integrated Microbial Genomes feature within the Joint Genome Institute (61). Glycoside hydrolase and carbohydrate esterase enzymes annotated in the *A. acidocaldarius* strain DSM 446 were found in the Carbohydrate Active Enzyme Database (CAZy) (62). Homology determinations for *A. acidocaldarius* proteins were accomplished using the Basic Local Alignment Search Tool (BLAST) for protein sequences using the 'blastp' algorithm (63, 64). The non-redundant protein sequence database was used for searches and uncultured and environmental sample sequences

were excluded from the search. Phylogenetic trees comparing protein sequences were generated using the Molecular Evolutionary Genetics Analysis (MEGA6) software program (65). Protein sequences were aligned using the MUSCLE program within MEGA6 (66, 67). Phylogenetic reconstruction was accomplished using the Maximum Likelihood statistical method and distances between sequences were determined using 1000 bootstrap replicates.

RESULTS

Glycoside hydrolase inventory of *A. acidocaldarius*.

Analysis of the *A. acidocaldarius* genome shows 20 gene loci that are annotated as glycoside hydrolases (EC 3.2.1.-) (Table 2). The broad substrate range of *A. acidocaldarius* related to oligo- and polysaccharides is supported by the wide variety of genes that act on cellulose and hemicellulose. Interestingly, only three (Aaci_0048, Aaci_2457 and Aaci_2874) of the loci encode proteins that contain a signal peptide indicating extracellular, or at least membrane bound, activity. The Carbohydrate Active enZYme (CAZY) database lists other GH family enzymes, but most are associated with hydrolysis of cell wall constituents, such as peptidoglycan, or are related to spore germination.

Overall down-regulation of proposed xylan utilization genes.

A. acidocaldarius cells were grown to mid-exponential phase on WAX, and then samples were taken for extraction of RNA and analysis, just prior to and following the addition of glucose or xylose. Growth on WAX would require the expression of a number of glycoside hydrolases (See Table 1and Figure 1). Following

initial exposure of the *A. acidocaldarius* growing on WAX to the monomer sugars, microarray analysis showed that transcription of 13 of the 20 genes that encode putative glycoside hydrolases were, for the most part, down-regulated, regardless of whether glucose or xylose was added (Figure 2). In general, down-regulation of glycoside hydrolase gene transcription was greater when xylose was added to the culture than when glucose was added. This is puzzling because in most Gram-positive bacteria, the opposite has been shown (40, 68). Two glycoside hydrolase genes were up-regulated when glucose was added (Aaci_0912 and Aaci_2891), but up-regulation was less than 2-fold, while these same genes were down-regulated when xylose was added. Likewise, two glycoside hydrolase genes (Aaci_2869 and Aaci_2887) were up-regulated when xylose was added but down-regulated with glucose. One of these genes, Aaci_2887, was up-regulated nearly 4-fold. Transcription of the remaining 15 glycoside hydrolase genes was down-regulated following addition of xylose or glucose. In analyzing transcription of glycoside hydrolase genes, the assumption has been made that if a gene was regulated when the monosaccharide was added, a sensory cascade occurred that led to the regulation of gene transcription (69, 70). If a gene was down-regulated then it was being expressed when growing only on WAX; therefore, if the gene was up-regulated, transcription increased upon exposure to the sugar added. *In silico* analysis of glycoside hydrolase genes that were regulated was performed to determine the possible function of the coded proteins in hydrolysis of WAX. Analysis was based on the annotated product of the gene, rather than apparent importance.

Gene locus Aaci_0048, that encodes a 959 amino acid protein that has been annotated as an α-L-arabinofuranosidase-like protein, was down-regulated 5.6-fold when xylose was added and 2.8-fold when glucose was added. Analysis of conserved domains from the amino acid sequence indicates the presence of domains homologous to Family 44 glycoside hydrolases, which demonstrate activity toward cellulose (i.e., endoglucanase activity). The N-terminal domain of the protein also contains a sequence with close homology to carbohydrate binding domains of numerous glycoside hydrolases. Prior to publication of the *A. acidocaldarius* genome, Eckert and Schneider (9) expressed and characterized a 959 amino acid thermophilic endoglucanase (CelB; Accession AJ551527.1) from *A. acidocaldarius* with 100% identity to the gene product discussed from Aaci_0048. Activity testing of the recombinant CelB enzyme demonstrated endo-acting activity toward carboxymethylcellulose, steam-exploded cellulose and oat spelt xylan, demonstrating a broad substrate range for the enzyme. Sequencing and characterization of the enzyme classified it as an extracellular endoglucanase, with high sequence similarity to GH family 51 of arabinofuranosidases. While this enzyme has been annotated as a α-L-arabinofuranosidase-like protein, no activity was demonstrated using p-nitrophenyl-α-l-arabinofuranoside. These results have been verified in unrelated studies at the Idaho National Laboratory for the native enzyme from *A. acidocaldarius* and a recombinant enzyme expressed from *Pichia pastoris* (data not shown). In total, these data indicate that the gene product from Aaci_0048 is a glycoside hydrolase protein that catalyzes hydrolysis of β-1,4-linked xylose subunits in the WAX xylan backbone. It should be

noted that the glycoside hydrolase encoded by Aaci_0048 shares 43% identity with a β-1,4-glucanase expressed from *Alicyclobacillus* sp. A4 (71).

The Homology Toolkit of the Integrated Microbial Genomes (IMG) Tool at JGI was used to compare the α-L-arabinofuranosidase-like protein (Aaci_0048) from *A. acidocaldarius* to other genes within IMG. As would be expected, the protein showed the highest percent identity to similarly annotated genes in other species of *Alicyclobacillus*. The most closely related protein sequence from non-*Alicyclobacillus* was a cellulose binding family II protein from *Ktedonobacter racemifer* SOSP1-21. A distance tree (Figure 3) generated from sequence information shows that based on percent identity, Aaci_0048 had the highest homology to α-arabinofuranosidases or cellulose binding family II proteins from numerous species of bacteria from the Order *Actinomycetales*. Differences in activity between this enzyme and other α-arabinofuranosidases found in the database may be due to the additional 200 to 400 amino acids found in Aaci_0048 and the other similar proteins found in other strains of *A. acidocaldarius*. Alignment of the predicted protein encoded by Aaci_0048 with other similarly annotated genes, some made up of greater than 1,000 amino acids and those containing fewer than 700 amino acids, is shown in Figure 4. The β-1,4-glucanase expressed from *Alicyclobacillus* sp. A4 still has fewer amino acids than the product of Aaci_0048, which contains an additional ~250 amino acids at the C-terminus of the protein sequence. There is very little homology between Aaci_0048 and another annotated α-arabinofuranosidase encoded by Aaci_2894 (discussed below). Evolutionarily, while the exact mechanism of catalysis is not known, an α-

arabinofuranosidase could have been transferred from an *Actinomycete*, followed by fusion with a protein that supplied the endoglucanase and endoxylanase activity. Additional amino acids in the *A. acidocaldarius* α-L-arabinofuranosidase-like protein may cause the enzyme to fold in a manner that occludes the α-L-arabinofuranosidase activity.

Gene transcription from locus Aaci_0060 from *A. acidocaldarius* was down-regulated 2.5-fold when glucose was added and 1.7-fold when xylose was added. The putative protein product is annotated as an α-1,2-glucuronidase, which is most likely involved in hydrolysis of 4-*O*-methyl-D-glucuronic acid or glucuronic acid, both of which are found as modifications of the xylan backbone of WAX. Multi-domain analysis of the protein product of Aaci_0060 supports the annotation as an α-1,2-glucuronidase, and more detailed analysis shows the presence of C-terminal and middle domains (probable catalytic region) found in glycoside hydrolase family 67 proteins (72). Aaci_0060 and Aaci_0048 may act in concert, since their loci are in the same gene neighborhood of the *A. acidocaldarius* genome; however the relationship of transcription of these two genes has not been thoroughly studied. Figure 5 shows the comparison of the *A. acidocaldarius* strain DSM 446 genome neighborhood genomes of *A. acidocaldarius* subsp. *acidocaldarius* (Tc-4-1) and *A. hesperidum* URH17-3-68. The proximity of these two genes appears to be conserved in a variety of *Alicyclobacillus* species.

Phylogenetic analysis of the protein encoded by Aaci_0060 indicates relatedness to proteins from a number of other cellulolytic bacteria found in the

phylum *Firmicutes* (Figure 6). This protein from *A. acidocaldarius* shows greater than 50% homology to proteins that have been annotated as α-glucuronidases in both aerobic and anaerobic bacteria in the genera *Bacillus*, *Clostridium*, *Paenibacillus*, *Geobacillus* and *Thermoanaerobacterium*.

A second neighborhood in the *A. acidocaldarius* genome appears to be involved in WAX depolymerization. Transcription of gene loci Aaci_0786, Aaci_0789 and Aaci_0797 was down-regulated during this experiment; greater when xylose was supplied as the inducing sugar. The first of these genes, Aaci_0786, has been annotated as a glycoside hydrolase clan GH-D protein, specifically an α-galactosidase. A conserved domain comparison with other glycoside hydrolases in GenBank indicates specific hits to melibiase, with non-specific hits to glycoside hydrolase family 31 (GH31) enzymes. Enzymes in the GH31 superfamily are present in all 3 domains of life, and activities include α-glucosidase, α-xylosidase, 6-α-glucosyltransferase, 3-α-isomaltosyltransferase, and α-1,4-glucan lyase. While this enzyme has been annotated as an α-galactosidase, and could be involved in removal of galactose from the xylan backbone of WAX, expression of this enzyme during growth on WAX may also indicate α-xylosidase activity.

The second genome locus in this cluster, Aaci_0789, which encodes a 320 amino acid protein annotated as a hypothetical protein, was down regulated 2.9-fold when xylose was added and 1.5-fold when glucose was added. Protein sequence analysis and classification (IPR013781) indicates the presence of a catalytic TIM beta/alpha barrel common to many different families of glycoside hydrolases. This

protein shares greater than 50% identity with hypothetical proteins containing glycoside hydrolase catalytic domains found in other species of *Alicyclobacillus*. Figure 7 is a phylogenetic tree constructed to display that the proposed protein product for Aaci_0789 shows homology to an endo-β-1,4-mannanase that was characterized in *Alicyclobacillus* species A4 and *A. acidocaldarius* strains Tc-4-1 and Tc-12-31 (29, 73). The glycoside hydrolase encoded by this gene locus also demonstrates up to 50% homology to endo-β-1,4-xylanases from a number of other *Firmicutes*, as well as hypothetical proteins from *Firmicutes* known to degrade xylan. *In silico* analysis of this gene along with expression results indicate a possible broad substrate range for this enzyme that would include mannose- and xylose-containing polysaccharides.

The final gene locus in this genome neighborhood that was down-regulated was Aaci_0797, which has been annotated as a glycoside hydrolase family 4, α-galactosidase. The protein encoded by Aaci_0797 shares 99% sequence identity to a β-galactosidase cloned from *A. acidocaldarius* (22). This recombinant enzyme hydrolyzed *o*-nitrophenyl-β-D-galactopyranoside and *o*-nitrophenyl-β-D-fucopyranoside, but did not catalyze hydrolysis of *o*-nitrophenyl-β-D-glucopyranoside, *o*-nitrophenyl-β-D-xylopyranoside or *o*-nitrophenyl-β-D-arabinopyranoside. These results indicate that Aaci_0797 is likely a β-galactosidase active toward galactose moieties on side chains of the WAX. Aaci_0797 also has greater than 70% similarity to α-galactosidase from a number of other Gram-positive bacteria, including numerous species of *Paenibacillus*.

Other genes co-located with these three glycoside hydrolase encoding genes in the *A. acidocaldarius* strain DSM 446 genome are shown in Figure 8. Annotation of protein products from these genes indicates that this area of the genome is active in carbohydrate metabolism (Table 3). During the chemostat experiments, five of the sixteen genes were differentially expressed when glucose was added to the culture growing on WAX, and 13 of 16 were differentially expressed when xylose was added to the culture growing on WAX. Along with the three glycoside hydrolase genes regulated when xylose was added (Aaci_0786, Aaci_0789 and Aaci_0797), gene loci Aaci_0782 and Aaci_0783 encode glycoside transferase proteins and were up-regulated nearly 2-fold when xylose was added, but not when glucose was added. Aaci_0788, down-regulated when xylose was added, encodes a protein annotated as a carbohydrate kinase, possibly involved in phosphorylation and transport of sugars. Aaci_0791 which encodes a putative carbohydrate binding protein associated with a glycosyl transferase was likewise down-regulated with xylose but not glucose. Aaci_0793, which encodes a LacI family transcriptional regulator, was down-regulated when glucose was added, but not when xylose was added. Finally, two apparent sugar transport systems, a multifacilitator superfamily transporter (Aaci_0792) and an ATP-binding cassette (ABC) transporter (Aaci_0794 through Aaci_0796), were down-regulated when xylose and glucose were added to the *A. acidocaldarius* culture growing on WAX.

Analysis of this genome neighborhood indicates conservation of genes among sequenced *Alicyclobacillus* species (Figure 9). Arrangement of the genes encoding the

glycoside hydrolase clan GH-D (Aaci_0786) and the hypothetical protein containing a glycoside hydrolase catalytic domain (Aaci_0789) are nearly identical in *A. acidocaldarius* subsp. *acidocaldarius* (DSM 446), *A. acidocaldarius* subsp. *acidocaldarius* (Tc-4-1), and *A. hesperidum* URH17-3-68. However, this gene arrangement is not conserved in *A. herbarius* DSM 12609. When comparing the two subspecies of *A. acidocaldarius*, DSM 446 appears to have a cassette containing the glycoside hydrolase family 4 α-galactosidase (Aaci_0797), genes encoding ABC-transporter components (Aaci_0794-0796) and a LacI family transcriptional regulator (Aaci_0793) that is not present in the Tc-4-1 genome. Two β-galactosidase genes can be found in the Tc-4-1 genome, but translated protein sequences show low homology to Aaci_0797, indicating that Tc-4-1 may lack α-galactosidase activity.

Gene locus Aaci_1218 was the most highly down-regulated gene for which the product has been annotated as a glycoside hydrolase enzyme. When xylose was added, transcription of this gene locus was down-regulated nearly 14-fold, but less than 2-fold when glucose was added. Annotation within the Joint Genome Institute's (JGI) Integrated Microbial Genome (IMG) gives a product name of a glycoside hydrolase family 2 TIM barrel. Gene Ontology (GO:0004565), clusters of orthologous groups (COG3250) and KEGG Orthology (KO:01190) indicate that this protein may be a β-galactosidase/β-glucuronidase. This 1,041 amino acid protein shows the highest homology to glycoside hydrolases found in other *Bacilli.* This enzyme most likely acts on either galactose or 4-O-methyl-D-glucuronic acid side chains found in WAX. These results indicate that *A. acidocaldarius* was expressing genes related to

side chain hydrolysis of the WAX which were regulated upon addition of xylose. As with many other genes, transcription of this gene was only slightly down-regulated when glucose was added. Aaci_1218 appears to be in an operon with genes for an ABC-transport system and a LacI transcriptional regulator (Figure 10). All of the genes in this operon were highly down-regulated, suggesting that genes from this apparent operon are involved in hydrolysis of WAX, as well as transport of some products from this hydrolysis.

A second gene (Aaci_1895) that has been annotated as a β-galactosidase/ β-glucosidase in glycoside hydrolase family 1 was down-regulated 7.9-fold when xylose was added and 2.3-fold when glucose was added. The 453 amino acid *A. acidocaldarius* protein encoded by this gene has greater than 60% identity to β-galactosidase/ β-glucosidase enzymes found in other *Firmicutes,* namely numerous species of *Thermoanaerobacter* (Figure 11). Di Lauro *et al*. (74) characterized a recombinant β-glycosidase from *A. acidocaldarius* that showed wide substrate specificity and was able to hydrolyze β-D-gluco-, -galacto-, and fucosides. The protein encoded by Aaci_1895has 98% identity with the β-glycosidase that was characterized, so is likely the same protein. Like many other glycoside hydrolase encoding genes in the *A. acidocaldarius* genome, Aaci_1895 is arranged in an operon with an ABC-type transport system; however, this specific ABC-type transporter has been annotated as an oligopeptide/dipeptide transporter. Utilization of oligopeptide/dipeptide ABC-type transporters for carbohydrate transport is not uncommon in other thermophilic lignocelluloses degrading bacteria, including

Thermotoga maritima and *Caldicellulosiruptor saccharolyticus* (75, 76). This gene arrangement, including the oligopeptide/dipeptide ABC-type transporter genes, is also common in a number of *Thermoanaerobacter* species. As shown in Figure 12, the primary difference between *A. acidocaldarius* and the *Thermoanaerobacter* species is the presence of an additional gene, which encodes a Family 2 glycoside hydrolase, situated between the β-galactosidase/ β-glucosidase and the transporter genes. The Family 2 glycoside hydrolase gene encoded in the *Thermoanaerobacter* genomes may have a similar function to the Family 2 glycoside hydrolase encoded by Aaci_1218, which was also highly down-regulated in *A. acidocaldarius*, as discussed above. Comparing the Family 2 glycoside hydrolase from *Thermoanaerobacter mathranii* against the *A. acidocaldarius* subsp. *acidocaldarius* DSM 446 genome yielded the closest homology to Aaci_1218 (E-value = 9e-10); other proteins had E-values greater than 1.

A gene encoding a Family 9 glycoside hydrolase (Aaci_2475) was down-regulated equally (~2.8-fold) when the exponentially growing *A. acidocaldarius* was exposed to xylose or glucose. Domain analysis of this 537 amino acid protein indicates that the enzyme may have endoglucanase activity, but since it appears to be active on WAX, the enzyme may have an expanded substrate range, and act on substrates other than cellulose or cellobiose. Endoglucanases with broad substrate specificity, which included activity toward xylans, have been demonstrated in other thermophiles, including *Pyrococcus furiosus* and *Thermotoga maritima* (77, 78). The glycoside hydrolase encoded by Aaci_2475 is associated with an ABC-type

oligopeptide transport system, which was also highly down-regulated when xylose was added, as was the case with Aaci_1895 and its neighboring genes. Analysis of the gene neighborhood where this Family 9 glycoside hydrolase is found indicates that this apparent operon structure is common in numerous *Alicyclobacillus* species, including *A. acidocaldarius* subsp. *acidocaldarius* (DSM 446), *A. acidocaldarius* subsp. *acidocaldarius* (Tc-4-1), and *A. hesperidum* URH17-3-68 (Figure 13). Xylan-hydrolyzing activity by an endoglucanase is not unprecedented; Hall *et al*. (1988)(79) characterized an endoglucanase from *Clostridium thermocellum* with xylanase activity. More recently, a bifunctional xylanase-glucanase from *Paenibacillus* sp. Strain E18 was characterized and showed activity toward xylan and glucan (80). Likewise, bifunctional xylanase/endoglucanase enzymes have been generated from the metagenome libraries from a yak and bovine rumen microbial community and showed activity toward a variety of xylans and glucans (81, 82).

The transcript for a 782-amino acid protein, encoded by genome locus Aaci_2630, was down-regulated nearly 2-fold when glucose was added and greater than 4-fold when xylose was added. This gene encodes a glycoside hydrolase family 3 domain protein with β-glucosidase or β-xylosidase activity. Regulation during growth on WAX further supports the possibility of β-xylosidase activity. Figure 14 shows the alignment of Aaci_2630 with related β-xylosidases in lignocellulose-degrading bacteria. Homology analysis of this glycoside hydrolase to other genes deposited in the NCBI database shows greater than 50% similarity to family 3 glycoside hydrolases from other thermophilic, lignocellulose-degrading bacteria, including

Thermoanaerobacter, *Caldicellulosiruptor* and *Thermotoga* species. This proposed β-xylosidase also has ~60% identity with three enzymes expressed from recombinant DNA showing specificity toward xylo-oligosaccharides for which activity assays have been performed (83-85). Xyl3A from *Caldanaerobius polysaccharolyticus* demonstrated both β-glucosidase and β-xylosidase activity, but activity toward xylooligosaccharides was the highest (84). When tested against a variety of polysaccharides that included various xylans, glucans, mannan and glucomannans, the primary activity detected was β-xylosidase activity, which was supported by production of xylose from the assays. Enzymes that showed homology to Aaci_2630 from two species of *Thermoanaerobacter* have also been characterized as β-xylosidases. A bifunctional xylosidase-arabinosidase (XarB) from *T. ethanolicus* JW200 demonstrated activity toward a variety of substrates, but the highest activity/affinity was toward xylo- and arabinopyranoside molecules (85). Similarly, a xylodextrin-hydrolyzing xylo-β-glucosidase from *T. brockii* was shown to possess both β-glucosidase and β-xylosidase activity (83). Homology of Aaci_2630 with these other proteins that have demonstrated β-xylosidase activity and the down-regulation in these experiments support classification of the encoded enzyme as a β-xylosidase.

A section of the *A. acidocaldarius* genome from DNA coordinate 2,926,031 to 2,962,624 (gene loci Aaci_2868 through Aaci_2894) appears to be a 'hot spot' for glycoside hydrolase activity because 6 of the 20 glycoside hydrolase encoding genes in *A. acidocaldarius* are found in this region (Figure 15). Annotation of the genome in this region indicates the presence of genes encoding both cellulose-and hemicellulose-

hydrolyzing enzymes. Half of the glycoside hydrolase genes were regulated during the experiment, maintaining the trend of higher regulation when xylose was added to the exponentially growing culture of *A. acidocaldarius*.

While transcription of genes encoding enzymes that would be active toward glucose polymers would not be expected during growth on a xylan substrate, gene loci Aaci_2874, which encodes a protein annotated as an α-amylase, was down-regulated nearly 3-fold when xylose was added to the exponentially growing culture, but not with glucose. The translated protein from Aaci_2874 shows over 99% homology to an enzyme cloned from *Alicyclobacillus acidocaldarius* (B*acillus acidocaldarius*) that had α-amylase and pullulanase catalytic activity (23). A thermoacidophilic α-amylase expressed from *Alicyclobacillus* sp. A4 which showed 65% identity with the protein encoded by Aaci_2874 also showed high activity toward starch compounds, but the substrate range of the enzyme was not determined (86). Testing was not performed to determine the substrate range of this enzyme. Aaci_2874 is located in what may be an operon containing two other glycoside hydrolases, Aaci_2868 and Aaci_2869, neither of which was regulated to the extent of Aaci_2874 when xylose was added. Since there have been no reports of bifunctional α-amylases, the relationship of this enzyme to WAX depolymerization is not known, but regulation of this gene when *A. acidocaldarius* was grown on WAX indicates that this enzyme has adapted for hydrolysis of xylan polysaccharides.

A gene annotated to encode an α-arabinofuranosidase (Aaci_2894) was down-regulated 3.4-fold when xylose was added to the logarithmically growing *A.*

acidocaldarius culture, but less than 2-fold when glucose was added. During metabolism of WAX, α-arabinofuranosidase catalyzes the hydrolysis of terminal α-arabinofuranosidic linkages to xylose residues in the xylan backbone. This gene is associated with an operon involved in arabinose metabolism, for which all genes were down-regulated, regardless of whether xylose or glucose was used as the inducing sugar. The protein product from this gene has greater than 60% homology to α-arabinofuranosidases found in a number of Gram-positive bacteria, including *Geobacillus, Paenibacillus* and *Thermoanaerobacterium*. As with other genes previously discussed, this or similar operons appear to be conserved among *Alicyclobacillus* species.

Gene Aaci_2887 was up-regulated 3.8-fold when xylose was added and 1.7-fold when glucose was added, and has been annotated as a glycoside hydrolase family 31enzyme. Domain analysis for the encoded protein is consistent with α-xylosidase/α-glucosidase activity, which consists of cleaving the terminal carbohydrate moiety from a variety of substrates. Since the gene was up-regulated upon the addition of xylose, activity of this 779 amino acid glycoside hydrolase may be involved in xylose metabolism, or even cleaving xylose that is linked to other sugars present in WAX. Comparison to other genes in the NCBI database indicates that Aaci_2887 50% identical to α-xylosidase/α-glucosidase enzymes in other Gram-positive lignocellulose utilizing bacteria, including numerous *Paenibacillus* and *Clostridium* species (Figure 16). The protein sequence from Aaci_2887 is 99% similar to an enzyme expressed from recombinant DNA, thermostable α-glucosidase (ABI81478.1) from *A.*

acidocaldarius that was deposited into GenBank, but no information on enzyme activity or specificity has been published. Other glycoside hydrolase family 31 enzymes that display α-xylosidase activity have been found in *Sulfolobus solfataricus* (i.e., XylS) and the Gram-positive *Cellovibrio japonicus* (87, 88).

As shown in Figure 1, the xylose backbone and sugar moieties attached to this backbone can also have acetyl and ferulic acid groups chemically bound in the arabinoxylan structure. For optimal liberation of the sugars in arabinoxylan, a number of carbohydrate esterases (CE) or deacetylase enzymes are also required for removal of these groups. Putative CE enzymes attributed to *A. acidocaldarius* strain DSM 446 as annotated in the CAZy database are listed in Table 4. Typically, CE activity is attributed to families 1-7 and 16, acting on fragments generated by the activity of endo-β-1,4-xylanases (89). Proteins found in CE family 14, such as LmbE family proteins, have broad substrate specificity and can act as carbohydrate esterases (90, 91). Deacetylation activity by this group of enzymes is primarily associated with cell wall rearrangement and sporulation in bacteria, but the broad substrate specificity may allow these enzymes to act on acetyl moieties found in arabinoxylan or even chitin (92). Six of the eight esterase-encoding genes were regulated during the experiments (Figure 17).

Two (Aaci_1372 and Aaci_1443) of the four genes encoding CE family 4 enzymes were up-regulated during the experiments. Aaci_1372 was up-regulated to a greater extent when xylose was added to the *A. acidocaldarius* strain DSM 446 culture growing on WAX, while the converse was true for Aaci_1443, where greater up-

regulation was seen when glucose was added. The protein encoded by Aaci_1372 also contains a signal peptide indicating extracellular localization of this enzyme. The protein encoded by Aaci_1372 is homologous to putative xylanase and chitin deacetylase enzymes found in *Desulfosporosinus* species and to polysaccharide deacetylase enzymes in other Alicyclobacilli (Figure 18). The putative CE family 4 esterase encoded by Aaci_1443 is homologous to polysaccharide deacetylases in other Alicyclobacilli, as well as xylanase/chitin deacetylase enzymes found in a variety of *Anoxybacillus* species. While substrate specificity of the proteins encoded by these two loci has not been determined, up-regulation during the experiment and homology to other CE family 4 proteins may indicate that these enzymes are involved in deacetylation of arabinoxylan. One possibility is that these genes were expressed to remove the acetyl groups from small oligosaccharides, allowing further metabolism of the arabinoxylan fractions.

All three genes encoding LmbE family proteins found in the *A. acidocaldarius* strain DSM 446 genome were regulated during the experiments (See Table 4). Aaci_0881 was up-regulated to similar levels when either glucose or xylose was added, while Aaci_0954 was only up-regulated when xylose was added. In contrast, Aaci_2070 was down-regulated during the experiments, 8.3-fold when xylose was added and 3-fold when glucose was added. The phylogenetic tree in Figure 19 illustrates the homology of the proteins encoded by Aaci_0881 and Aaci_0954 to annotated deacetylases in other Alicyclobacilli and a number of *Geobacillus* species. Aaci_2070 shows homology with deacetylases found in many hyperthermophiles.

While this group of enzymes has been annotated as deacetylases, their specific function in arabinoxylan depolymerization, if any, is not known.

DISCUSSION

Lignocellulose has three components: a hexose-containing component (cellulose); a primarily pentose-containing component (hemicellulose); and an aromatic-containing component (lignin), and accounts for a vast amount of carbon in the Earth's surface environment (17, 93). Due to the massive amount of this material in the environment, many microorganisms have developed the ability to hydrolyze the various components into oligomer and monomer units that are further metabolized for cellular processes, or fermented to produce alcohols and organic acids (14). An important first step in metabolism of lignocellulose is the production of glycoside hydrolases that hydrolyze chemical linkages between the sugars, establishing the secondary structure of the polymeric compounds (i.e., cellulose, hemicellulose and lignin). The primary objective of the current study was to understand gene transcription during growth on xylan (i.e., hemicellulose) by the Gram-positive thermoacidophile, *A. acidocaldarius*. To accomplish this objective, a physiological, molecular and *in silico* approach was carried out to identify the specific glycoside hydrolases active when *A. acidocaldarius* is grown on hemicellulose provided in the form of WAX.

Molecular analysis was carried out using high density oligonucleotide microarray studies, comparing gene expression of *A. acidocaldarius* during logarithmic growth on WAX, to gene expression after a monosaccharide, glucose or xylose, was spiked into the growth medium. If genes were down-regulated when the inducing sugar was

added, the assumption was made that the protein product of the gene was being expressed during growth on WAX. Conversely, if genes were up-regulated when the monosaccharide was added, the assumption was that the gene product was induced by the sugar. *In silico* analysis of the glycoside hydrolases expressed by *A. acidocaldarius* was used to determine how xylan metabolism in *A. acidocaldarius* is related to hemicellulose metabolism in other Bacteria and Archaea. Finally, all of the information was combined to propose a model for xylan metabolism by *A. acidocaldarius,* which is shown in Figure 20.

Steps in arabinoxylan metabolism hypothesized in this model include an initial extracellular depolymerization, followed by numerous steps inside the cell. The first step in the process is extracellular hydrolysis of β-1,4-bonds between internal xylopyranose residues in the arabinoxylan xylose backbone by the α-arabinofuranosidase-like enzyme encoded by Aaci_0048. Studies by Eckert and Schneider have shown xylanase activity by this enzyme (9). Since the remainder of the enzymes that were down-regulated during the experiment do not contain signal sequences, these enzymes are most likely active in the cytoplasm. Inside the cell, smaller β-D-xylopyranose oligosaccharides, including β-D-xylobiose, would be depolymerized by an endo-acting xylosidase (Aaci_0789 and Aaci_2328) and Aaci_2630 and Aaci_2887 for xylobiose molecules. The up-regulation of Aaci_2887 indicates a shift in metabolism to hydrolyze xylobiose residues already inside the cell when the second sugar was added. Removal of α-L-arabinofuranose side groups would then be catalyzed by the α-L-arabinofuranosidase encoded by Aaci_2894.

Approximately six enzymes that are annotated for putative activity toward 4-*O*-methyl glucuronic acid or galactose side groups were regulated during the experiments. While galactose is not shown in the schematic (Figure 20), galactose is often found in arabinoxylan molecules (94). Finally a number of proposed esterase enzymes are thought to be active on acetyl- and feruloyl- (not shown in model) groups on the xylan backbone. Polysaccharide deacetylase (Aaci_1372 and Aaci_1443) and LmbE-like proteins (Aaci_0881, Aaci_0952 and Aaci_2070) were regulated during the experiment. Phylogenetic analysis of enzymes encoded by these genes indicated homology to other xylanase type enzymes and polysaccharide deacetylase, which would be involved in removing acetyl side groups from the arabinoxylan molecule.

In general, *A. acidocaldarius* strain DSM 446 appears to have enzymes necessary for the complete depolymerization of WAX. Likewise, these enzymes were expressed when *A. acidocaldarius* strain DSM 446 was grown on WAX. A number of the enzymes also appear to have a broad substrate range, allowing for hydrolysis of various bonds present in WAX even though the annotated activity of the enzyme would not predict this hydrolysis.

Contrary to classical Gram-positive carbon catabolite repression (CCR), which is stimulated by glucose, regulation of glycoside hydrolase genes in *A. acidocaldarius* was more responsive to xylose than glucose. During CCR in the model Gram-positive bacterium, *Bacillus subtilis*, glucose is the preferred carbon source, and when present in the growth medium, components of CCR regulate expression of genes associated with metabolism of other carbon sources (95, 96). Genes encoding glycoside

hydrolases would typically be negatively regulated through this process (44). Genome analysis indicates the presence of all cellular components associated with CCR, including genes for; catabolite control protein A (*ccpA*), histidine protein (*hpr*), HPr kinase/phosphorylase (*hprK*), and catabolite repression HPr (*crh*). When either glucose or xylose was added to the *A. acidocaldarius* culture, xylose appeared to induce down-regulation of more glycoside hydrolases than was the case for glucose, diverging from classical CCR. A common characteristic of CCR in Gram-positive bacteria is participation of phosphoenolpyruvate-dependent phosphotransferase system (PTS) for sugars (97-99). Analysis of the *A. acidocaldarius* genome indicates that most sugars are transported by the activity of ATP-binding cassette (ABC) type sugar transporters, or proton-type symport systems. The response of *A. acidocaldarius* to sugars appears to be much like the response seen in some lactic acid bacteria, such as *Lactobacillus buchneri* and *L. brevis*, which also have been shown to use non-PTS sugar transporters for sugar metabolism (100, 101).

Glycoside hydrolases, for the most part, appear to be found in clusters, throughout the *A. acidocaldarius* genome. Not all of the glycoside hydrolase genes found at loci within these clusters were regulated during the experiment, indicating that a specific subset of the total arsenal of glycoside hydrolase genes found in *A. acidocaldarius* were used during metabolism of WAX. While specific functions of the glycoside hydrolases was not tested as part of the research discussed, many of the glycoside hydrolases found in *A. acidocaldarius* strain DSM 446 appear to have a broader

substrate range than represented by the glycoside hydrolase family in which the enzymes were placed.

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Table 1. Glycoside hydrolase genes required for complete depolymerization of the cellulose and hemicellulose fractions of lignocellulose and enzymes encoded by genes in the *A. acidocaldarius* genome.

Table 2. Identification of genome loci in *A. acidocaldarius* strain DSM 446 genome that encode putative glycoside hydrolases. Classification of Enzyme Commission and glycoside hydrolase family numbers were obtained from the Carbohydrate-Active enZYme database (CAZY). Signal sequences and transmembrane helices were taken from JGI-IMG, and signal sequences were verified using the SignalP program.

Table 3. Summary of gene products from a genome neighborhood (nucleotides 844681..870620) of the *A. acidocaldarius* strain DSM 446 genome.

Table 4. Identification of genome loci in *A. acidocaldarius* strain DSM 446 genome that encode putative carbohydrate deacetylase enzymes. Classification of Enzyme Commission and carbohydrate deacetylase family numbers were obtained from the Carbohydrate-Active enZYme database (CAZY). Signal sequences and transmembrane helices were taken from JGI-IMG, and signal sequences were verified using the SignalP program.

Figure 1. Enzymes involved in arabinoxylan utilization. Schematic showing basic structure of arabinoxylan and enzymes required to hydrolyze specific bonds leading to liberation of smaller oligosaccharides and sugar monomers, which can then be transported into the bacterial cell.

Figure 2. Regulated glycoside hydrolase genes. Regulation of glycoside hydrolase genes in *A. acidocaldarius* when grown on WAX alone compared to regulation when growing on WAX and either glucose or xylose. See Table 2 for annotation of genes listed on x-axis.

Figure 3. Phylogenetic tree showing distance of α-L-arabinofuranosidase-like protein encoded by gene locus Aaci_0048 in *A. acidocaldarius* strain DSM 446 genome to similarly annotated genes in other Alicyclobacilli and a number of *Actinomycetes*. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 4. Alignment of protein sequence translated from gene locus Aaci_0048 (encodes α-L-arabinofuranosidase-like protein) with similar proteins encoded in genomes of other Alicyclobacilli and *Actinomycetes*.

Figure 5. Schematic showing proximity of Aaci_0048 (encoding an α-L-arabinofuranosidase-like protein) and Aaci_0060 (which encodes a xylan α-1,2-glucuronosidase) from *A. acidocaldarius* strain DSM 446 and other *Alicyclobacillus* species. A) *A. acidocaldarius* strain DSM 446, B) *A. acidocaldarius* strain Tc-4-1, C) *A. hesperidium* strain URH17-3-68. Gene neighborhoods were retrieved using the Gene Ortholog Neighborhood Tool within the JGI-IMG database. Blue arrows identify gene loci in *A. acidocaldarius* strain DSM 446, and red arrows identify genes encoding similar proteins in other *Alicyclobacillus* species.

Figure 6. Phylogenetic tree showing distance of α-1,2-glucuronosidase encoded by gene locus Aaci_0060 in *A. acidocaldarius* strain DSM 446 genome to similarly

annotated genes *Firmicutes*. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 7. A phylogenetic distance tree of the glycoside hydrolase encoded by Aaci_0789, and homology to an endo-β-1,4-mannanase from another *A. acidocaldarius* strain, and the endo-β-1,4-xylanase from a number of other *Firmicutes*. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 8. *A. acidocaldarius* strain DSM 446 genome neighborhood containing glycoside hydrolase encoding genes Aaci_0786, Aaci_0789 and Aaci_0797 has numerous other genes encoding translation products annotated as carbohydrate active enzymes and proteins. Annotated products for each gene are listed in Table 3.

Figure 9. Genome neighborhood showing conserved genes encoding glycoside hydrolases (red boxes) in two *A. acidocaldarius* species, and *A. hesperidum* species URH17-3-68. Green box indicates genes for a glycoside hydrolase, an ABC-transporter and a transcriptional regulator that are present in the *A. acidocaldarius* strain DSM 446 genome but not in *A. acidocaldarius* strain Tc-4-1. (A) *A. acidocaldarius* subsp. *acidocaldarius* (DSM 446), (B) *A. acidocaldarius* subsp. *acidocaldarius* (Tc-4-1), and (C) *A. hesperidum* URH17-3-68

Figure 10. Schematic of apparent operon involved in hydrolysis and transport of wheat arabinoxylan-like poly- and oligosaccharides. Annotated gene products: Aaci_1214 – transcriptional regulator, LacI family; Aaci_1215 – extracellular solute binding protein, family 1; Aaci_1216 – binding protein-dependent transport systems,

inner membrane component; Aaci_1217 - binding protein-dependent transport systems, inner membrane component; and Aaci_1218 – glycoside hydrolase family 2 TIM barrel.

Figure 11. A phylogenetic distance tree showing glycoside hydrolase encoded by Aaci_1895, and homology to a β-galactosidase/β-glucosidase from other *Firmicutes*, as well as a number of *Thermoanaerobacter* species. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 12. Schematic of the structure of an apparent operon associated with a β-galactosidase/β-glucosidase gene (Aaci_1895) in *A. acidocaldarius* strain DSM 446, and comparison to a similar operon in a number of *Thermoanaerobacter* species. A) *A. acidocaldarius* strain DSM 446, B) *T. siderophilis* strain SR4, C) *T. italicus* strain Ab9, D) *T. ethanolicus* strain JW 200. Dashed lines show glycoside hydrolase bearing similarity to Aaci_1895. Opp indicates various components of annotated ATP-binding cassette transporter for dipeptides, and GH indicates glycoside hydrolases.

Figure 13. Schematic showing apparent operon structure associated with Aaci_2475, a gene encoding an endoglucanase, and comparison to other *Alicyclobacillus* species. A) *A. acidocaldarius* strain DSM 446, B) *A. acidocaldarius* strain Tc-4-1, C) *A. hesperidum* strain URH17-3-68, D) general operon structure showing proposed components associated with gene loci for each bacterium.

Figure 14. A phylogenetic distance tree showing glycoside hydrolase encoded by Aaci_2630, and homology to a β-xylosidase enzymes from other thermophilic

lignocellulose degrading bacteria. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 15. Schematic showing glycoside hydrolase genes from the *A. acidocaldarius* strain DSM 446 genome spanning DNA coordinates 2,926,031 to 2,962,624. Short arrows without gene locus are shown to demonstrate the numbers of genes between each glycoside hydrolase and directionality of genes.

Figure 16. A phylogenetic distance tree showing glycoside hydrolase encoded by Aaci_2887, and homology to a α-xylosidase/α-glucosidase enzymes from other lignocellulose degrading *Firmicutes*. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 17. Regulation of carbohydrate deacetylase genes in *A. acidocaldarius* strain DSM 446 when grown on WAX alone compared to regulation when growing on WAX and either glucose or xylose. See Table 4 for annotation of genes listed on x-axis.

Figure 18. A phylogenetic distance tree showing alignment of CE family 4 deacetylase enzymes and homology to deacetylase enzymes from other bacteria. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 19. A phylogenetic distance tree showing three LmbE family proteins encoded by gene loci in *A. acidocaldarius* strain DSM 446 and comparison to other *Firmicutes* and hyperthermophiles. Each gene in tree is listed by Accession number and bacterium carrying gene.

Figure 20. Schematic of arabinoxylan showing chemical structure and proposed *A. acidocaldarius* strain DSM 446 genes annotated to produce enzymes catalyzing hydrolysis of specific bonds in the xylan backbone as well as functional groups attached to the backbone. See Tables 2 and 4 for description of gene products. GH family numbers listed are given to demonstrate common genes associated with hydrolysis of representative bonds, but does not take into account increased specificity of other enzymes that may hydrolyze these bonds also.

Table 1.

Glycosyl Hydrolase Activity Required for Lignocellulose Depolymerization	**Presence in *A. acidocaldarius***
Cellulose	
endo-β-1,4-glucanase (EC 3.2.1.4)	X
1,4-β-glucan glucanohydrolase (EC 3.2.1.74)	
1,4-β-glucan cellobiohydrolase (EC 3.2.1.91)	
β-glucosidase (EC 3.2.1.21)	X
Hemicellulose	
endo-β-1,4-xylanase (EC 3.2.1.8)	X
exo-β-1,4-xylosidase or β-xylosidase (EC 3.2.1.37)	X
α-L-arabinofuranosidase (EC 3.2.1.55)	X
endo-α-1,5-arabinanase (EC 3.2.1.99)	
α-glucuronidase (EC 3.2.1.139)	X
endo-β-1,4-mannanase (EC 3.2.1.78)	
exo-β-1,4-mannosidase (EC 3.2.1.25)	
α-galactosidase (EC 3.2.1.22)	
β-glucosidase (EC 3.2.1.21)	X
endo-β-1,4-galactanase (EC 3.2.1.89)	
acetylxylan esterase (EC 3.1.1.72)	X
acetylmannan esterase (EC 3.1.1.6)	
ferulic and *p*-coumaric acid esterases (EC 3.1.1.73)	X

Table 2.

Gene Locus	Product Name	Enzyme Commission Number	Fam.	Signal Seq.	TM Helices
Aaci_0048	α-L-arabinofuranosidase-like protein			Yes (24/25)	Yes
Aaci_0060	Xylan α-1,2-glucuronosidase	EC:3.2.1.131	67	No	No
Aaci_0332	β-glucosidase	EC:3.2.1.21	3	No	No
Aaci_0786	α-galactosidase	EC:3.2.1.22	36	No	No
Aaci_0789	β-1,4-mannanase	EC:3.2.1.78	113	No	No
Aaci_0797	α-galactosidase	EC:3.2.1.22	4	No	No
Aaci_0912	Amylo-α-1,6-glucosidase	EC:3.2.1.33	13	No	No
Aaci_1218	β-galactosidase	EC:3.2.1.23	2	No	No
Aaci_1895	β-galactosidase/ β-glucosidase	EC:3.2.1.21	1	No	No
Aaci_2328	Endo-1,4-β-xylanase	EC:3.2.1.8	10	No	No
Aaci_2457	Polygalacturonase	EC:3.2.1.15	28	Yes (33/34)	Yes
Aaci_2475	Endoglucanase C	EC:3.2.1.4	9	No	No
Aaci_2630	β-glucosidase / β-xylosidase	EC:3.2.1.21 EC:3.2.1.37	3	No	No
Aaci_2868	α-glucosidase	EC:3.2.1.20	31	No	No
Aaci_2869	Neopullulanase / Cyclomaltodextrinase/ Maltogenic α-amylase	EC:3.2.1.135 EC:3.2.1.54 EC:3.2.1.133	13	No	No
Aaci_2874	α-amylase	EC:3.2.1.1	13	Yes (23/24)	No
Aaci_2887	α-xylosidase	EC:3.2.1.177	31	No	No
Aaci_2891	β-galactosidase	EC:3.2.1.23	42	No	No
Aaci_2894	α-L-arabinofuranosidase	EC:3.2.1.55	51	No	No

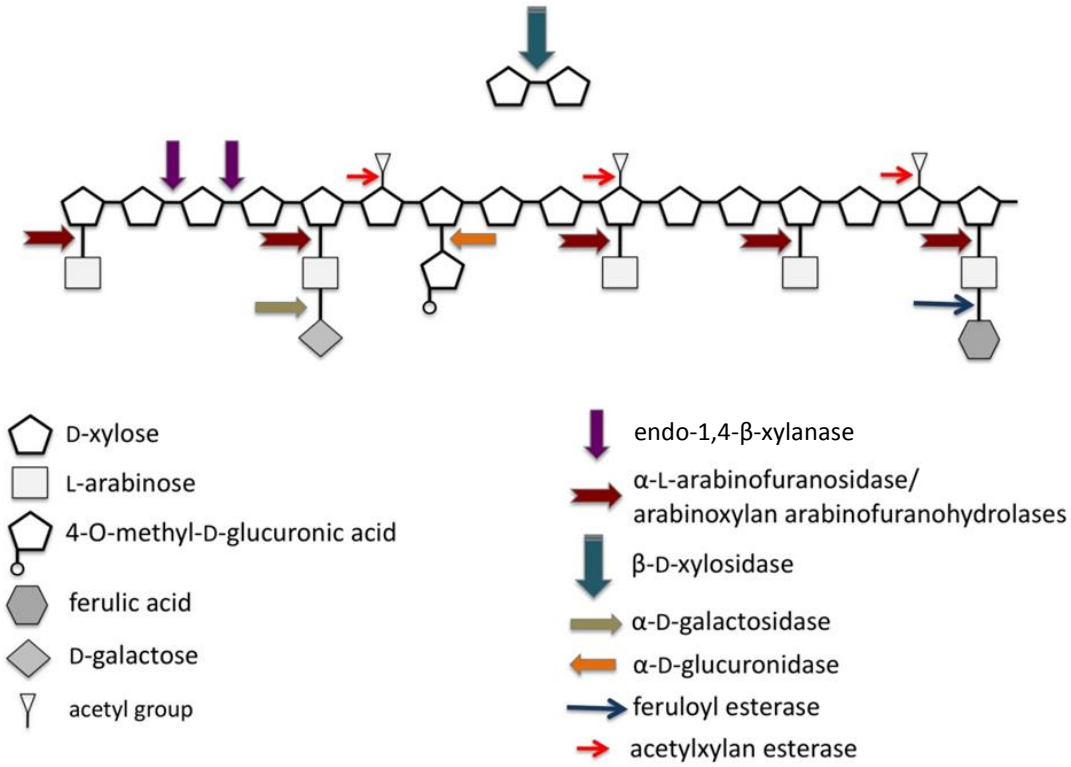
Table 3.

Gene Locus	Product Name	Strand	Size bp	Size – amino acids	Regulated
Aaci_0782	Glycosyl transferase, Group 1	+	1194	397	X-Yes ↑ G-No
Aaci_0783	Glycosyl transferase	+	1215	404	X-Yes ↑ G-No
Aaci_0784	Hypothetical protein	-	498	165	X-No G-No
Aaci_0785	NAD-dependent epimerase/dehydratase	+	849	282	X-Yes ↓ G-No
Aaci_0786	Glycoside hydrolase clan GH-D	+	2205	734	X-Yes ↓ G-No
Aaci_0787	Oxidoreductase domain protein	+	1125	374	X-Yes ↓ G-No
Aaci_0788	Carbohydrate kinase, FGGY-like protein	+	1326	441	X-Yes ↓ G-No
Aaci_0789	Hypothetical protein, glycoside hydrolase superfamily	-	963	320	X-Yes ↓ G-No
Aaci_0790	Hypothetical protein, glyoxalase	-	459	152	X-Yes ↓ G-No
Aaci_0791	Putative carbohydrate binding protein	+	8151	2716	X-No G-No
Aaci_0792	Major facilitator superfamily transporter	-	1377	458	X-Yes ↓ G-Yes ↓
Aaci_0793	Transcriptional regulator, LacI family	+	1023	340	X-No G-Yes ↓
Aaci_0794	Binding protein-dependent transport system, inner membrane component	+	822	273	X-Yes ↓ G-Yes ↓
Aaci_0795	Extracellular solute-binding protein, Family 1	+	1383	460	X-Yes ↓ G-Yes ↓
Aaci_0796	Binding protein-dependent transport system, inner membrane component	+	939	312	X-Yes ↓ G-Yes ↓
Aaci_0797	Glycoside hydrolase, Family 4	+	1299	432	X-Yes ↓ G-No

Table 4.

Gene Locus	Product Name	Enzyme Commission Number	Fam.	Signal Sequence	TM Helices
Aaci_0255	Polysaccharide deacetylase	EC:3.1.1.72 3.5.1.-	4	No	No
Aaci_0863	N-acetylglucosamine-6-phosphate deacetylase	EC:3.5.1.25 3.5.1.80	9	No	No
Aaci_0881	LmbE family protein	EC:3.5.1.-	14	No	No
Aaci_0954	LmbE family protein	EC:3.5.1.-	14	No	No
Aaci_1372	Polysaccharide deacetylase	EC:3.1.1.72 3.5.1.-	4	Yes (28/29)	No
Aaci_1443	Polysaccharide deacetylase	EC:3.1.1.72 3.5.1.-	4	No	No
Aaci_1912	Polysaccharide deacetylase	EC:3.1.1.72 3.5.1.-	4	No	No
Aaci_2070	LmbE family protein	EC:3.5.1.-	14	No	No

Figure 1.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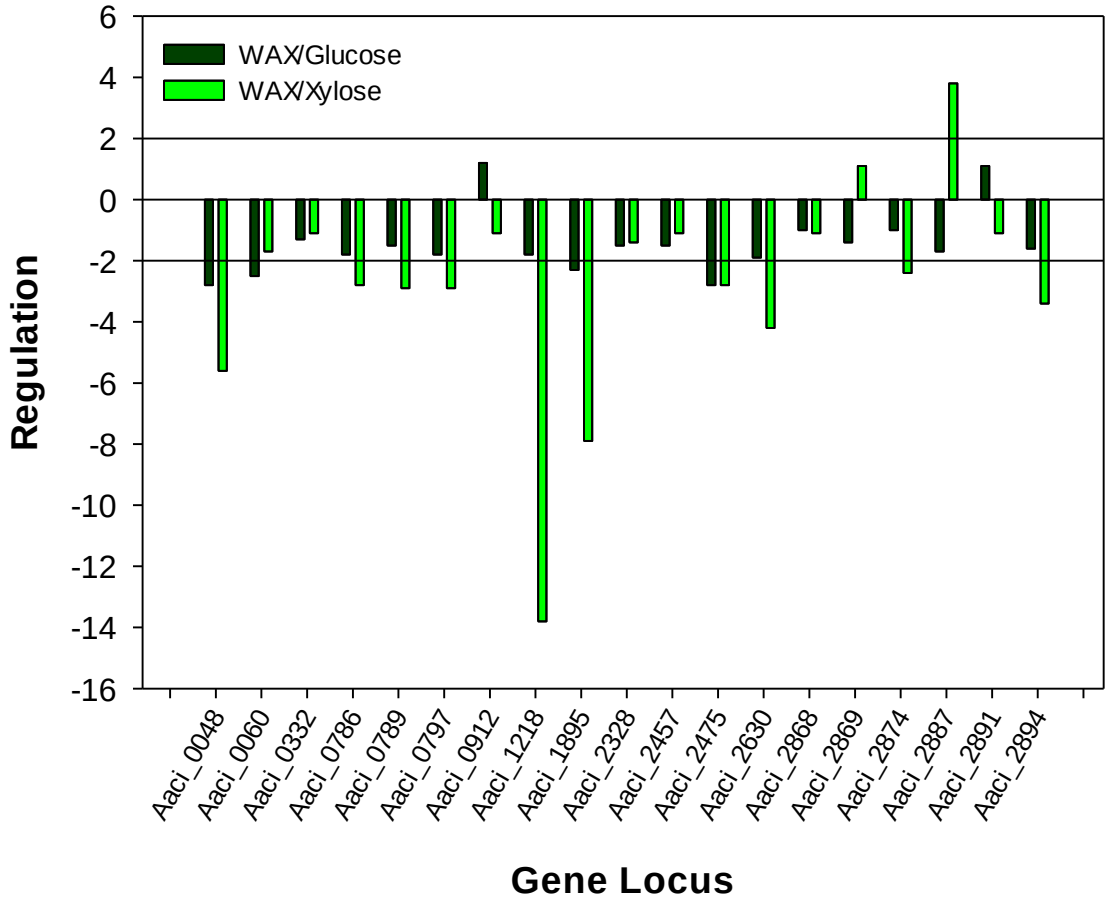
α-D-galactosidase
α-D-glucuronidase
feruloyl esterase
acetylxylan esterase

Figure 2.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Gene Locus

Figure 3.

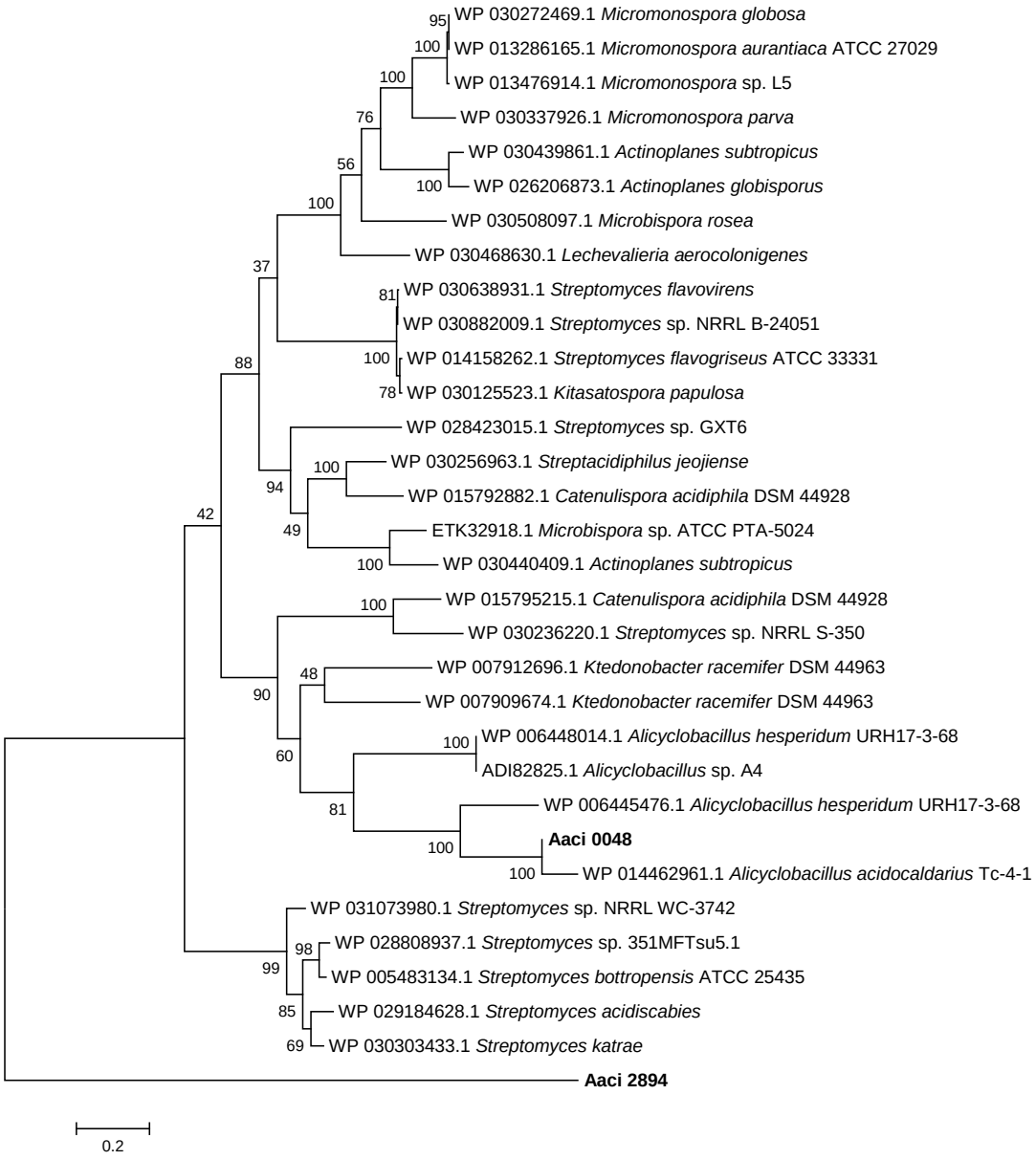
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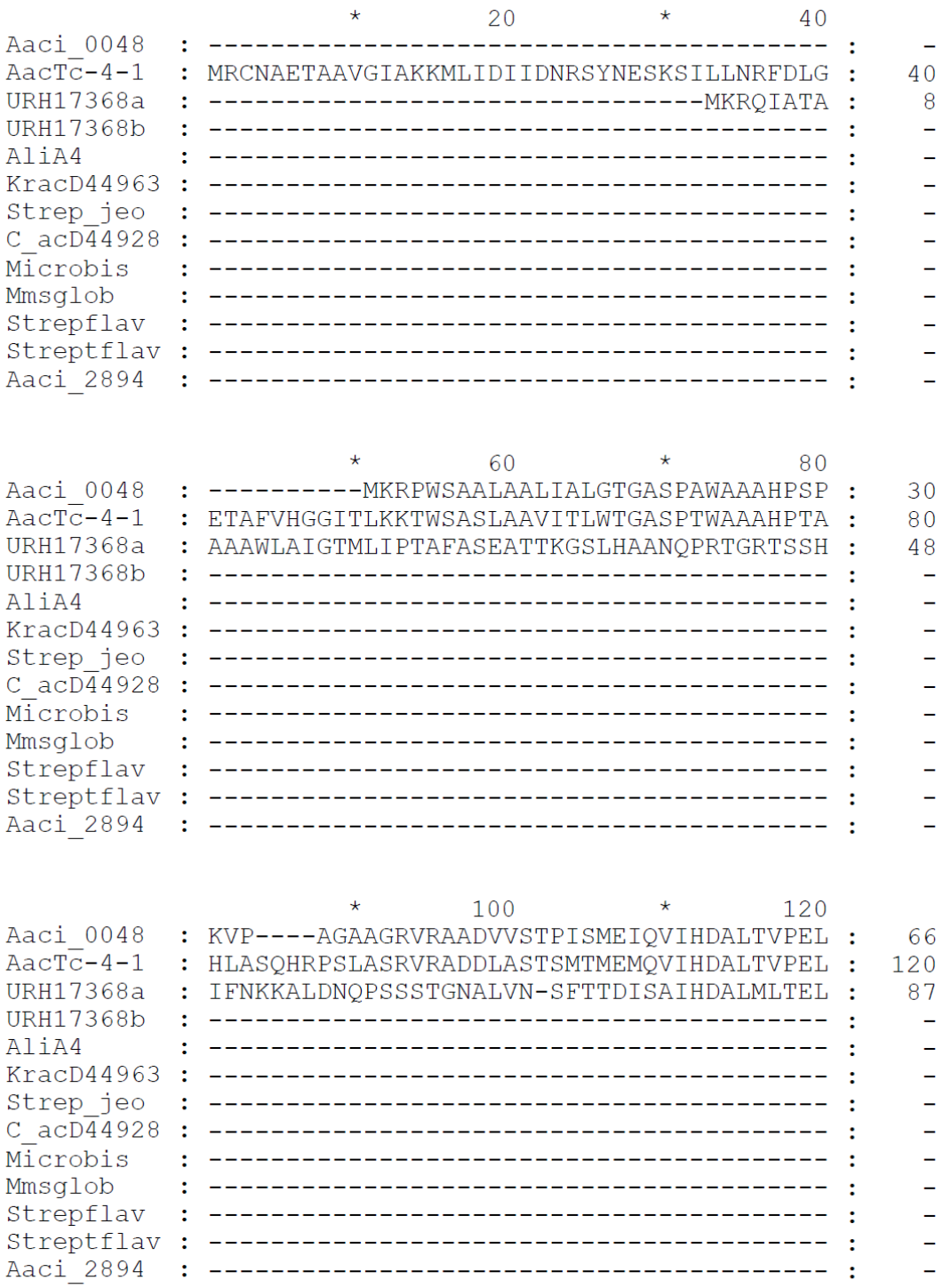
Figure 4.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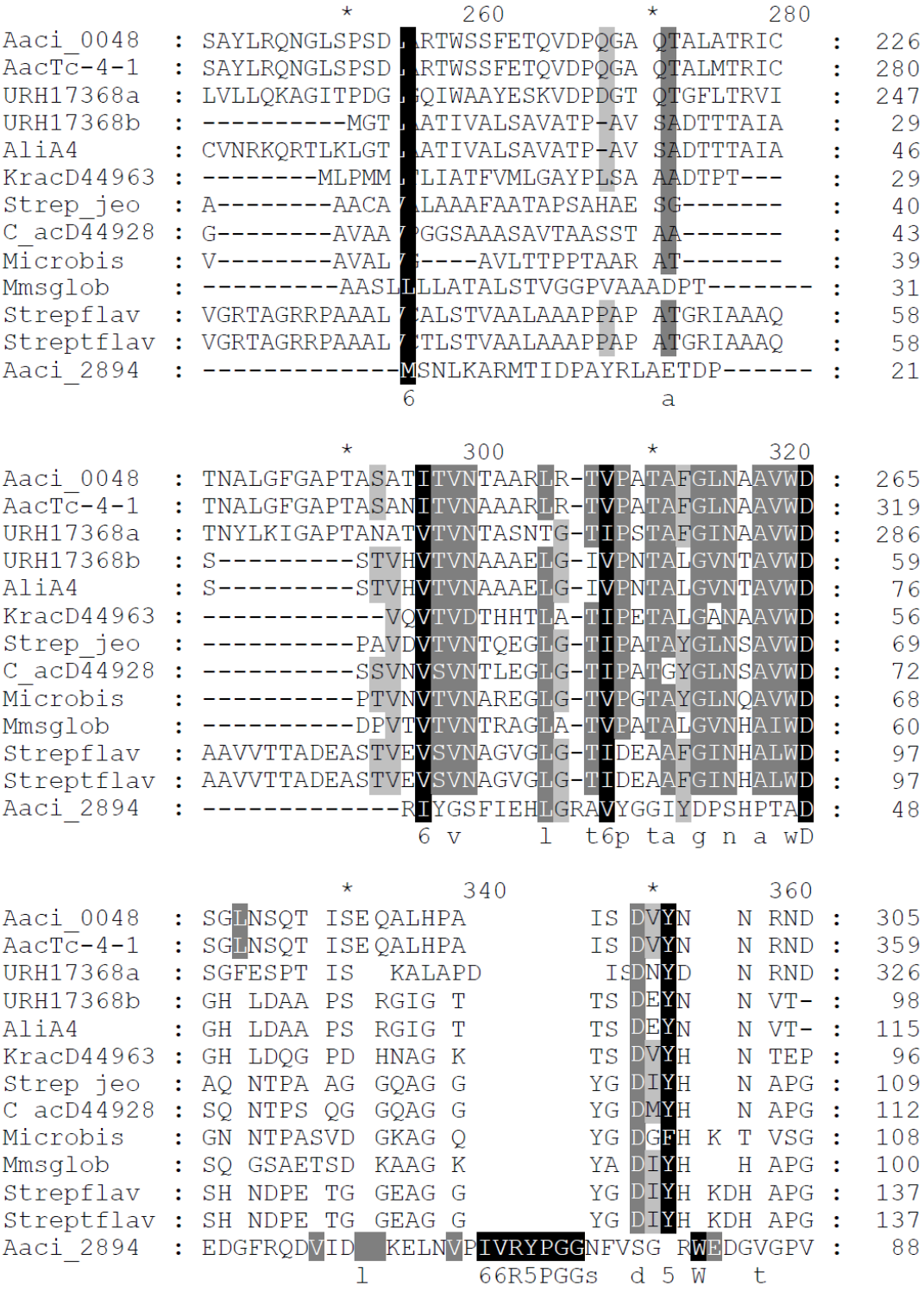
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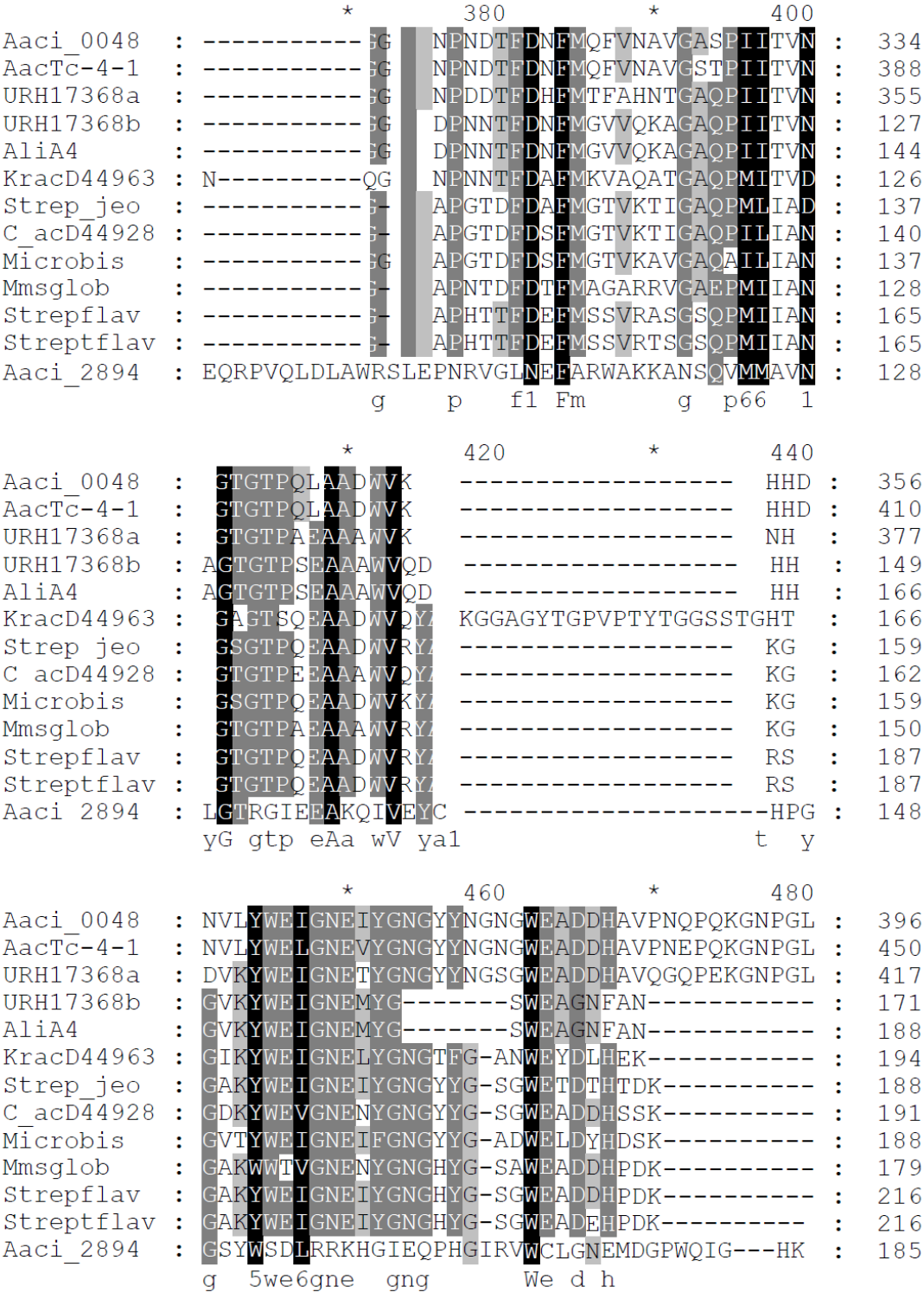
AliA4 : S---------STVHVTVNAAAELG-IVPNTALGVNTAVWD : 76
KracD44963 : ----------VQVTVDTHHTLA-TIPETALGANAAVWD : 56
Strep_jeo : ----------PAVDVTVNTQEGLG-TIPATAYGLNSAVWD : 69
C_acD44928 : ----------SSVNVSVNTLEGLG-TIPATGYGLNSAVWD : 72
Microbis : ----------PTVNVTVNAREGLG-TVPGTAYGLNQAVWD : 68
Mmsglob : ----------DPVTVTVNTRAGLA-TVPATALGVNHAIWD : 60
Strepflav : AAVVTTADEASTVEVSVNAGVGLG-TIDEAAFGINHALWD : 97
Streptflav : AAVVTTADEASTVEVSVNAGVGLG-TIDEAAFGINHALWD : 97
Aaci_2894 : ----------RIYGSFIEHLGRAVYGGIYDPSHPTAD : 48
6 v l t6p ta g n a wD
* 340 * 360
Aaci_0048 : SGLNSQT ISEQALHPA IS DVYN N RND : 305
AacTc-4-1 : SGLNSQT ISEQALHPA IS DVYN N RND : 359
URH17368a : SGFESPT IS KALAPD ISDNYD N RND : 326
URH17368b : GH LDAA PS RGIG T TS DEYN N VT- : 98
AliA4 : GH LDAA PS RGIG T TS DEYN N VT- : 115
KracD44963 : GH LDQG PD HNAG K TS DVYH N TEP : 96
Strep jeo : AQ NTPA AG GQAG G YG DIYH N APG : 109
C acD44928 : SQ NTPS QG GQAG G YG DMYH N APG : 112
Microbis : GN NTPASVD GKAG Q YG DGFH K T VSG : 108
Mmsglob : SQ GSAETSD KAAG K YA DIYH H APG : 100
Strepflav : SH NDPE TG GEAG G YG DIYH KDH APG : 137
Streptflav : SH NDPE TG GEAG G YG DIYH KDH APG : 137
Aaci_2894 : EDGFRQDVID KELNVPIVRYPGGNFVSG RWEDGVGPV : 88
l 66R5PGGs d 5 W t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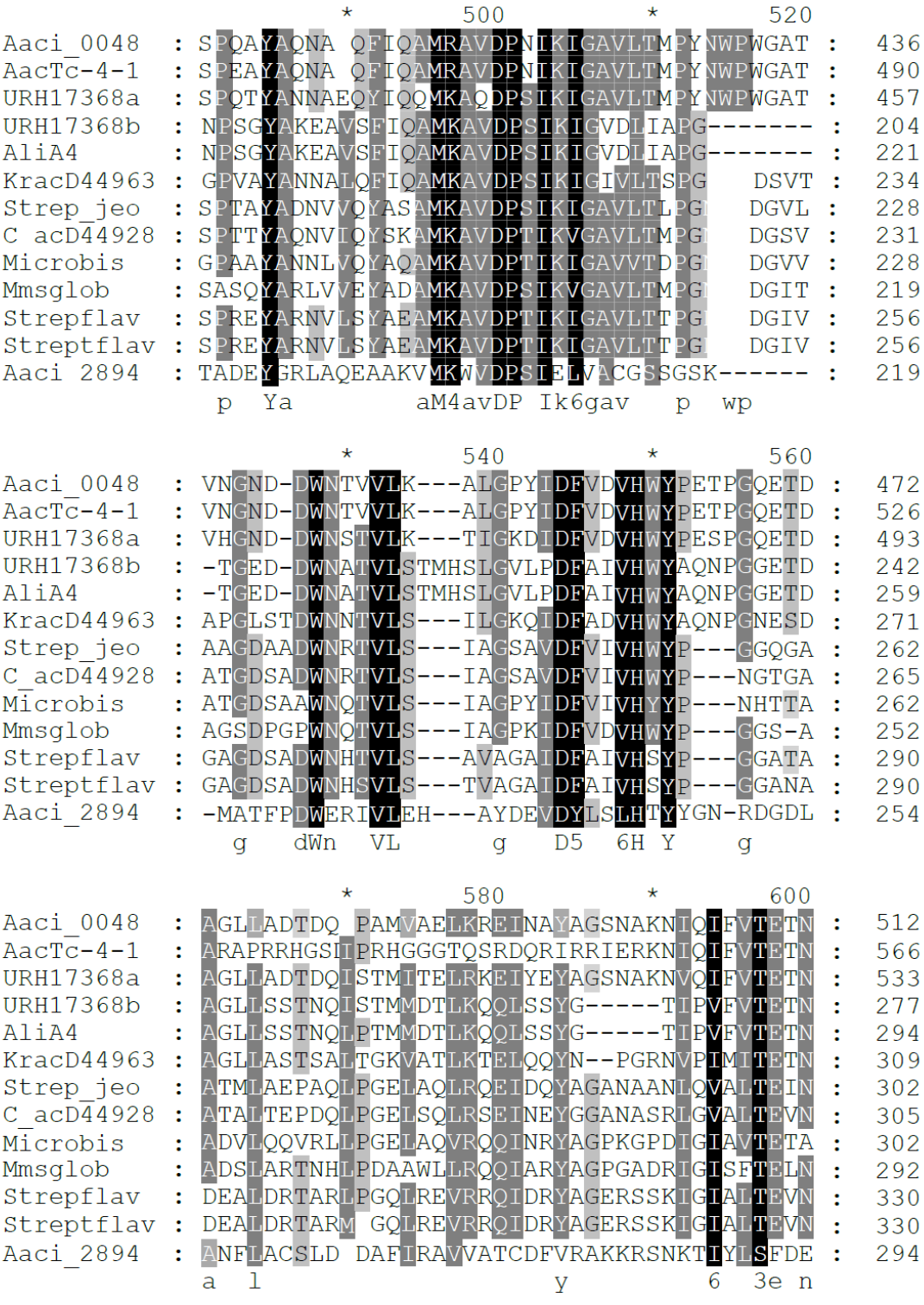
Aaci_0048 : ----------GG NPNDTFDNFMQFVNAVGASPIITVN : 334
AacTc-4-1 : ----------GG NPNDTFDNFMQFVNAVGSTPIITVN : 388
URH17368a : ----------GG NPDDTFDHFMTFAHNTGAQPIITVN : 355
URH17368b : ----------GG DPNNTFDNFMGVVQKAGAQPIITVN : 127
AliA4 : ----------GG DPNNTFDNFMGVVQKAGAQPIITVN : 144
KracD44963 : N----------QG NPNNTFDAFMKVAQATGAQPMITVD : 126
Strep_jeo : ----------G- APGTDFDAFMGTVKTIGAQPMLIAD : 137
C_acD44928 : ----------G- APGTDFDSFMGTVKTIGAQPILIAN : 140
Microbis : ----------GG APGTDFDSFMGTVKAVGAQAILIAN : 137
Mmsglob : ----------G- APNTDFDTFMAGARRVGAEPMIIAN : 128
Strepflav : ----------G- APHTTFDEFMSSVRASGSQPMIIAN : 165
Streptflav : ----------G- APHTTFDEFMSSVRTSGSQPMIIAN : 165
Aaci_2894 : EQRPVQLDLAWRSLEPNRVGLNEFARWAKKANSQVMMAVN : 128
g p f1 Fm g p66 1
* 420 * 440
Aaci_0048 : GTGTPQLAADWVK ---------- HHD : 356
AacTc-4-1 : GTGTPQLAADWVK ---------- HHD : 410
URH17368a : GTGTPAEAAAWVK ---------- NH : 377
URH17368b : AGTGTPSEAAAWVQD ---------- HH : 149
AliA4 : AGTGTPSEAAAWVQD ---------- HH : 166
KracD44963 : GAGTSQEAADWVQYI KGGAGYTGPVPTYTGGSSTGHT : 166
Strep jeo : GSGTPQEAADWVRYI ---------- KG : 159
C acD44928 : GTGTPEEAAAWVQYI ---------- KG : 162
Microbis : GSGTPQEAADWVKYI ---------- KG : 159
Mmsglob : GTGTPAEAAAWVRYI ---------- KG : 150
Strepflav : GTGTPQEAADWVRYI ---------- RS : 187
Streptflav : GTGTPQEAADWVRYI ---------- RS : 187
Aaci 2894 : LGTRGIEEAKQIVEYC ----------HPG : 148
yG gtp eAa wV ya1 t y
* 460 * 480
Aaci_0048 : NVLYWEIGNEIYGNGYYNGNGWEADDHAVPNQPQKGNPGL : 396
AacTc-4-1 : NVLYWELGNEVYGNGYYNGNGWEADDHAVPNEPQKGNPGL : 450
URH17368a : DVKYWEIGNETYGNGYYNGSGWEADDHAVQGQPEKGNPGL : 417
URH17368b : GVKYWEIGNEMYG-------SWEAGNFAN---------- : 171
AliA4 : GVKYWEIGNEMYG-------SWEAGNFAN---------- : 188
KracD44963 : GIKYWEIGNELYGNGTFG-ANWEYDLHEK---------- : 194
Strep_jeo : GAKYWEIGNEIYGNGYYG-SGWETDTHTDK---------- : 188
C_acD44928 : GDKYWEVGNENYGNGYYG-SGWEADDHSSK---------- : 191
Microbis : GVTYWEIGNEIFGNGYYG-ADWELDYHDSK---------- : 188
Mmsglob : GAKWWTVGNENYGNGHYG-SAWEADDHPDK---------- : 179
Strepflav : GAKYWEIGNEIYGNGHYG-SGWEADDHPDK---------- : 216
Streptflav : GAKYWEIGNEIYGNGHYG-SGWEADEHPDK---------- : 216
Aaci_2894 : GSYWSDLRRKHGIEQPHGIRVWCLGNEMDGPWQIG---HK : 185
g 5we6gne gng We d h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Aaci_0048 : SPQAYAQNA QFIQAMRAVDPNIKIGAVLTMPYNWPWGAT : 436
AacTc-4-1 : SPEAYAQNA QFIQAMRAVDPNIKIGAVLTMPYNWPWGAT : 490
URH17368a : SPQTYANNAEQYIQQMKAQDPSIKIGAVLTMPYNWPWGAT : 457
URH17368b : NPSGYAKEAVSFIQAMKAVDPSIKIGVDLIAPG------- : 204
AliA4 : NPSGYAKEAVSFIQAMKAVDPSIKIGVDLIAPG------- : 221
KracD44963 : GPVAYANNALQFIQAMKAVDPSIKIGIVLTSPG DSVT : 234
Strep_jeo : SPTAYADNVVQYASAMKAVDPSIKIGAVLTLPG DGVL : 228
C acD44928 : SPTTYAQNVIQYSKAMKAVDPTIKVGAVLTMPG DGSV : 231
Microbis : GPAAYANNLVQYAQAMKAVDPTIKIGAVVTDPG DGVV : 228
Mmsglob : SASQYARLVVEYADAMKAVDPSIKVGAVLTMPG DGIT : 219
Strepflav : SPREYARNVLSYAEAMKAVDPTIKIGAVLTTPG DGIV : 256
Streptflav : SPREYARNVLSYAEAMKAVDPTIKIGAVLTTPG DGIV : 256
Aaci 2894 : TADEYGRLAQEAAKVMKWVDPSIELVACGSSGSK------ : 219
p Ya aM4avDP Ik6gav p wp
* 540 * 560
Aaci_0048 : VNGND-DWNTVVLK---ALGPYIDFVDVHWYPETPGQETD : 472
AacTc-4-1 : VNGND-DWNTVVLK---ALGPYIDFVDVHWYPETPGQETD : 526
URH17368a : VHGND-DWNSTVLK---TIGKDIDFVDVHWYPESPGQETD : 493
URH17368b : -TGED-DWNATVLSTMHSLGVLPDFAIVHWYAQNPGGETD : 242
AliA4 : -TGED-DWNATVLSTMHSLGVLPDFAIVHWYAQNPGGETD : 259
KracD44963 : APGLSTDWNNTVLS---ILGKQIDFADVHWYAQNPGNESD : 271
Strep_jeo : AAGDAADWNRTVLS---IAGSAVDFVIVHWYP---GGQGA : 262
C_acD44928 : ATGDSADWNRTVLS---IAGSAVDFVIVHWYP---NGTGA : 265
Microbis : ATGDSAAWNQTVLS---IAGPYIDFVIVHYYP---NHTTA : 262
Mmsglob : AGSDPGPWNQTVLS---IAGPKIDFVDVHWYP---GGS-A : 252
Strepflav : GAGDSADWNHTVLS---AVAGAIDFAIVHSYP---GGATA : 290
Streptflav : GAGDSADWNHSVLS---TVAGAIDFAIVHSYP---GGANA : 290
Aaci_2894 : -MATFPDWERIVLEH---AYDEVDYLSLHTYYGN-RDGDL : 254
g dWn VL g D5 6H Y g
* 580 * 600
Aaci_0048 : AGLLADTDQ PAMVAELKREINAYAGSNAKNIQIFVTETN : 512
AacTc-4-1 : ARAPRRHGSIIPRHGGGTQSRDQRIRRIERKNIQIFVTETN : 566
URH17368a : AGLLADTDQISTMITELRKEIYEYAGSNAKNVQIFVTETN : 533
URH17368b : AGLLSSTNQISTMMDTLKQQLSSYG-----TIPVFVTETN : 277
AliA4 : AGLLSSTNQLPTMMDTLKQQLSSYG-----TIPVFVTETN : 294
KracD44963 : AGLLASTSALTGKVATLKTELQQYN--PGRNVPIMITETN : 309
Strep_jeo : ATMLAEPAQLPGELAQLRQEIDQYAGANAANLQVALTEIN : 302
C_acD44928 : ATALTEPDQLPGELSQLRSEINEYGGANASRLGVALTEVN : 305
Microbis : ADVLQQVRLLPGELAQVRQQINRYAGPKGPDIGIAVTETA : 302
Mmsglob : ADSLARTNHLPDAAWLLRQQIARYAGPGADRIGISFTELN : 292
Strepflav : DEALDRTARLPGQLREVRRQIDRYAGERSSKIGIALTEVN : 330
Streptflav : DEALDRTARM GQLREVRRQIDRYAGERSSKIGIALTEVN : 330
Aaci_2894 : ANFLACSLD DAFIRAVVATCDFVRAKKRSNKTIYLSFDE : 294
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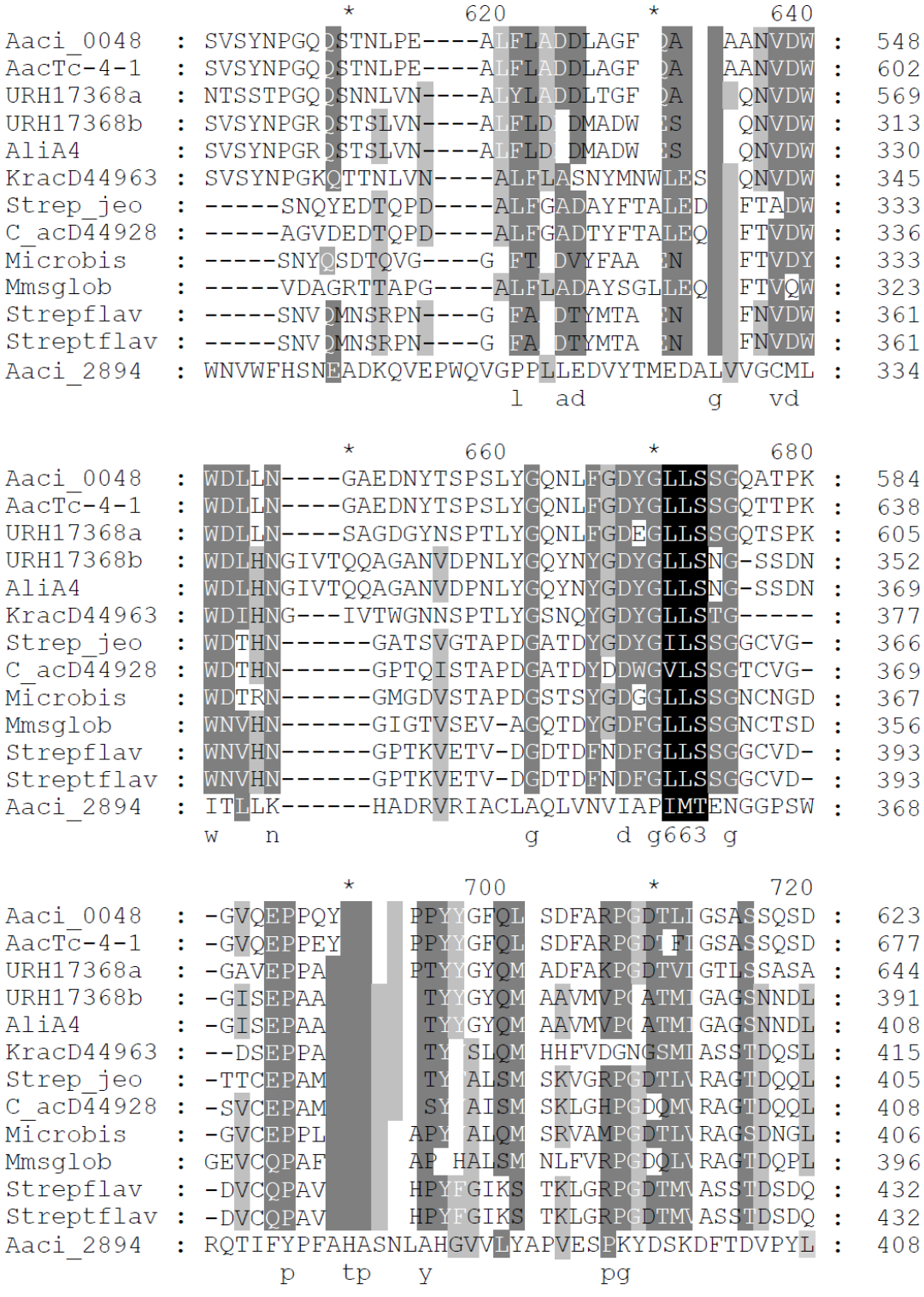
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Aaci_0048 : SVSYNPGQQSTNLPE----ALFLADDLAGF QA AANVDW : 548
AacTc-4-1 : SVSYNPGQQSTNLPE----ALFLADDLAGF QA AANVDW : 602
URH17368a : NTSSTPGQQSNNLVN----ALYLADDLTGF QA QNVDW : 569
URH17368b : SVSYNPGRQSTSLVN----ALFLD DMADW ES QNVDW : 313
AliA4 : SVSYNPGRQSTSLVN----ALFLD DMADW ES QNVDW : 330
KracD44963 : SVSYNPGKQTTNLVN----ALFLASNYMNWLES QNVDW : 345
Strep_jeo : -----SNQYEDTQPD----ALFGADAYFTALED FTADW : 333
C_acD44928 : -----AGVDEDTQPD----ALFGADTYFTALEQ FTVDW : 336
Microbis : -----SNYQSDTQVG----G FT DVYFAA EN FTVDY : 333
Mmsglob : -----VDAGRTTAPG----ALFLADAYSGLLEQ FTVQW : 323
Strepflav : -----SNVQMNSRPN----G FA DTYMTA EN FNVDW : 361
Streptflav : -----SNVQMNSRPN----G FA DTYMTA EN FNVDW : 361
Aaci_2894 : WNVWFHSNEADKQVEPWQVGPPLLEDVYTMEDALVVGCML : 334
l ad g vd
660
680
Aaci_0048 : WDLLN----GAEDNYTSPSLYGQNLFGDYGLLSSGQATPK : 584
AacTc-4-1 : WDLLN----GAEDNYTSPSLYGQNLFGDYGLLSSGQTTPK : 638
URH17368a : WDLLN----SAGDGYNSPTLYGQNLFGDEGLLSSGQTSPK : 605
URH17368b : WDLHNGIVTQQAGANVDPNLYGQYNYGDYGLLSNG-SSDN : 352
AliA4 : WDLHNGIVTQQAGANVDPNLYGQYNYGDYGLLSNG-SSDN : 369
KracD44963 : WDIHNG---IVTWGNNSPTLYGSNQYGDYGLLSTG----- : 377
Strep_jeo : WDTHN------GATSVGTAPDGATDYGDYGILSSGGCVG- : 366
C_acD44928 : WDTHN------GPTQISTAPDGATDYDDWGVLSSGTCVG- : 369
Microbis : WDTRN------GMGDVSTAPDGSTSYGDGGLLSSGNCNGD : 367
Mmsglob : WNVHN------GIGTVSEV-AGQTDYGDFGLLSSGNCTSD : 356
Strepflav : WNVHN------GPTKVETV-DGDTDFNDFGLLSSGGCVD- : 393
Streptflav : WNVHN------GPTKVETV-DGDTDFNDFGLLSSGGCVD- : 393
Aaci_2894 : ITLLK------HADRVRIACLAQLVNVIAPIMTENGGPSW : 368
w n g d g663 g
700
720
Aaci_0048 : -GVQEPPQY PPYYGFQL SDFARPGDTLIGSASSQSD : 623
AacTc-4-1 : -GVQEPPEY PPYYGFQL SDFARPGDTFIGSASSQSD : 677
URH17368a : -GAVEPPA PTYYGYQM ADFAKPGDTVIGTLSSASA : 644
URH17368b : -GISEPAA TYYGYQM AAVMVPQATMIGAGSNNDL : 391
AliA4 : -GISEPAA TYYGYQM AAVMVPQATMIGAGSNNDL : 408
KracD44963 : --DSEPPA TY SLQM HHFVDGNGSMIASSTDQSL : 415
Strep_jeo : -TTCEPAM TY ALSM SKVGRPGDTLVRAGTDQQL : 405
C_acD44928 : -SVCEPAM SY AISM SKLGHPGDQMVRAGTDQQL : 408
Microbis : -GVCEPPL APY ALQM SRVAMPGDTLVRAGSDNGL : 406
Mmsglob : GEVCQPAF AP HALSM NLFVRPGDQLVRAGTDQPL : 396
Strepflav : -DVCQPAV HPYFGIKS TKLGRPGDTMVASSTDSDQ : 432
Streptflav : -DVCQPAV HPYFGIKS TKLGRPGDTMVASSTDSDQ : 432
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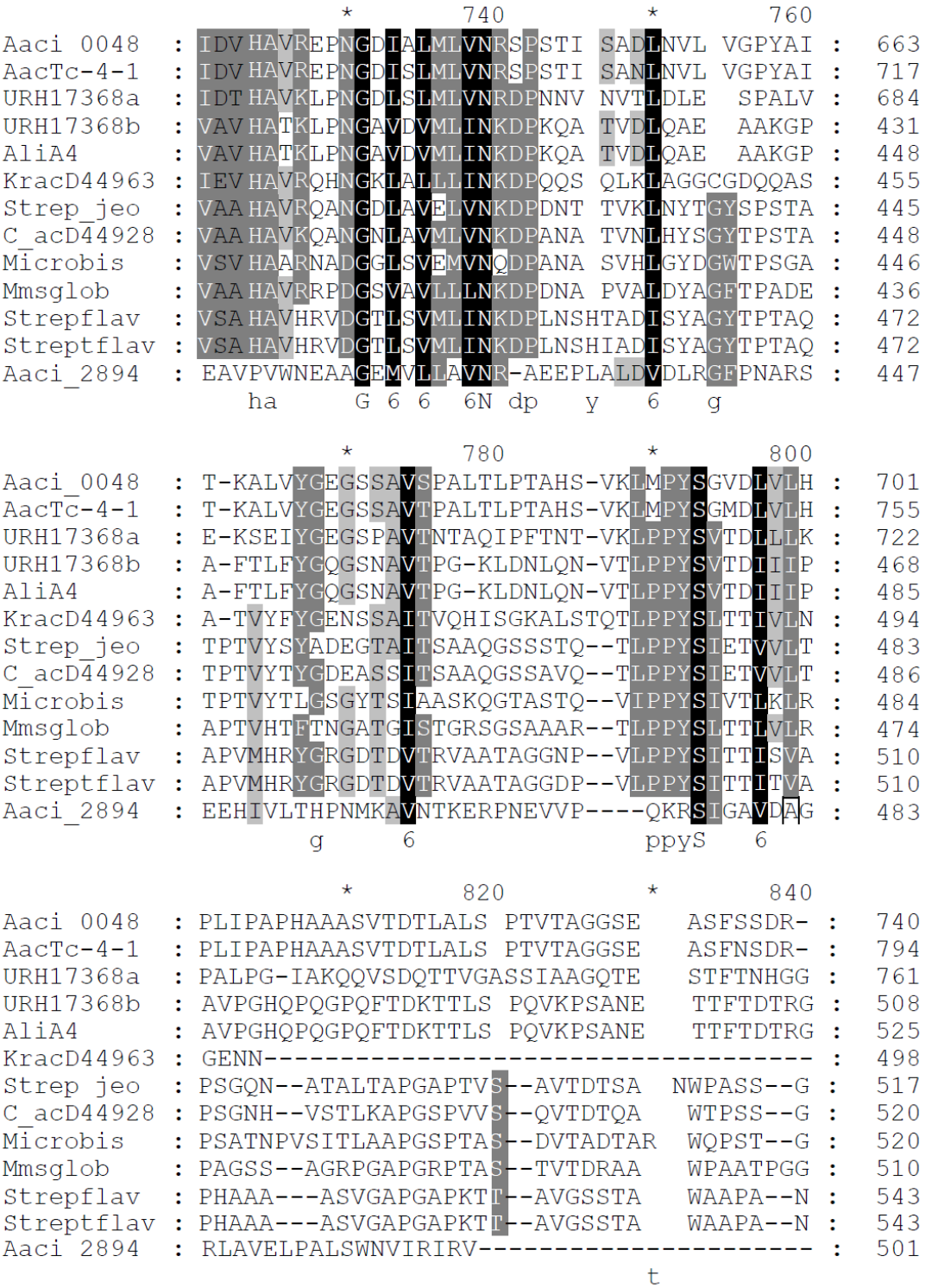
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AacTc-4-1 : IDVHAVREPNGDISLMLVNRSPSTI SANLNVL VGPYAI : 717
URH17368a : IDTHAVKLPNGDLSLMLVNRDPNNV NVTLDLE SPALV : 684
URH17368b : VAVHATKLPNGAVDVMLINKDPKQA TVDLQAE AAKGP : 431
AliA4 : VAVHATKLPNGAVDVMLINKDPKQA TVDLQAE AAKGP : 448
KracD44963 : IEVHAVRQHNGKLALLLINKDPQQS QLKLAGGCGDQQAS : 455
Strep_jeo : VAAHAVRQANGDLAVELVNKDPDNT TVKLNYTGYSPSTA : 445
C_acD44928 : VAAHAVKQANGNLAVMLVNKDPANA TVNLHYSGYTPSTA : 448
Microbis : VSVHAARNADGGLSVEMVNQDPANA SVHLGYDGWTPSGA : 446
Mmsglob : VAAHAVRRPDGSVAVLLLNKDPDNA PVALDYAGFTPADE : 436
Strepflav : VSAHAVHRVDGTLSVMLINKDPLNSHTADISYAGYTPTAQ : 472
Streptflav : VSAHAVHRVDGTLSVMLINKDPLNSHIADISYAGYTPTAQ : 472
Aaci_2894 : EAVPVWNEAAGEMVLLAVNR-AEEPLALDVDLRGFPNARS : 447
ha G 6 6 6N dp y 6 g
* 780 * 800
Aaci_0048 : T-KALVYGEGSSAVSPALTLPTAHS-VKLMPYSGVDLVLH : 701
AacTc-4-1 : T-KALVYGEGSSAVTPALTLPTAHS-VKLMPYSGMDLVLH : 755
URH17368a : E-KSEIYGEGSPAVTNTAQIPFTNT-VKLPPYSVTDLLLK : 722
URH17368b : A-FTLFYGQGSNAVTPG-KLDNLQN-VTLPPYSVTDIIIP : 468
AliA4 : A-FTLFYGQGSNAVTPG-KLDNLQN-VTLPPYSVTDIIIP : 485
KracD44963 : A-TVYFYGENSSAITVQHISGKALSTQTLPPYSLTTIVLN : 494
Strep_jeo : TPTVYSYADEGTAITSAAQGSSSTQ--TLPPYSIETVVLT : 483
C_acD44928 : TPTVYTYGDEASSITSAAQGSSAVQ--TLPPYSIETVVLT : 486
Microbis : TPTVYTLGSGYTSIAASKQGTASTQ--VIPPYSIVTLKLR : 484
Mmsglob : APTVHTFTNGATGISTGRSGSAAAR--TLPPYSLTTLVLR : 474
Strepflav : APVMHRYGRGDTDVTRVAATAGGNP--VLPPYSITTISVA : 510
Streptflav : APVMHRYGRGDTDVTRVAATAGGDP--VLPPYSITTITVA : 510
Aaci_2894 : EEHIVLTHPNMKAVNTKERPNEVVP----QKRSIGAVDAG : 483
g 6 ppyS 6
* 820 * 840
Aaci_0048 : PLIPAPHAAASVTDTLALS PTVTAGGSE ASFSSDR- : 740
AacTc-4-1 : PLIPAPHAAASVTDTLALS PTVTAGGSE ASFNSDR- : 794
URH17368a : PALPG-IAKQQVSDQTTVGASSIAAGQTE STFTNHGG : 761
URH17368b : AVPGHQPQGPQFTDKTTLS PQVKPSANE TTFTDTRG : 508
AliA4 : AVPGHQPQGPQFTDKTTLS PQVKPSANE TTFTDTRG : 525
KracD44963 : GENN---------- : 498
Strep_jeo : PSGQN--ATALTAPGAPTVS--AVTDTSA NWPASS--G : 517
C_acD44928 : PSGNH--VSTLKAPGSPVVS--QVTDTQA WTPSS--G : 520
Microbis : PSATNPVSITLAAPGSPTAS--DVTADTAR WQPST--G : 520
Mmsglob : PAGSS--AGRPGAPGRPTAS--TVTDRAA WPAATPGG : 510
Strepflav : PHAAA---ASVGAPGAPKTT--AVGSSTA WAAPA--N : 543
Streptflav : PHAAA---ASVGAPGAPKTT--AVGSSTA WAAPA--N : 543
Aaci_2894 : RLAVELPALSWNVIRIRV---------- : 501
t

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                        *       860         *       880
Aaci_0048   : PVRDATVELELYDSTGDLVANHEMTGVDIAPGQP-VSE W :  779
AacTc-4-1   : PVHGATVELELYDSTGDLVASHQVTGVDIAPGAP-ASE W :  833
URH17368a   : YLPDATLDLEVYDATGD VAQQTVPHV  SLPAGGS-KLV W :  800
URH17368b   : AVKDGTLDVEIYNPAGQLVGQQVQ GVTFTPGQSSQPI W :  548
AliA4       : AVKDGTLDVEIYNPAGQLVGQQVQ GVTFTPGQSSQPI W :  565
KracD44963  : ---------------------------------------- :    -
Strep jeo   : GAVTRYGVYRQVGTVSELLAESTSTGATLHNLVPGTSY V :  557
C acD44928  : GTATRYEIYRQFGTTSELLAESTSTSATIANLVPGTGY F :  560
Microbis    : GDVTRYAVYQQFGTNSVLLGESTSTSFTARNLVPGTAY L :  560
Mmsglob     : SPIAKYEVHRQYGAVSEQLGETAGTSLTVGNLEPGARY V :  550
Strepflav   : GKAVRYEVYERLGSNAQLLATSTSTSAMLHNLPPGSEH V :  583
Streptflav  : GKAVRYEVYERLGSNAQLLATSTSTSATLHNLPPGSEH V :  583
Aaci 2894   : ---------------------------------------- :    -
                          l

                        *       900         *       920
Aaci_0048   : TFAAPAANGTYTVEAFAFDPATGATYDADTTGATITVNQP :  819
AacTc-4-1   : SFAAPSANGTYSVEAFAFDPATGATYDADTAGATLTVNQP :  873
URH17368a   : NWVAPNNQQTFTVEAYAFSGIGGFVYDADRNAATFTVTPP :  840
URH17368b   : NWTAPDSP TYTVKAFVFSQDGTSVYAADPSAATFTVTQP :  588
AliA4       : NWTAPDSP TYTVKAFVFSQDGTSVYAADPSAATFTVTQP :  605
KracD44963  : ---------------------------------------- :    -
Strep_jeo   : NVLATDQAGHLSQPSAPLTFTTGTPSSSTCAASYQFSDG- :  596
C_acD44928  : NVLATDQSGNLSPPSDPLTFTTGTPAASTCAVDYQVTSG- :  599
Microbis    : NVLATDQKGYLSMPSKPVTFITGTPRNSTCAVTYDLATG- :  599
Mmsglob     : NVLARDTAGRVSWSSPPLTFATGSPAESSCAVRFDDDND- :  589
Strepflav   : NVLARDADGRLSRPSEPLTFATTAPRDSSCLVNYRVDSG- :  622
Streptflav  : NVLARDAEGRLSRPSEPLTFATTAPRDSSCLVNYRVDSG- :  622
Aaci 2894   : ---------------------------------------- :    -
               a    g

                        *       940         *       960
Aaci_0048   : ---------------------------------------- :    -
AacTc-4-1   : ---------------------------------------- :    -
URH17368a   : PLAKYGDIVATGTQVTVSGADQGTYNIPDNNGQYQNGPTI :  880
URH17368b   : ---------------------------------------- :    -
AliA4       : ---------------------------------------- :    -
KracD44963  : ---------------------------------------- :    -
Strep_jeo   : ---------------------------------------- :    -
C_acD44928  : ---------------------------------------- :    -
Microbis    : ---------------------------------------- :    -
Mmsglob     : ---------------------------------------- :    -
Strepflav   : ---------------------------------------- :    -
Streptflav  : ---------------------------------------- :    -
Aaci_2894   : ---------------------------------------- :    -
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                        *       980          *      1000
Aaci_0048   : ---------------------------------------- :    -
AacTc-4-1   : ---------------------------------------- :    -
URH17368a   : KISPGDTVTITTTFKNVSASDFLQNAILDMEIDPGAFQYF :  920
URH17368b   : ---------------------------------------- :    -
AliA4       : ---------------------------------------- :    -
KracD44963  : ---------------------------------------- :    -
Strep_jeo   : ---------------------------------------- :    -
C_acD44928  : ---------------------------------------- :    -
Microbis    : ---------------------------------------- :    -
Mmsglob     : ---------------------------------------- :    -
Strepflav   : ---------------------------------------- :    -
Streptflav  : ---------------------------------------- :    -
Aaci_2894   : ---------------------------------------- :    -

                        *      1020          *      1040
Aaci_0048   : ---------------------------------------- :    -
AacTc-4-1   : ---------------------------------------- :    -
URH17368a   : TQSNNLAPGQSITLTKTWTVPSTLASGTYQLGFQADNNNT :  960
URH17368b   : ---------------------------------------- :    -
AliA4       : ---------------------------------------- :    -
KracD44963  : ---------------------------------------- :    -
Strep_jeo   : ---------------------------------------- :    -
C_acD44928  : ---------------------------------------- :    -
Microbis    : ---------------------------------------- :    -
Mmsglob     : ---------------------------------------- :    -
Strepflav   : ---------------------------------------- :    -
Streptflav  : ---------------------------------------- :    -
Aaci_2894   : ---------------------------------------- :    -

                        *      1060          *      1080
Aaci_0048   : ------------------------PAAKYGDIVTKNTV  :  834
AacTc-4-1   : ------------------------PAAKYGDIVTQNTV  :  888
URH17368a   : WGGSNNCYYQPNVATFAMTNTSAAKPAAKYGDIVTGNTT  : 1000
URH17368b   : -------------------------------DPPTISAT  :  597
AliA4       : -------------------------------DPPTISAT  :  614
KracD44963  : ---------------------------------------- :    -
Strep_jeo   : ----------------------------WGSGWVANIT  :  607
C_acD44928  : ----------------------------WGSGYVTAIT  :  610
Microbis    : ----------------------------WGSGFVANIS  :  610
Mmsglob     : ----------------------------WGNGYVANVE  :  600
Strepflav   : ----------------------------WGNGFVATVT  :  633
Streptflav  : ----------------------------WGNGFVATVT  :  633
Aaci_2894   : ---------------------------------------- :    -
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                      *      1100          *      1120
Aaci_0048   : TVNGTTYTVPAPDASGHYPSGTNISIAPGDTVTIQTT AN :   874
AacTc-4-1   : TVNGTTYTVPAPDANGHYPSGTNISIAPGDTVTIQTT AN :   928
URH17368a   : SVNGTTYNVPAPDGSGHYPSGTNISVQPGDTVTITTT QN : 1040
URH17368b   : QLSATTVKVGTP-------------------VTITTT TE :   618
AliA4       : QLSATTVKVGTP-------------------VTITTT TE :   635
KracD44963  : ---------------------------------------- :     -
Strep_jeo   : TDTGPAA-----------------------IDGWTLN SF :   624
C_acD44928  : TDTGPAP-----------------------INGWSLT TF :   627
Microbis    : TNTGTTP-----------------------ITGWTLA SF :   627
Mmsglob     : INTGAKA-----------------------VDGWTLT TW :   617
Strepflav   : SNVGDSP-----------------------INGWTLD TW :   650
Streptflav  : SNVGDSP-----------------------IDGWTLD TW :   650
Aaci_2894   : ---------------------------------------- :     -

                      *      1140          *      1160
Aaci_0048   : VSSTDA QNGLIDMEVDGQNGAIFQKYWPSTTLLPGQTET :   914
AacTc-4-1   : VSSTDA QNGLIDVEVDGQSGAIFQKYWPSTTLLPGQTQT :   968
URH17368a   : VSSSDY QNGLLDIEVDGSSGSVFQQTAQDVTLNPGDTKT : 1080
URH17368b   : TAPTGY NNGLLVQYAVYNNWTSSQQSNPTATLTPG--QS :   656
AliA4       : TAPTGY NNGLLVQYAVYNNWTSSQQSNPTATLTPG--QS :   673
KracD44963  : ---------------------------------------- :     -
Strep_jeo   : PDAGESVS-SGWNGNWTTTGTSVRVTSLDSNAQLAPYSGN :   663
C_acD44928  : PSTSETLS-SGWNATWTGTGQNIEATSLSWNANLAANGGN :   666
Microbis    : PATTESVSDSTWNATFAGDGQHVVVTPAGSNTYLAANGGN :   667
Mmsglob     : PTAWQQVS-SGWSATWDQQGRDVRVTPTADNRRLAADGG- :   655
Strepflav   : PSTGQTVQ-SFWNANVTSSGTRVHVTDNGANAKLAPRSGS :   689
Streptflav  : PSTGQTVQ-SFWNANVTSSGTRVHVTDNGANAKLAPRSGS :   689
Aaci_2894   : ---------------------------------------- :     -

                      *      1180          *      1200
Aaci_0048   : VTATWQVPSSVSAGTYPLNFQAFD SNW GNCYFTNGGVV :   954
AacTc-4-1   : VTATWQVPSSVTAGTYPLNFQAFD SNW GNCYFTNGGVV : 1008
URH17368a   : VSVTWNVPSSQASGTYTLMFQAFDNSDW GNCYYTNGGVA : 1120
URH17368b   : VTETWTFTP-EQAGTYTFPEGIF- SGW -QLQWINQNVT :   693
AliA4       : VTETWTFTP-EQAGTYTFPEGIF- SGW -QLQWINQNVT :   710
KracD44963  : ---------------------------------------- :     -
Strep_jeo   : STTVGFVGSNTGAYGPPTAISLNGTVCT TYTS------- :   696
C_acD44928  : SASIGFVGNNTGAYPSPAAISLNGTVCS TYSS------- :   699
Microbis    : TVSFGFVGNQTGANPPPASFTLNGTVCT TYSS------- :   700
Mmsglob     : STTVGFVGAYSGPNVPVGAFRLNGTVCTLR---------- :   685
Strepflav   : TAIFGFVGANDDANPTPTTFTLNGTVCR S---------- :   719
Streptflav  : TATFGFVGANDGANPTPTTFTLNGTVCR S---------- :   719
Aaci_2894   : ---------------------------------------- :     -
                                        t   t
```

```
Aaci_0048   : NFVVN :  959
AacTc-4-1   : NFVVS : 1013
URH17368a   : DIDVG : 1125
URH17368b   : LTVTN :  698
AliA4       : LTVTN :  715
KracD44963  : ----- :    -
Strep_jeo   : ----- :    -
C_acD44928  : ----- :    -
Microbis    : ----- :    -
Mmsglob     : ----- :    -
Strepflav   : ----- :    -
Streptflav  : ----- :    -
Aaci_2894   : ----- :    -
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Figure 5.

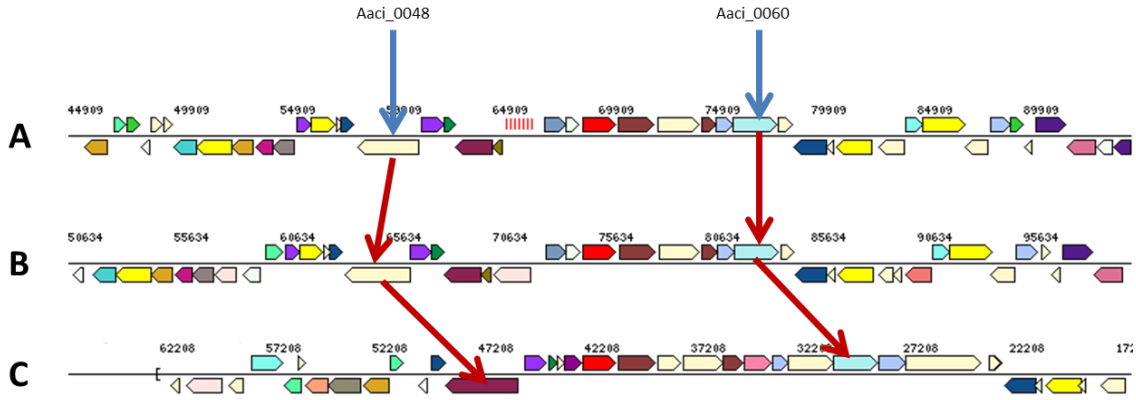
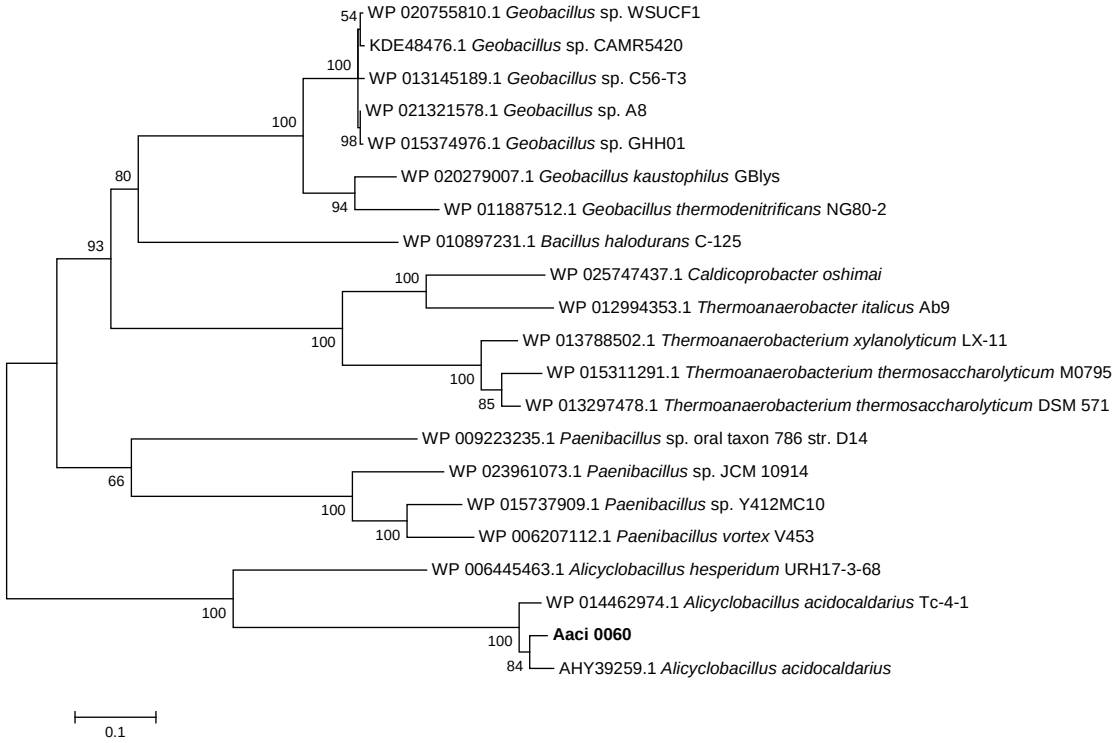


Figure 6.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WP 021321578.1 *Geobacillus* sp. A8
WP 015374976.1 *Geobacillus* sp. GHH01
WP 020279007.1 *Geobacillus kaustophilus* GBlys
WP 011887512.1 *Geobacillus thermodenitrificans* NG80-2
WP 010897231.1 *Bacillus halodurans* C-125
WP 025747437.1 *Caldicoprobacter oshimai*
WP 012994353.1 *Thermoanaerobacter italicus* Ab9
WP 013788502.1 *Thermoanaerobacterium xylanolyticum* LX-11
WP 015311291.1 *Thermoanaerobacterium thermosaccharolyticum* M0795
WP 013297478.1 *Thermoanaerobacterium thermosaccharolyticum* DSM 571
WP 009223235.1 *Paenibacillus* sp. oral taxon 786 str. D14
WP 023961073.1 *Paenibacillus* sp. JCM 10914
WP 015737909.1 *Paenibacillus* sp. Y412MC10
WP 006207112.1 *Paenibacillus vortex* V453
WP 006445463.1 *Alicyclobacillus hesperidum* URH17-3-68
WP 014462974.1 *Alicyclobacillus acidocaldarius* Tc-4-1
Aaci 0060
AHY39259.1 *Alicyclobacillus acidocaldarius*
54
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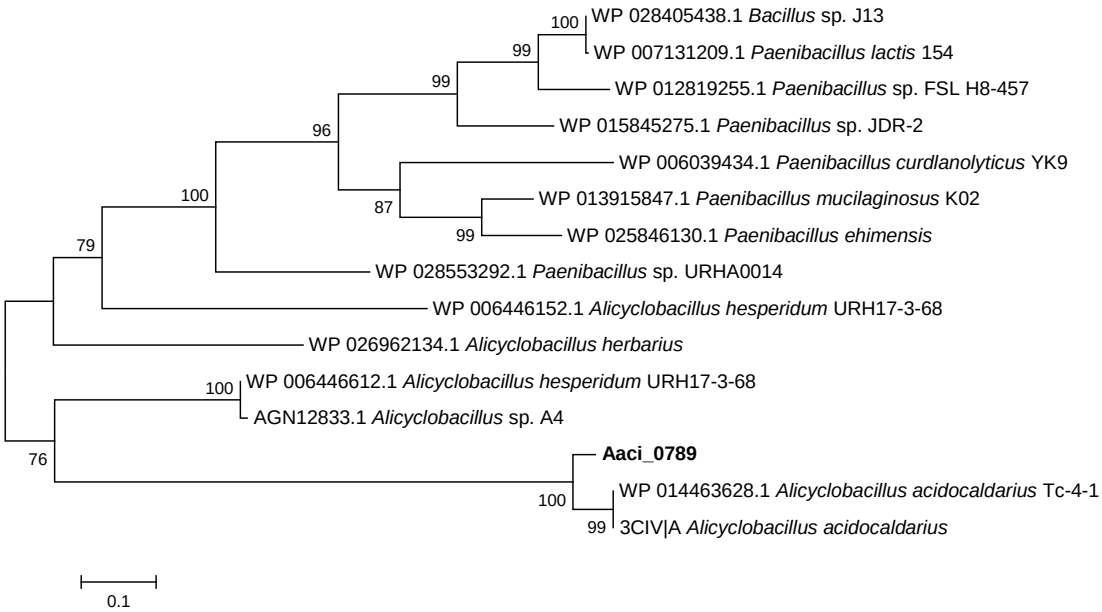
98
80
94
93
100
100
100
85
66
100
100
100
84
0.1

Figure 7.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WP 007131209.1 Paenibacillus lactis 154
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WP 012819255.1 Paenibacillus sp. FSL H8-457
WP 015845275.1 Paenibacillus sp. JDR-2
96
WP 006039434.1 Paenibacillus curdlanolyticus YK9
100
87
WP 013915847.1 Paenibacillus mucilaginosus K02
99
WP 025846130.1 Paenibacillus ehimensis
79
WP 028553292.1 Paenibacillus sp. URHA0014
WP 006446152.1 Alicyclobacillus hesperidum URH17-3-68
WP 026962134.1 Alicyclobacillus herbarius
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WP 006446612.1 Alicyclobacillus hesperidum URH17-3-68
AGN12833.1 Alicyclobacillus sp. A4
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Aaci_0789
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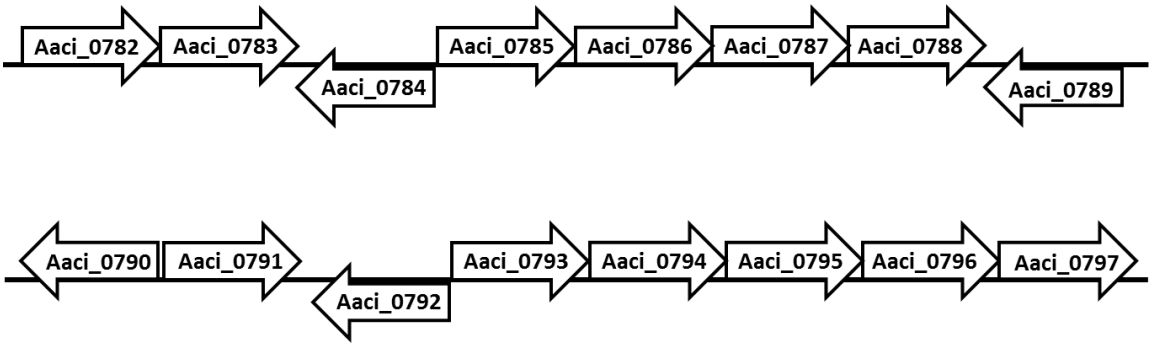
WP 014463628.1 Alicyclobacillus acidocaldarius Tc-4-1
99
3CIV|A Alicyclobacillus acidocaldarius
0.1

Figure 8.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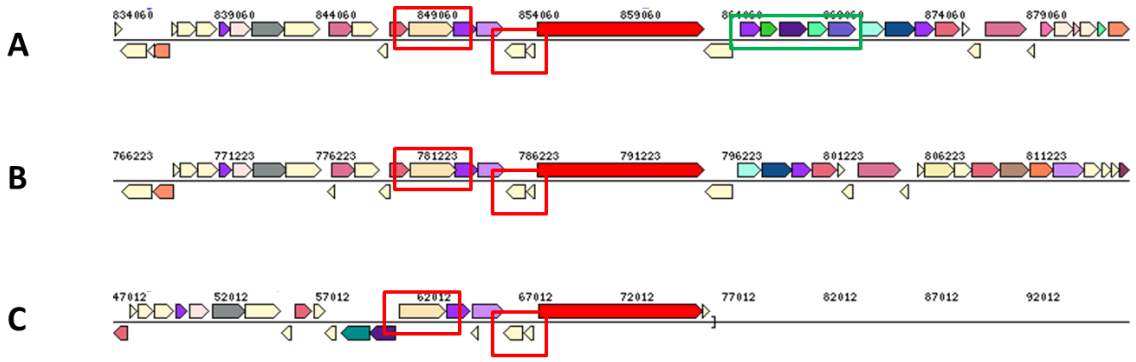
Aaci_0784
Aaci_0785
Aaci_0786
Aaci_0787
Aaci_0788
Aaci_0789
Aaci_0790
Aaci_0791
Aaci_0792
Aaci_0793
Aaci_0794
Aaci_0795
Aaci_0796
Aaci_0797

Figure 9.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834060
839060
844060
849060
854060
859060
874060
879060
B
766223
771223
776223
781223
786223
791223
796223
801223
806223
811223
C
47012
52012
57012
62012
67012
72012
77012
82012
87012
92012

Figure 10.

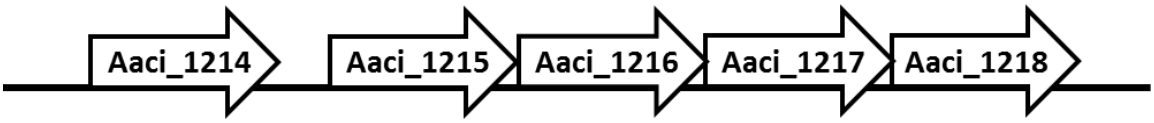
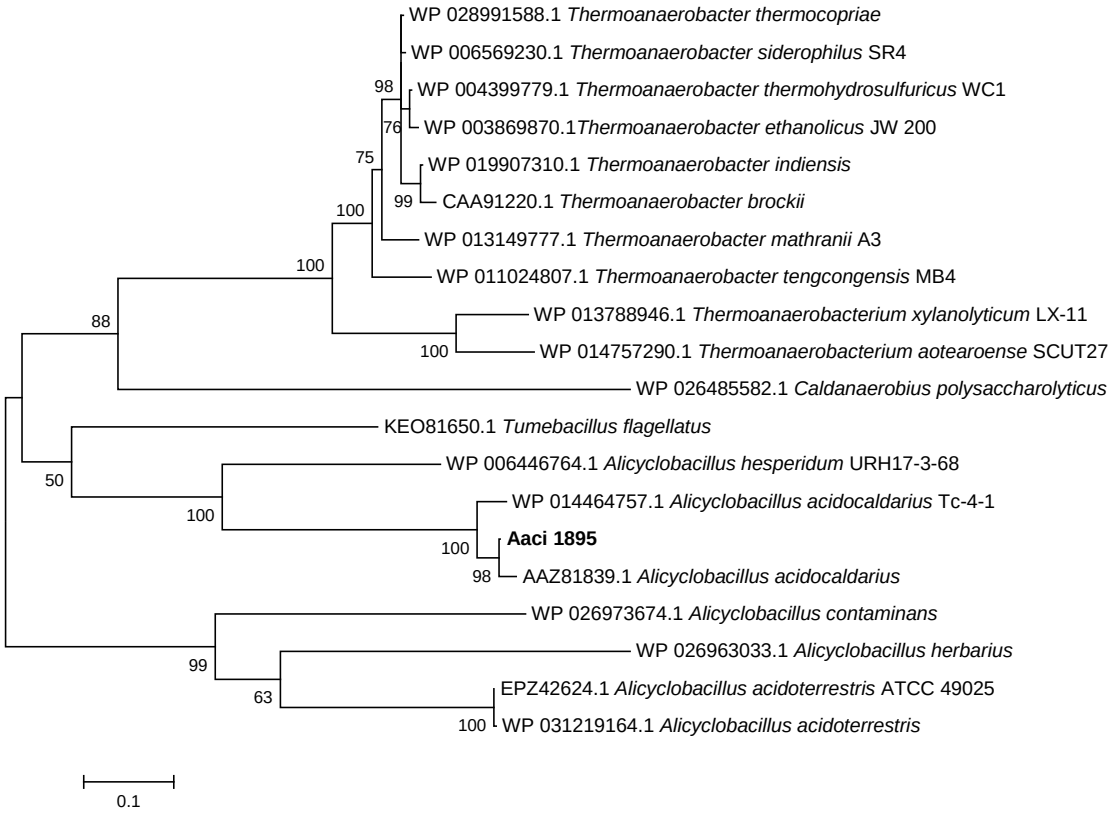


Figure 11.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WP 006569230.1 Thermoanaerobacter siderophilus SR4
98
WP 004399779.1 Thermoanaerobacter thermohydrosulfuricus WC1
76
WP 003869870.1Thermoanaerobacter ethanolicus JW 200
75
WP 019907310.1 Thermoanaerobacter indiensis
99
CAA91220.1 Thermoanaerobacter brockii
100
WP 013149777.1 Thermoanaerobacter mathranii A3
100
WP 011024807.1 Thermoanaerobacter tengcongensis MB4
88
WP 013788946.1 Thermoanaerobacterium xylanolyticum LX-11
100
WP 014757290.1 Thermoanaerobacterium aotearoense SCUT27
WP 026485582.1 Caldanaerobius polysaccharolyticus
KEO81650.1 Tumebacillus flagellatus
WP 006446764.1 Alicyclobacillus hesperidum URH17-3-68
50
WP 014464757.1 Alicyclobacillus acidocaldarius Tc-4-1
100
Aaci 1895
100
98
AAZ81839.1 Alicyclobacillus acidocaldarius
WP 026973674.1 Alicyclobacillus contaminans
WP 026963033.1 Alicyclobacillus herbarius
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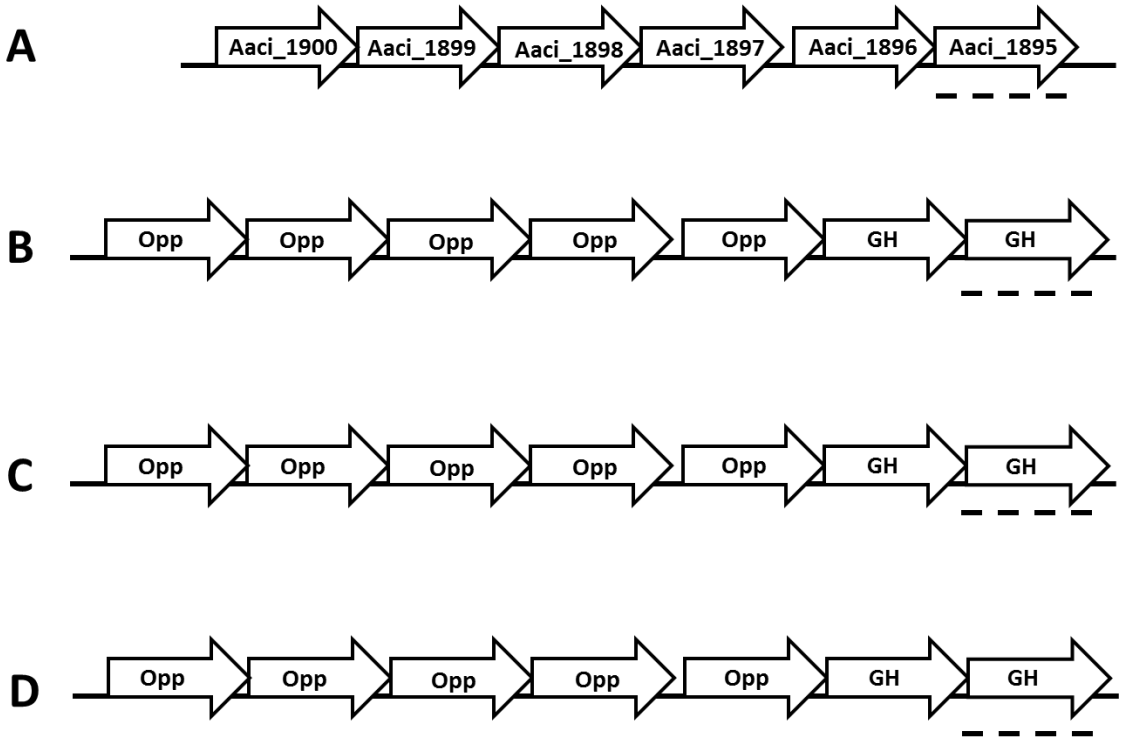
EPZ42624.1 Alicyclobacillus acidoterrestris ATCC 49025
63
100
WP 031219164.1 Alicyclobacillus acidoterrestris
0.1

Figure 12.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Aaci_1899
Aaci_1898
Aaci_1897
Aaci_1896
Aaci_1895
B
Opp
Opp
Opp
Opp
Opp
GH
GH
C
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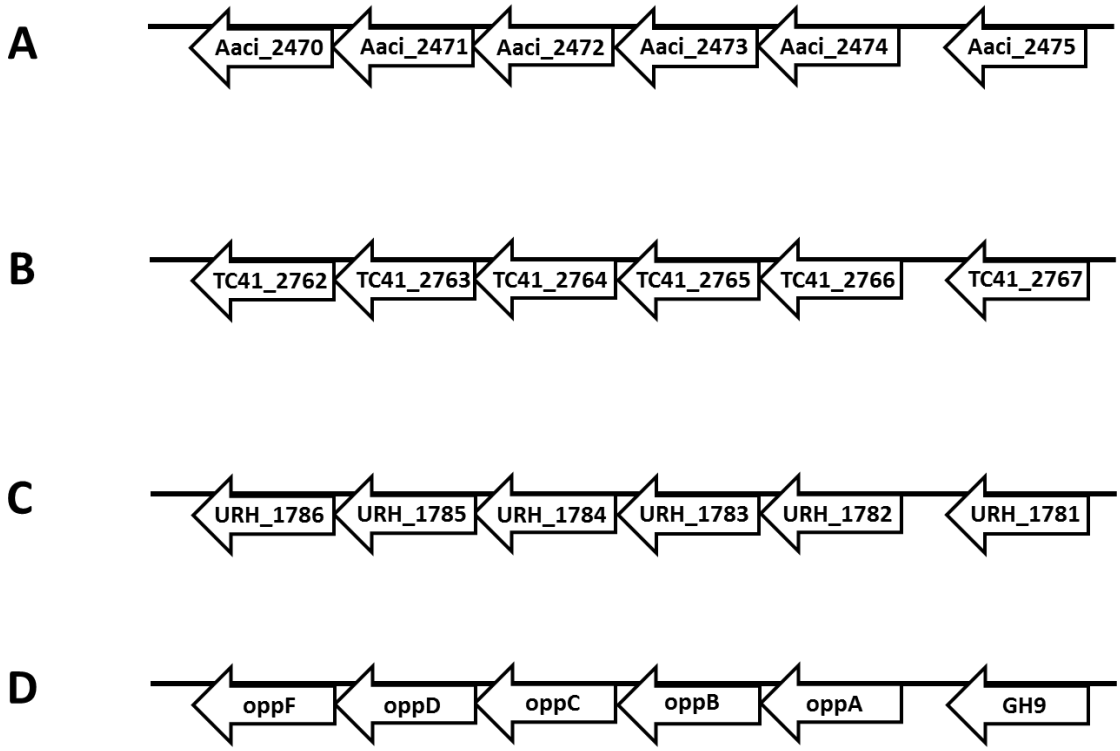
Opp
Opp
Opp
Opp
GH
GH
D
Opp
Opp
Opp
Opp
Opp
GH
GH

Figure 13.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Aaci_2474
Aaci_2475
B
TC41_2762
TC41_2763
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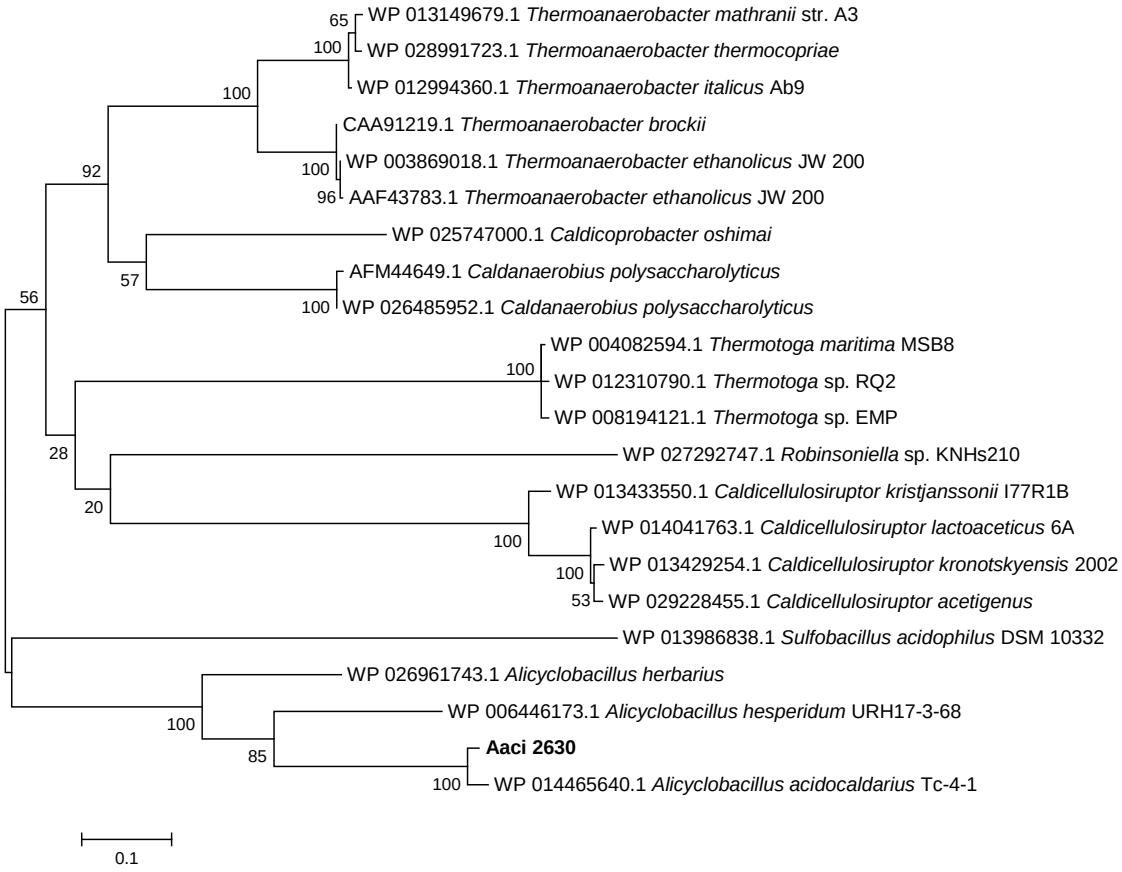
TC41_2765
TC41_2766
TC41_2767
C
URH_1786
URH_1785
URH_1784
URH_1783
URH_1782
URH_1781
D
oppF
oppD
oppC
oppB
oppA
GH9

Figure 14.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WP 012994360.1 Thermoanaerobacter italicus Ab9
CAA91219.1 Thermoanaerobacter brockii
WP 003869018.1 Thermoanaerobacter ethanolicus JW 200
AAF43783.1 Thermoanaerobacter ethanolicus JW 200
WP 025747000.1 Caldicoprobacter oshimai
AFM44649.1 Caldanaerobius polysaccharolyticus
WP 026485952.1 Caldanaerobius polysaccharolyticus
WP 004082594.1 Thermotoga maritima MSB8
WP 012310790.1 Thermotoga sp. RQ2
WP 008194121.1 Thermotoga sp. EMP
WP 027292747.1 Robinsoniella sp. KNHs210
WP 013433550.1 Caldicellulosiruptor kristjanssonii I77R1B
WP 014041763.1 Caldicellulosiruptor lactoaceticus 6A
WP 013429254.1 Caldicellulosiruptor kronotskyensis 2002
WP 029228455.1 Caldicellulosiruptor acetigenus
WP 013986838.1 Sulfobacillus acidophilus DSM 10332
WP 026961743.1 Alicyclobacillus herbarius
WP 006446173.1 Alicyclobacillus hesperidum URH17-3-68
Aaci 2630
WP 014465640.1 Alicyclobacillus acidocaldarius Tc-4-1
65
100
100
92
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96
57
56
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100
28
20
100
100
53
100
85
0.1

Figure 15.

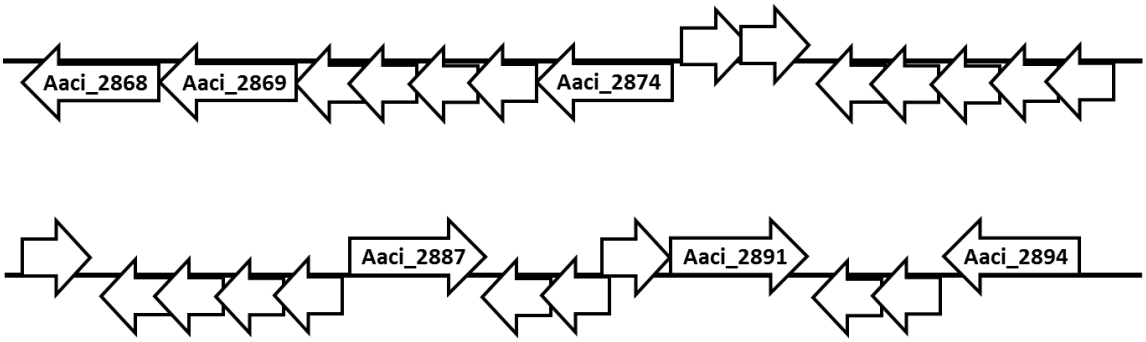
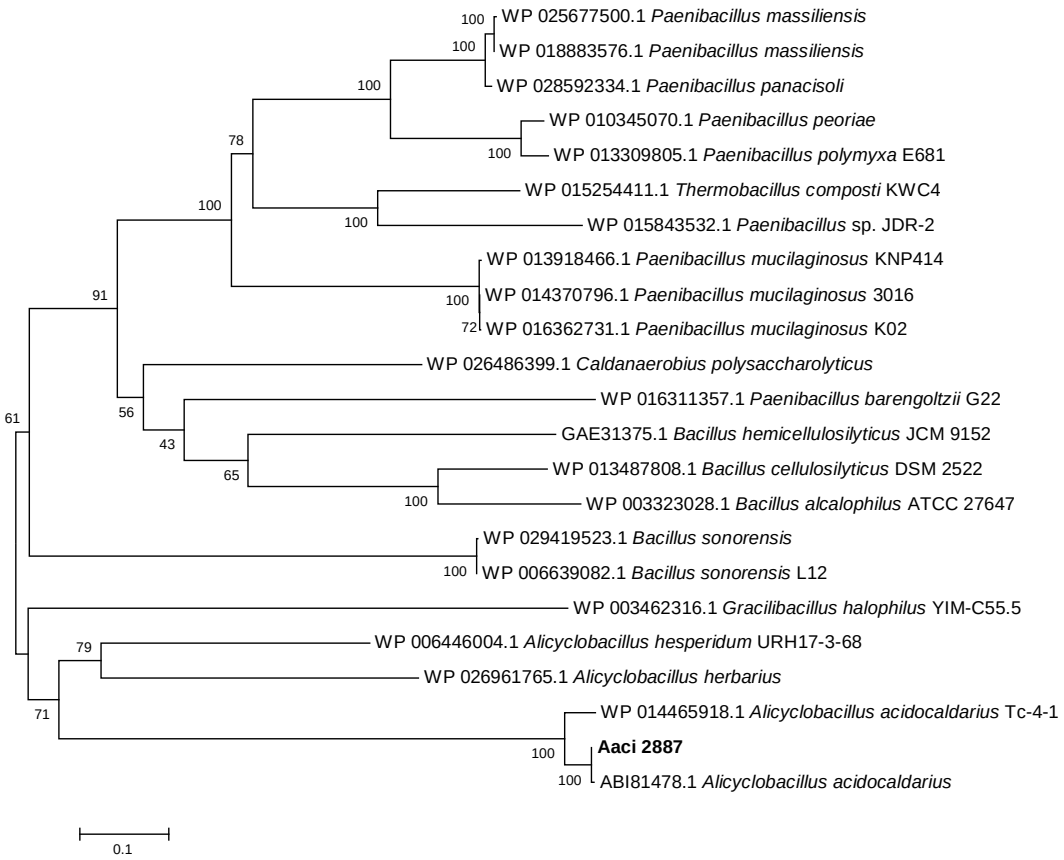


Figure 16.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WP 010345070.1 *Paenibacillus peoriae*
WP 013309805.1 *Paenibacillus polymyxa* E681
WP 015254411.1 *Thermobacillus composti* KWC4
WP 015843532.1 *Paenibacillus* sp. JDR-2
WP 013918466.1 *Paenibacillus mucilaginosus* KNP414
WP 014370796.1 *Paenibacillus mucilaginosus* 3016
WP 016362731.1 *Paenibacillus mucilaginosus* K02
WP 026486399.1 *Caldanaerobius polysaccharolyticus*
WP 016311357.1 *Paenibacillus barengoltzii* G22
GAE31375.1 *Bacillus hemicellulosilyticus* JCM 9152
WP 013487808.1 *Bacillus cellulosilyticus* DSM 2522
WP 003323028.1 *Bacillus alcalophilus* ATCC 27647
WP 029419523.1 *Bacillus sonorensis*
WP 006639082.1 *Bacillus sonorensis* L12
WP 003462316.1 *Gracilibacillus halophilus* YIM-C55.5
WP 006446004.1 *Alicyclobacillus hesperidum* URH17-3-68
WP 026961765.1 *Alicyclobacillus herbarius*
WP 014465918.1 *Alicyclobacillus acidocaldarius* Tc-4-1
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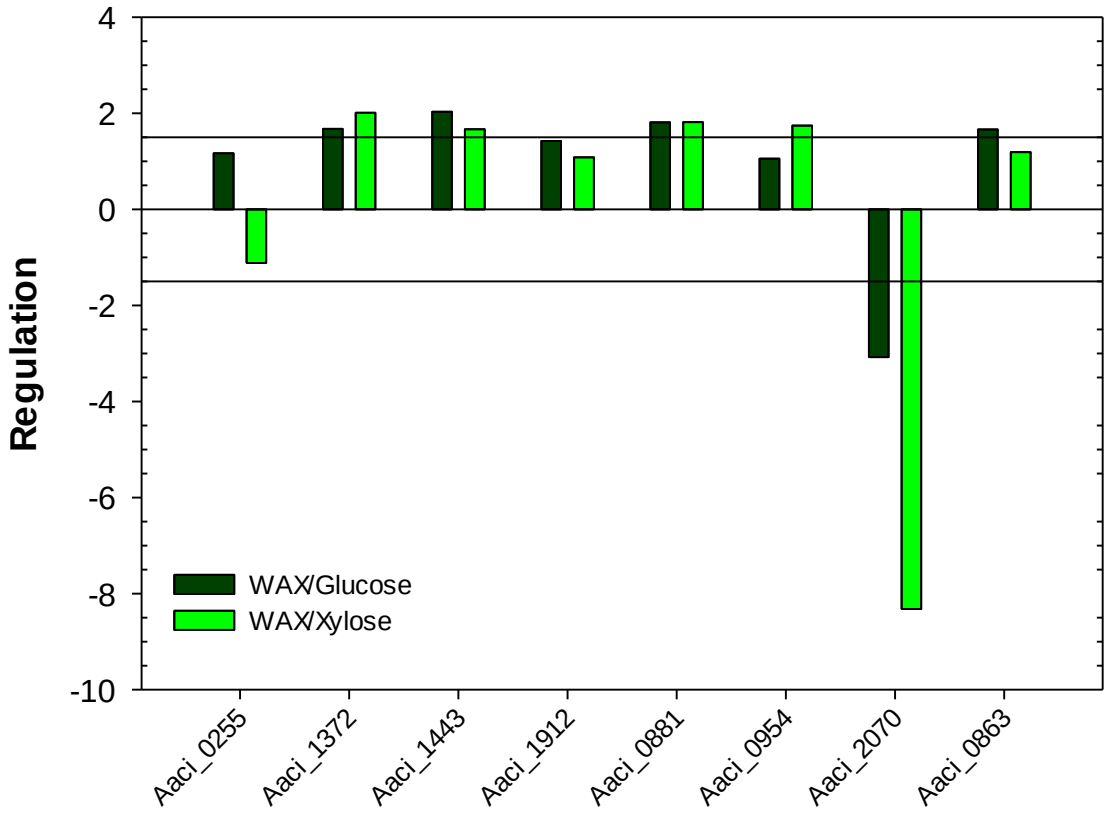
Figure 17.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-2
-4
-6
-8
-10
WAX/Glucose
WAX/Xylose
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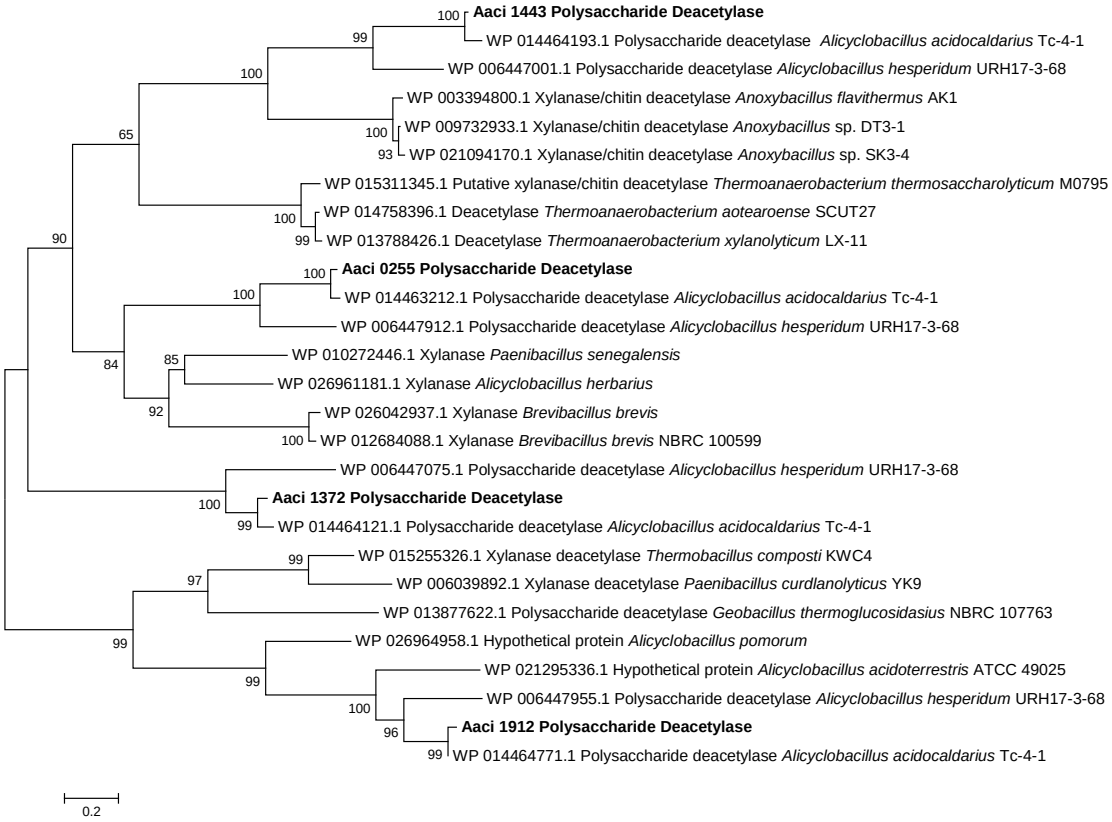
Aaci_1443
Aaci_1912
Aaci_0881
Aaci_0954
Aaci_2070
Aaci_0863

Figure 18.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WP 006447001.1 Polysaccharide deacetylase Alicyclobacillus hesperidum URH17-3-68
WP 003394800.1 Xylanase/chitin deacetylase Anoxybacillus flavithermus AK1
WP 009732933.1 Xylanase/chitin deacetylase Anoxybacillus sp. DT3-1
WP 021094170.1 Xylanase/chitin deacetylase Anoxybacillus sp. SK3-4
WP 015311345.1 Putative xylanase/chitin deacetylase Thermoanaerobacterium thermosaccharolyticum M0795
WP 014758396.1 Deacetylase Thermoanaerobacterium aotearoense SCUT27
WP 013788426.1 Deacetylase Thermoanaerobacterium xylanolyticum LX-11
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WP 010272446.1 Xylanase Paenibacillus senegalensis
WP 026961181.1 Xylanase Alicyclobacillus herbarius
WP 026042937.1 Xylanase Brevibacillus brevis
WP 012684088.1 Xylanase Brevibacillus brevis NBRC 100599
WP 006447075.1 Polysaccharide deacetylase Alicyclobacillus hesperidum URH17-3-68
Aaci 1372 Polysaccharide Deacetylase
WP 014464121.1 Polysaccharide deacetylase Alicyclobacillus acidocaldarius Tc-4-1
WP 015255326.1 Xylanase deacetylase Thermobacillus composti KWC4
WP 006039892.1 Xylanase deacetylase Paenibacillus curdlanolyticus YK9
WP 013877622.1 Polysaccharide deacetylase Geobacillus thermoglucosidasius NBRC 107763
WP 026964958.1 Hypothetical protein Alicyclobacillus pomorum
WP 021295336.1 Hypothetical protein Alicyclobacillus acidoterrestris ATCC 49025
WP 006447955.1 Polysaccharide deacetylase Alicyclobacillus hesperidum URH17-3-68
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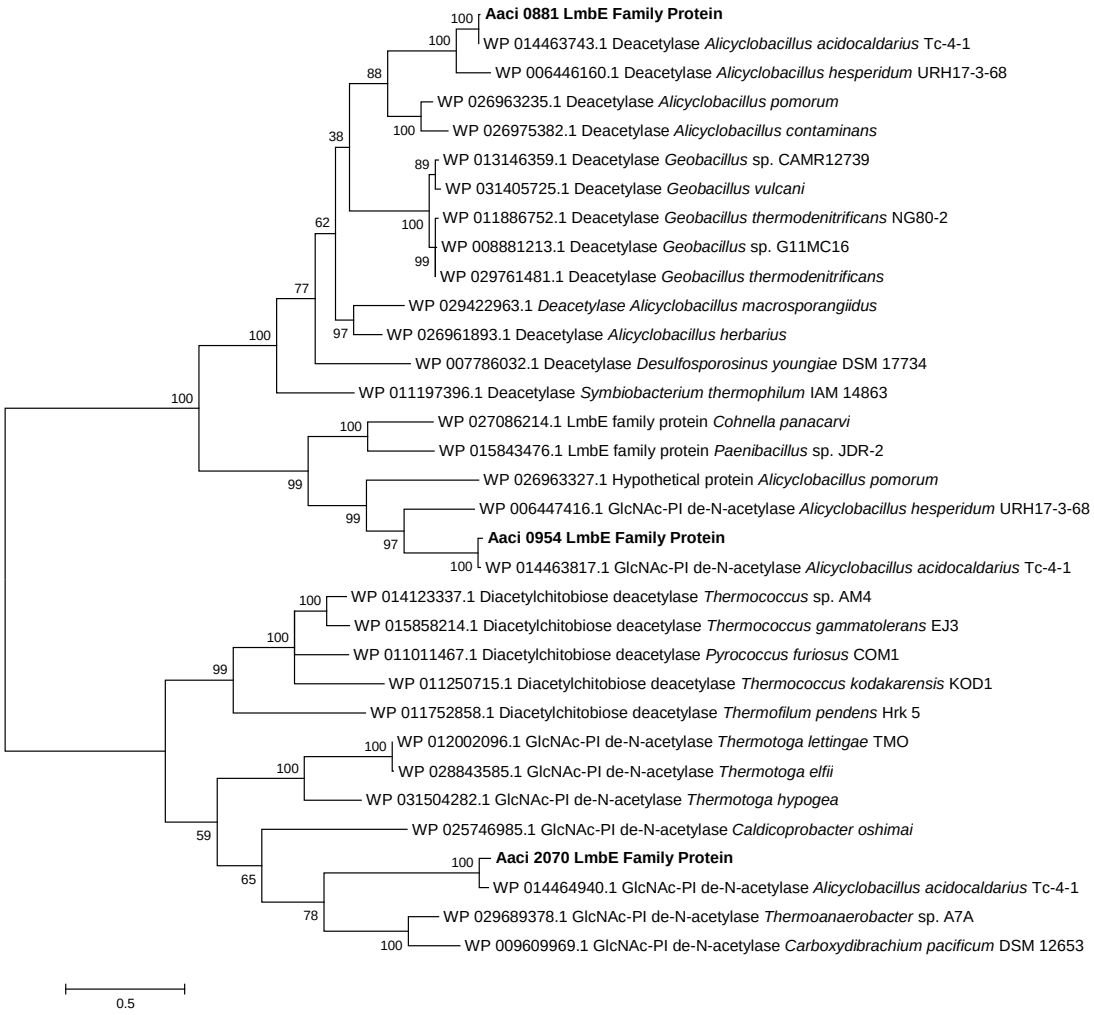
Figure 19.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WP 006446160.1 Deacetylase Alicyclobacillus hesperidum URH17-3-68
WP 026963235.1 Deacetylase Alicyclobacillus pomorum
WP 026975382.1 Deacetylase Alicyclobacillus contaminans
WP 013146359.1 Deacetylase Geobacillus sp. CAMR12739
WP 031405725.1 Deacetylase Geobacillus vulcani
WP 011886752.1 Deacetylase Geobacillus thermodenitrificans NG80-2
WP 008881213.1 Deacetylase Geobacillus sp. G11MC16
WP 029761481.1 Deacetylase Geobacillus thermodenitrificans
WP 029422963.1 Deacetylase Alicyclobacillus macrosporangiidus
WP 026961893.1 Deacetylase Alicyclobacillus herbarius
WP 007786032.1 Deacetylase Desulfosporosinus youngiae DSM 17734
WP 011197396.1 Deacetylase Symbiobacterium thermophilum IAM 14863
WP 027086214.1 LmbE family protein Cohnella panacarvi
WP 015843476.1 LmbE family protein Paenibacillus sp. JDR-2
WP 026963327.1 Hypothetical protein Alicyclobacillus pomorum
WP 006447416.1 GlcNAc-PI de-N-acetylase Alicyclobacillus hesperidum URH17-3-68
Aaci 0954 LmbE Family Protein
WP 014463817.1 GlcNAc-PI de-N-acetylase Alicyclobacillus acidocaldarius Tc-4-1
WP 014123337.1 Diacetylchitobiose deacetylase Thermococcus sp. AM4
WP 015858214.1 Diacetylchitobiose deacetylase Thermococcus gammatolerans EJ3
WP 011011467.1 Diacetylchitobiose deacetylase Pyrococcus furiosus COM1
WP 011250715.1 Diacetylchitobiose deacetylase Thermococcus kodakarensis KOD1
WP 011752858.1 Diacetylchitobiose deacetylase Thermofilum pendens Hrk 5
WP 012002096.1 GlcNAc-PI de-N-acetylase Thermotoga lettingae TMO
WP 028843585.1 GlcNAc-PI de-N-acetylase Thermotoga elfii
WP 031504282.1 GlcNAc-PI de-N-acetylase Thermotoga hypogea
WP 025746985.1 GlcNAc-PI de-N-acetylase Caldicoprobacter oshimai
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WP 014464940.1 GlcNAc-PI de-N-acetylase Alicyclobacillus acidocaldarius Tc-4-1
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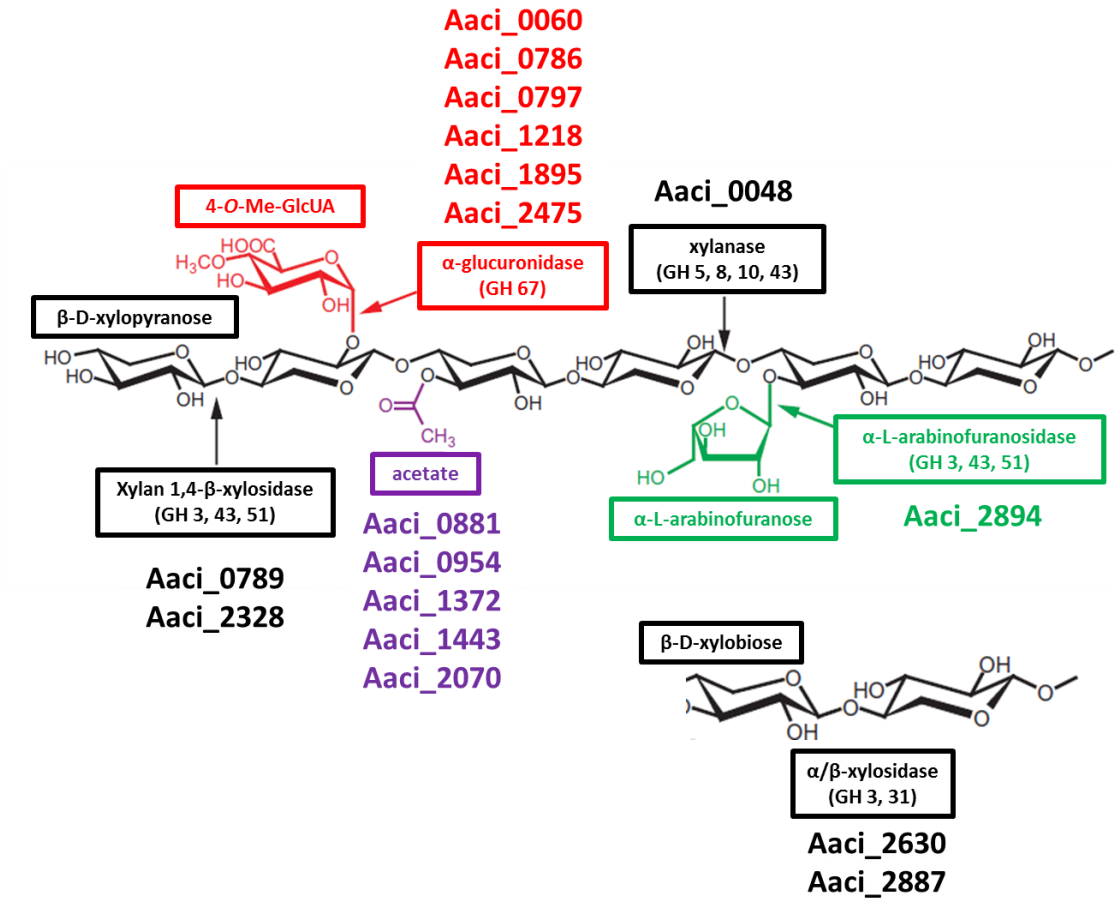
Figure 20.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Aaci_2475
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(GH 67)
Aaci_0048
xylanase
(GH 5, 8, 10, 43)
β-D-xylopyranose
α-L-arabinofuranosidase
(GH 3, 43, 51)
Aaci_2894
α-L-arabinofuranose
acetate
Xylan 1,4-β-xylosidase
(GH 3, 43, 51)
Aaci_0789
Aaci_2328
Aaci_0881
Aaci_0954
Aaci_1372
Aaci_1443
Aaci_2070
β-D-xylobiose
α/β-xylosidase
(GH 3, 31)
Aaci_2630
Aaci_2887

CHAPTER IV: An Expression-Driven Approach to Predict Carbohydrate Transport in the Themoacidophilic Bacterium *Alicyclobacillus acidocaldarius* During Growth on Wheat Arabinoxylan

Brady D. Lee[1,3], William A. Apel[2], Linda C. DeVeaux[4], and Peter P. Sheridan[3]

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ABSTRACT

Bacterial metabolism of carbon sources, whether in the form of simple sugars, di-, oligo- or polysaccharides, requires transport from the extracellular environment to the cytoplasm. In bacteria, this transport is accomplished by a variety of mechanisms including multifacilitator superfamily (MFS) transporters, ATP binding cassette (ABC) type transporters and phosphoenolpyruvate phosphotransferase system (PTS) transporters. *Alicyclobacillus acidocaldarius* is a Gram positive thermo- and acid tolerant bacterium capable of growth on carbohydrates ranging from simple sugars to complex polysaccharides such as xylan. Molecular analysis of *A. acidocaldarius* strain DSM 446 when growing on wheat arabinoxylan (WAX) was performed using high density oligonucleotide microarrays. When either glucose or xylose was added to induce changes in gene transcription, 83 genes associated with 28 transporters were regulated. With the exception of a few transport genes, most genes were down-regulated, indicating that proteins encoded by these genes were expressed when *A. acidocaldarius* was

growing on sugars derived from WAX. Results and genome analysis also indicated that transport of carbohydrates by *A. acidocaldarius* is primarily accomplished by MFS-type and ABC-type transporters. In addition, three oligopeptide/dipeptide transporters appear to be active in carbohydrate transport as has been demonstrated in other thermophiles. Finally, WAX metabolism by *A. acidocaldarius* is hypothesized to occur by hydrolysis of the polysaccharide by glycoside hydrolases exported to the surface of the cell, followed by uptake of mono-, di-, oligo- and polysaccharides, by the transporters described. Intracellular glycoside hydrolases further hydrolyze these simpler constituents to sugars that are then processed through glycolysis or the pentose phosphate pathway.

INTRODUCTION

Alicyclobacillus acidocaldarius ATCC 27009/DSM 446 is a Gram-positive thermoacidophilic bacterium capable of growth on a variety of mono-, di-, oligo- and polysaccharides, including cellulose and hemicellulose. *A. acidocaldarius* is spore-forming and grows optimally in strictly aerobic conditions at 60°C and at pH between 3 and 4 (1, 2). Following isolation from a hot spring in Yellowstone National Park, this bacterium was originally classified as *Bacillus acidocaldarius*, but it was reclassified as *A. acidocaldarius* due to the prevalence of ω-cyclic fatty acids in the cell wall and an abbreviated helix 6 of the 16S rRNA (3, 4). Phenotypically acid and thermal tolerant, *A. acidocaldarius* has been isolated from diverse habitats including water and soil from geothermal sites, submarine hot springs, orchard soils, and also as a contaminant in heat processed foods (e.g., fruit juices) (5-10).

The potential for use of plant derived oligo- and polysaccharides by *A. acidocaldarius* is supported by the numerous genes encoding glycoside hydrolase enzymes that have

been found through genome sequencing, or direct characterization. To date, 19 glycoside hydrolase genes have been found encoded in the *A. acidocaldarius* genome (11), and β-galactosidase, α-amylase, cellulase, neopullulanase, exo-pectinase, mannanase, β-glycosidase, and endoglucanase enzymes have been expressed and characterized (1, 2, 8, 12-26).

Some of these acid- and thermostable hydrolases catalyze degradation of cellulose and hemicellulose providing soluble oligomers and monomers that are small enough to be transported into the cell as sources of carbon and energy. Transport of carbohydrates, including mono-, di-, oligo-, and polysaccharides into a bacterial cell is typically accomplished using three general families of transporters (Figure 1) (27). Specifically, these families are: primary transporters, such as the ATP-binding cassette (ABC) type transporter superfamily; secondary transporters consisting primarily of major facilitator superfamily (MFS) transporters; and phosphoenolpyruvate (PEP): carbohydrate phosphotransferase systems (PTS). ABC-Type transporters related to carbohydrate transport are of two primary types: 1) carbohydrate utilization transporter 1 (CUT1), which transport oligosaccharides, and 2) carbohydrate utilization transporter 2 (CUT2), which transport monosaccharides. MFS type transporters can be further divided into uniport-, symport- and antiport-type transport proteins. Primary transporters, such as ABC-transporters, are 'active' transporters that transport solutes across the cell membrane at the expense of a molecule of ATP (28). Relative to carbohydrate transport, proton gradients generated in the extracellular medium allow the flow of protons down a chemical and charge concentration gradient, facilitating uptake of sugars or other carbohydrates, which are examples of secondary transport. These types of transport

systems are important because the bacterial cell wall represents a barrier between the cytoplasm and the external environment; therefore, the cytoplasmic membrane represents the primary permeability barrier for the passage of most, if not all, nutrients, including carbohydrates, into the cell.

The relevance of *A. acidocaldarius* for lignocellulose depolymerization is underscored by this bacterium's ability to use a wide range of monosaccharides for growth. An important aspect of understanding this metabolism will be characterizing transporters used during growth, on a variety of saccharides. A thorough understanding of polysaccharide, monosaccharide and mixed saccharide metabolism by *A. acidocaldarius* will help in determining the utility of this bacterium for lignocellulose depolymerization. The first step in this process is to understand the effect of transcriptional regulation (i.e., CCR as one example) on expression of genes encoding glycoside hydrolase enzymes, which represents the first step in metabolism of lignocellulose. The purpose of this study was to monitor gene transcription during growth on the polysaccharide WAX. In addition, the regulatory effects of monosaccharides on transcription of *A. acidocaldarius* genes related to carbohydrate transport were used to determine the range of transporter genes regulated during growth on WAX. High density oligonucleotide microarrays were used to analyze the global transcriptome of *A. acidocaldarius* during these batch chemostat studies. This research represents the first in-depth analysis of *A. acidocaldarius* sugar transporters while growing on plant polysaccharides, such as WAX.

MATERIALS AND METHODS

Inoculum Development.

The Type strain of *A. acidocaldarius* ATCC 27009/DSM 446 was purchased from the American Type Culture Collection (ATCC) and used for all experiments. To maintain generational integrity between chemostat experiments, stock cultures representing two generations from the culture collection stock were maintained in 5% DMSO and stored at -80 °C. Chemostat inoculum was prepared by dispensing 1 ml of the frozen stock into 25 ml of Modified 402 Medium, which contained the following (g/L): $(NH_4)_2SO_4$ (1.3), Fe(III) EDTA (0.047), $CaCl_2 \cdot 2H_2O$ (0.07), $MgSO_4 \cdot 7H_2O$ (0.25), KH_2PO_4 (3.0), and xylose (4.0). In addition, 1 ml of a mineral (Solutions A and B) and vitamin stock (Solution C) were added. Solution A (g/L): $MgCl_2$ (25), $CaCl_2 \cdot 2H_2O$ (6.6), H_3BO_3 (0.58), $FeCl_3 \cdot 6H_2O$ (5), $Co(NO_3)_2 \cdot 6H_2O$ (0.05), $NiCl_2 \cdot 6H_2O$ (0.02). Solution B (g/L): $MnSO_4 \cdot H_2O$ (2.0), $ZnSO_4 \cdot 7H_2O$ (0.5), $CuSO_4 \cdot 5H_2O$ (0.15), $Na_2MoO_4 \cdot 2H_2O$ (0.025). Solution C (g/L): pyridoxine hydrochloride (0.08), folic acid (0.012), thiamine hydrochloride (0.13), riboflavin (0.042), nicotinamide (0.084), *p*-aminobenzoate (0.088), biotin (0.01), cyanocobalamin (0.0004), D-pantothenic acid, calcium salt (0.086), *myo*-inositol (0.021), choline bromide (0.053), orotic acid, sodium salt (0.021), spermidine (0.1). Base medium was autoclaved (121 °C, 20 psi) for 30 min prior to use; KH_2PO_4 solution was adjusted to pH 4.0, autoclaved separately and added once bulk media had cooled. Solutions A, B and C were filter sterilized (0.22 μm) separately prior to addition to the base medium. Solutions A and B were autoclaved with the bulk medium, whereas Solution C was filter sterilized and added to the base medium once it had cooled. The 25 ml *A. acidocaldarius* starter culture was grown overnight and then used to inoculate 250

ml of Modified 402 Medium containing 4 g/L xylose. This culture was then used to inoculate the chemostat.

Chemostat Studies.

Experiments to monitor gene transcription by *A. acidocaldarius* when growing on WAX and xylose or glucose were performed in a BioFlo 3000 chemostat system (New Brunswick Scientific, Enfield, CT). Medium was added to the reactor, oxygen and DO probes were inserted into appropriate ports, and the entire reactor was autoclaved at 121 °C, and a pressure of 20 psi for 1 hour. The pH probe was calibrated using a two point calibration with pH 2 and 7 buffers, prior to autoclaving. Prior to heating the reactor for operation, the pH of the medium was measured using an external pH meter and the pH on the BioFlo control unit was adjusted accordingly. The DO probe was allowed to polarize for six hours and then the dynamic range of the probe was set first by purging nitrogen gas through the medium (0% oxygen) and then air (100% oxygen). A working volume of 2.0 L of Modified 402 Medium was used for experiments. The chemostat was run in batch mode and the temperature and pH of the growth medium were held at 60 °C and 4.0, respectively. Growth medium pH was controlled using 1 N NaOH. Dissolved oxygen was controlled to 10% using a cascade system based on changes in agitation along with the addition of pure oxygen to the inlet air stream. WAX, which is relatively insoluble, was prepared by first wetting 2 g with 95% ethanol. Once in a slurry, 190 ml of sterile distilled water (pH 4.0) was added and the solution was heated for 30 minutes to evaporated residual ethanol. This solution was then added to the 2 L of Modified 402 Medium in the chemostat, giving a final WAX concentration of 1 g/L. *A. acidocaldarius* was grown to mid-exponential phase (OD_{600} of ~0.5) and then either glucose or xylose

was spiked into the reactor at concentrations of 2 g/L to induce regulation of gene transcription.

Once the *A. acidocaldarius* culture reached an OD_{600} of 0.5 while growing at the expense of sugar liberated from the hydrolysis of WAX, a sample was taken for RNA extraction, either glucose or xylose was added, and then a second sample was immediately taken for RNA extraction. Three biological replicates for each condition were performed.

Isolation of Total RNA.

Samples for RNA extraction were taken from the chemostat and immediately mixed with RNA Protect Bacteria Reagent (Qiagen, Valencia, CA) at a 1:2 ratio. This mixture was incubated at room temperature for 5 minutes, centrifuged, the supernatant was discarded and the cell pellet was flash frozen in liquid nitrogen and then stored at -80 °C until the RNA was extracted. Total RNA was extracted from the *A. acidocaldarius* cells using an RNeasy Midi Kit (Qiagen, Valencia, CA) with slight modification of the manufacturer's protocol. *A. acidocaldarius* cells were thawed and lysis was accomplished by adding 200 µl of Tris-EDTA buffer containing 15 mg/ml lysozyme and 0.1 mg/ml proteinase K. Samples were vortexed for 10 seconds and then incubated at room temperature for 15 minutes with shaking. Buffer RLT (4 ml) containing β-mercaptoethanol was then added to each sample, the mixture was homogenized using a syringe with a 20G needle, and then incubated at room temperature for 10 minutes. Following incubation, 3.5 ml of 80% ethanol was added and the resulting solution was mixed vigorously. The lysate was then passed through a Midi spin filter via centrifugation at 5,000 x g for five minutes to capture the RNA. RNA on the spin filter

was washed sequentially with two 2.5 ml aliquots of Buffer RPE, followed by centrifugation at 5,000 x g for five minutes. RNA was eluted from the glass filter using 200 µl of DNase/RNase-free water; water was applied to the filter, incubated at room temperature for five minutes, and then centrifuged at 5,000 x g for three minutes. To increase RNA yield, the flow through was re-applied to the spin filter and centrifuged. Residual DNA in the samples was removed by treatment with Ambion TURBO DNA-free kit (Life Technologies, Grand Island, NY). RNA was purified to remove compounds that might interfere with cDNA synthesis and concentrated using ethanol precipitation. The pellet was dried using an Eppendorf Vacufuge Concentrator 5301 (Brinkmann Instruments, Westbury, NY), and then resuspended in 20 µl of DNase/RNase-Free water. To inhibit RNA degradation during storage, 1 µl of Ambion Superase-In RNase Inhibitor (Life Technologies, Grand Island, NY) was added. RNA concentration and purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was determined using an RNA Nano Chip Kit run on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Synthesis of cDNA.

Double stranded cDNA was synthesized from total RNA using the Invitrogen Superscript Double-Stranded cDNA Synthesis Kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Ten µg of total RNA was mixed with random hexamer primers in DNase/RNase-Free Water, heated for 10 minutes at 70 °C and then quenched in an ice-water slurry for five minutes. While on ice, First Strand Buffer, dithiothreitol, and dNTPs were added and then brought to the reaction temperature of 42 °C, prior to addition of SuperScript II. Following addition of the reverse transcriptase,

the reaction mixture was incubated at 42 °C for one hour. After this incubation the tubes were placed on ice and reaction components for second strand synthesis were added. The first strand reaction mixture was mixed with Second Strand Buffer, dNTPs, DNA ligase, DNA polymerase and RNase H; the reaction was mixed and then incubated at 16 °C for two hours. An additional five minute incubation was performed after T4 DNA polymerase was added to the reaction. Residual RNA was degraded by adding RNase A and incubating the reaction mixture at 37 °C for 10 minutes. Proteins were removed by treating with phenol:chloroform:isoamyl alcohol, with phase separation being accomplished using Phase Lock Tubes (5 Prime, Inc., Gaithersburg, MD). cDNA in the aqueous phase was then precipitated and concentrated by ethanol precipitation. The pellet was dried using an Eppendorf Vacufuge Concentrator 5301 (Brinkmann Instruments, Westbury, NY), and then resuspended in 20 µl of DNase/RNase-Free water and allowed to solubilize overnight. cDNA concentration in each reaction was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A DNA 7500 Chip Kit run on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) was used to verify that most of the cDNA was ≥ 400 bp.

Microarray Experiments and Data Analysis.

Microarrays were designed and synthesized by NimbleGen using their 4 x 72K Custom Gene Expression Array format. Complete genome sequence information for *A. acidocaldarius* ATCC 27009 (DSM 446) was provided to NimbleGen. Seven probes, each 60 nt in length, were designed for each of the 3,554 identified open reading frames (ORFs) in the genome. Each probe was synthesized on the microarray in triplicate.

Control probes were also included to ensure that there was no intra-quadrant contamination during the hybridization process.

Biological triplicate RNA samples isolated from *A. acidocaldarius* cells grown under the various conditions were used for microarray analysis. One color cDNA labeling using Cy3, hybridization to the *A. acidocaldarius* microarrays, array imaging and initial analysis of the array data was performed by NimbleGen. Data was normalized using NimbleScan software which normalizes probe response using quantile normalization and gene calls generation using Robust Multichip Averaging (RMA) (29, 30). Log_2-transformed RMA data files were imported into ArrayStar 4 software (DNASTAR, Inc., Madison, WI) and the mean expression levels of three replicate arrays for each condition were considered. For comparison between gene expression during growth on WAX, immediately upon addition of the glucose or xylose, and growth on WAX and second sugar, statistical significance was determined with a Bonferonni corrected moderate *t*-test and only genes that resulted in gene expression at 95% confidence ($p \leq 0.05$) were considered significant.

Bioinformatic Analysis

Comparative analysis of the *A. acidocaldarius* strain DSM 446 genome was performed using the Integrated Microbial Genomes (IMG) feature within the Joint Genome Institute (JGI) (31). Homology determinations for *A. acidocaldarius* proteins were accomplished using the Basic Local Alignment Search Tool (BLAST) for protein sequences using the 'blastp' algorithm (32, 33). The non-redundant protein sequence database was used for searches and uncultured and environmental sample sequences were excluded from the search. Alignments demonstrating variation between protein

sequences for the Hpr gene were generated using Genedoc (34). Phylogenetic trees comparing protein sequences were generated using the Molecular Evolutionary Genetics Analysis (MEGA6) software program (35). Protein sequences were aligned using the MUSCLE program within MEGA6 (36, 37). A phylogenetic tree was constructed based on the distance matrix data obtained with the Nearest-Neighbor-Interchange heuristic method. Robustness of the tree topology was evaluated by bootstrap resampling analysis with 1000 bootstraps and applying maximum-likelihood analysis using MEGA 6.

RESULTS AND DISCUSSION

A. acidocaldarius strain DSM 446 was grown to the mid-logarithmic phase of growth using WAX as the sole carbon source for growth. Transcriptional analysis was performed on the culture when growing on WAX alone, and after glucose or xylose was added to the culture. The monomer sugar was added in an attempt to induce changes in gene transcription in order to determine which genes were active when *A. acidocaldarius* was growing on WAX. Once the WAX polysaccharide is hydrolyzed, carbohydrate transporters required for uptake of sugar monomers, disaccharides and oligosaccharides are required to move these molecules from the external medium to the cytoplasm for further metabolism. The range of carbohydrate transporters hypothesized to be involved in transport of products of hydrolysis of WAX will be discussed.

Annotated Carbohydrate Transporters in the Genome of *A. acidocaldarius*.

Genome analysis of *A. acidocaldarius* strain DSM 446 shows 28 putative transporters for carbohydrates, as well as three annotated oligopeptide or dipeptide

transporters that may function as carbohydrate transporters. Since many of the proposed transporters consist of multiple subunits, approximately 83 total genes annotated to encode carbohydrate transporter subunits were regulated during the experiment. Families of carbohydrate transporters found in the *A. acidocaldarius* strain DSM 446 genome and the associated genome loci are shown in Table 1. This analysis shows that carbohydrate transport in *A. acidocaldarius* strain DSM 446 is primarily accomplished by MFS Superfamily or ABC-type transporters. Only two PTS transporters are found in the *A. acidocaldarius* strain DSM 446 genome, which is unusual because this type of transporter is used in many other bacteria, including *Bacillus subtilis* and *Escherichia coli,* for preferential uptake of carbohydrates (38-40). *A. acidocaldarius* was tested in batch chemostat studies in which WAX was the sole carbon source until the mid-logarithmic phase of growth, at which time glucose or xylose was added to stimulate gene regulation. In this way, genes encoding proteins used for growth on WAX would be revealed. Bioinformatic analysis for carbohydrate transporters annotated in the genome and regulated during the experiment will be discussed.

Multi-facilitator Super Family Transporters.

Following initial exposure of the *A. acidocaldarius* growing on WAX to monomer sugars, microarray analysis showed regulation of 12 genes for proposed MFS-type transporters annotated as probable sugar transporters (Figure 2). Eight of the 12 annotated MFS transporters were down-regulation when either sugar was added and typically down-regulation was more when xylose was added to the culture, than when glucose was added. Two MFS transporter encoding genes were upregulated when glucose and xylose were added (Aaci_0257 and Aaci_2665), while Aaci_2045 was only

upregulated when xylose was added to the exponentially growing *A. acidocaldarius* culture. Of the two annotated MFS transporters upregulated when either sugar was added, up-regulation was greater when glucose was added. Aaci_1578 was the only MFS transporter that was down-regulated only when xylose was added.

Homology of *A. acidocaldarius* MFS transporters with similar proteins from other bacteria in GenBank was determined using 'blastp'. Figure 3 shows a circular phylogenetic tree containing all 12 proposed MFS transporters and shows phylogenetic distance of these proteins to those from other Bacteria and Archaea. As would be expected, each protein product encoded by the genes appear to be conserved among Alicyclobacilli, but some showed homology to multiple *Alicyclobacillus* species while others showed only homology to proteins from *A. acidocaldarius* strain Tc-4-1 and *A. hesperidum* strain URH17-3-68. Proteins encoded by Aaci_0792, Aaci_1578, Aaci_2181, Aaci_2527, and Aaci_2622 show homology to Tc-4-1 and URH17-3-68, but also to *A. herbarius*, *A. acidoterrestris*, *A. contaminans*, *A. macrosporanglidus*, and *A. pomorum*. There are also differences between the MFS transporters when compared to other prokaryotes, indicating that evolutionarily, the MFS makeup of *A. acidocaldarius* strain DSM 446 had a variety of different sources. While many of the MFS transporters show homology to MFS transporters in other Firmicutes, Aaci_0335 shows homology to proteins from Archaeal species such as *Sulfolobus* and *Metallosphaera*.

Seven of the putative MFS proteins have been included within the cluster of orthologous groups of proteins as COG2814, which are annotated as arabinose efflux permease proteins. Probably the most well characterized protein in this family are the AraE transporters, from *E. coli*, *B. subtilis*, *Corynebacterium glutamicum*, as well as

some Clostridia (41-44). While annotated as an efflux permease, AraE has been shown to catalyze the import of arabinose, xylose and galactose.

Proteins from two of the up-regulated genes (Aaci_0257 and Aaci_2045) and annotated to encode AraE-like MFS transporters showed the most homology to MFS transporters from other *Firmicutes*, primarily different Clostridia. This pair of transporters may be involved in the transport of glucose and/or xylose, since these genes were upregulated when either glucose or xylose was added--as was the case for Aaci_0257-- or only when xylose was added (Aaci_2045). While the proposed uptake activity may be true for xylose, even with the demonstrated broad substrate specificity of these MFS transporters, no instances of glucose transport by AraE homologs is reported in the literature. For this reason, the up-regulation of Aaci_0257 may be a cellular response to scavenge residual pentose sugars liberated from WAX in the growth medium. In addition, Aaci_0257 is divergently transcribed from a glycoside hydrolase (Aaci_0258) and a CUT2 ABC-type transporter (Aaci_0259.. Aaci_0262) for monosaccharides, which will be described in greater detail in a section below. Figure 4A shows that similar grouping of MFS and ABC-type transporter genes are conserved in various Alicyclobacilli. In each case, the MFS transporter and ABC-type transporter genes are separated by a gene for a glycoside hydrolase involved in cell wall deconstruction. A similar arrangement of genes is also demonstrated with Aaci_2045 and another ABC-type transporter gene, but in this instance the ABC-type transporter is of the CUT1 type (Figure 4B). Gene organization shows the MFS and ABC-type transporter genes on the positive strand, and in this instance, the transporters are separated by a gene annotated to encode a metallo-β-lactamase superfamily-like

hydrolase on the negative strand. As with the genome region associated with Aaci_0257, the genome region associated with Aaci_2045 is conserved in *A. acidocaldarius* Tc-4-1. Although genome information for the MFS transporter in *A. hesperidum* was available, genome information for the metallo-β-lactamase superfamily-like hydrolase and ABC-type transporter genes was not available.

Gene locus Aaci_0335 was the most highly down-regulated of the MFS genes, regardless of the sugar used to induce regulation, but that induced by xylose was nearly double that by glucose. As discussed previously, this protein encoded by Aaci_0335 shows homology to MFS transporters found in a number of Archaeal species and is one of the aforementioned genes annotated to encode an arabinose efflux permease. This transporter is in close proximity (two genes downstream) to Aaci_0332, which has been annotated to encode a glycoside hydrolase. Unlike the transporter, the hydrolytic gene was not regulated during the experiment, indicating separate promoters.

Gene loci Aaci_2495, Aaci_2515, Aaci_2527, Aaci_2622 and Aaci_2900 were all down-regulated at similar levels, and in all cases these loci were regulated to a greater extent-- nearly double-- by xylose. With the exception of Aaci_2515, the annotated proteins from these genes are transporters ranging from 411-442 aa in length and have 11-12 transmembrane helices common to MFS transporters. Aaci_2515 has been annotated as a hypothetical protein, but contains homology to MFS transporters and appears to be truncated and is only 148 aa in length with only four transmembrane helices, so may not produce a fully functioning MFS transporter.

Aaci_2900 and Aaci_1578 were annotated in JGI-IMG to encode sugar transporters, described as D-xylose transporters. Conserved domain analysis of the proteins encoded by these genes shows similarity to XylE, which has been associated with transport of pentose and other sugars, such as glucose and galactose (45-49). SEED analysis of Aaci_2900 gives an annotation as an arabinose-proton symporter for L-arabinose utilization. Aaci_2900 is located within a section of the genome that contains a large number of genes annotated to encode proteins involved in carbohydrate transport in metabolism (Figure 5A). Just upstream from Aaci_2900 are genes (Aaci_2902 to Aaci_2904) that encode an ABC-type transporter that has been classified as a CUT2 transporter for monosaccharides. Genes for this transporter were also down-regulated during the experiment. While most of the genes found in *A. acidocaldarius* strain DSM446 appear to be present in other *Alicyclobacillus* species (Figure 5, B and C), gene order is different. In *A. acidocaldarius* strain Tc-4-1, the ABC-type transporter is downstream from a series of proposed arabinose metabolism genes, while in *A. hesperidum* strain URH7-3-68, genes for the ABC-type transporter are divergently transcribed from the other genes.

Aaci_2900 appears to be part of an arabinose operon that contains a gene that encodes α-arabinofuranosidase (Aaci_2894), and other genes for arabinose metabolism. Genes located between Aaci_2900 and Aaci_2894 appear to represent the *ara*-operon because they are annotated to encode genes common to this operon. A number of genes annotated as possible transcriptional regulators of arabinose metabolism are also present in this area of the genome and were regulated during the experiments. Table 2 shows the genes and the proposed *ara*-operon product and the proposed function of each gene, regulation

during the experiment, and the non-*Alicyclobacillus* species with proteins showing the most homology with the protein of interest. All of the genes for proteins associated with regulation and metabolism of arabinose were down-regulated during the experiments, with the exception of Aaci_2890, encoding an AraC Family regulator, which was regulated by xylose, but not by glucose. Three of remaining four genes encoding MFS-type transporters have been characterized as sugar phosphate permeases (Aaci_2495, Aaci_2622, Aaci_2665), while Aaci_0792 has been annotated as an MFS transporter, but only a general functional prediction has been given. Aaci_0792 was down-regulated approximately 2-fold by both sugars. Down-regulation of Aaci_2495 was nearly 4-fold with xylose, while less than 2-fold with glucose. Aaci_2622 was also more down-regulated by xylose, but less of a difference was noted between the two sugars. Aaci_2665 was up-regulated when both sugars were added, with slightly higher up-regulation on glucose.

Aaci_0792 encodes a 458 aa protein and is located in a section of the genome that appears to be a "hot spot" for carbohydrate processing and metabolism, and contains numerous annotated glycoside hydrolases and other sugar transporters. Aaci_0792 is located on the negative strand, while the other genes are on the positive strand. Conserved domains for this protein indicate similarity to general MFS sugar transporters, and specifically XylE, which is a proton symporter for xylose. These attributes suggest that this MFS transporter may be involved in xylose or arabinose uptake by *A. acidocaldarius* DSM 446.

Since genes for two of the annotated sugar phosphate permeases were down-regulated and one was up-regulated, no specific function can be suggested. Conserved domain

analysis of the predicted proteins indicates similarity to UhpC, which acts as a sensory protein for glucose-6-phosphate in some bacteria, while also maintaining some transport activity (50). Activity of UhpC is typically coupled to UhpT, which is also an MFS type transporter. No homologs of this are found in the *A. acidocaldarius* strain DSM 446 genome. How this activity relates to transport of pentose sugars is not known at this time.

Sodium Solute Transporter Superfamily.

Gene locus Aaci_2204, which has been annotated as a solute:sodium symporter (SSS) Superfamily transporter, was up-regulated 2.7-fold by addition of xylose, but not glucose.. SSS family transporters typically function to transport Na^+ with sugars, amino acids, inorganic ions or vitamins (51). Like previously described transporters, SSS transporters are also found in Prokaryotic and Eukaryotic cells (51). A structure with 13 transmembrane domains appears to be the most common arrangement, but others with 12, 14 and 15 exist (52). While these transporters use a similar transport mechanism to MFS transporters, no sequence or motif similarity have been detected between the two families (53). Unlike MFS transporters, SSS family proteins are only used for uptake, using a sodium symport mechanism. In a manner similar to proton coupled sugar transport, a Na^+-electrochemical gradient provides energy for transport to occur (54).

This transporter (2204—you need to remind us which one you are talking about) bears homology to a number of Na^+/galactose cotransporters in a number of *Alicyclobacillus* species, but also shows greater than 75% homology to a Na^+/galactose co-transporter in *Aneurinibacillus terranovensis*, a novel thermoacidophile that was isolated from geothermal soils (55). In addition, this annotated transporter shows greater

than 50% homology to Na^+/galactose cotransporters in a number of acidophilic bacteria, including a number of *Actinobacteria*.

ABC-Type Transporters – CUT1 Family.

In general, genes for ABC-type transporters were the most highly regulated type of transporter in these experiments, and genes for the CUT1 family, which transports di- and oligosaccharides, was the most represented family. Genes annotated to encode 11 CUT1 transporters and 29 total transporter components were regulated when glucose or xylose was added (Figure 6). For the majority of genes, regulation was typically in the same direction; however, genes for a few transporters were up-regulated on one sugar, but down-regulated with the other. One group of the CUT1 category of genes (Aaci_1215 through Aaci_1217) was also the most highly down-regulated gene responding to the addition of xylose. Levels of regulation for CUT1 transporter genes seen when xylose was added were typically greater than regulation seen when glucose was added.

Gene organization of typical CUT1 transporters is presented in Figure 7 (first line), showing the substrate binding protein, two integral membrane components, and the nucleotide binding domain, or ATPase subunit (56). All but one proposed transporter have genes for both integral membrane components next to one another, with the substrate binding protein gene either up- or down-stream. The transporter encoded by Aaci_0794 to Aaci_0796 has the integral membrane component genes separated by the SBP gene. One attribute of *A. acidocaldarius* strain DSM 446 CUT1 transporters is that none of the groups of genes have individual ATPase subunit genes present with the other transporter genes. The only CUT1 type ATPase gene (Aaci_2139) is found isolated away from genes for the other components. *Bacillus subtilis* and *Streptococcus pneumoniae*

species, which have been shown to use a variety of carbohydrates, were shown to have incomplete saccharide importers that were energized by a multitasking ATPase, similar to that seen in *A. acidocaldarius* strain DSM 446 (41, 57). Like *A. acidocaldarius*, the genome of the *S. pneumoniae* species was shown to encode a number of incomplete CUT1 transporters.

A phylogenetic tree showing homology of Aaci_2139 with ATP-binding proteins from other bacteria is shown in Figure 8. The protein encoded by this gene appears to be well conserved in numerous *Alicyclobacillus* species, ranging from 70 – 92% identity. This protein also appears to be closely related to ATP-binding proteins in numerous *Paenibacillus* species and other *Firmicutes*. Likewise, the encoded protein product from Aaci_2139 is 100% identical to a MalK protein in *A. acidocaldarius*, which was characterized by Scheffel *et al*. (58). In the characterization study, the ABC-type transporter was characterized and showed that genes encoding the sugar binding protein (MalE) and two membrane components (MalF and MalG) were clustered on the chromosome, but the gene encoding MalK was not in this cluster. The *malK* locus in *A. acidocaldarius* was identified by hybridization with *msiK* from *Streptomyces lividans*. Purified MalK expressed in E. coli showed spontaneous ATPase activity, and when co-expressed in *E. coli* with *malF* and *malG*, formed a complex. The MalFGK complex showed low intrinsic ATPase activity; however, when maltose loaded MalE was added ATPase activity increased sevenfold. Genes attributed to this genome locus in the current analysis are Aaci_2871 through Aaci_2873. Results from this study support the recruiting of a remotely located ATP-binding protein for energizing transport. While this operon has been characterized as maltose transporter, regulation during growth on WAX

indicates that the transporter may have a broader substrate range than previously determined.

While interaction of specific transporters with other carbohydrate active proteins, such as glycoside hydrolases, was not determined, proximity to genes for these types of proteins may provide insight into function. Similar functional linkages have been proposed for transporters and glycoside hydrolases found in *T. maritima* (59, 60). The CUT1 transporter encoded by Aaci_0075 to Aaci_0077 is in close proximity to both Aaci_0048 and Aaci_0060, both encode glycoside hydrolases annotated to catalyzed depolymerization of xylan and both loci were down-regulated during the experiments. A proposed CUT1 transporter encoded by genome loci Aaci_1215 through Aaci_1217 may be in an operon with Aaci_1218, which encodes a putative β-galactosidase. These results suggest that the transporter may transports products of depolymerization of WAX by Aaci_1218.

Specificity of ABC-type transporters is defined by the substrate binding protein associated with the transporter complex. CUT1-type transporters employ extracellular substrate-binding proteins from Family 1, which in Gram-positive bacteria are bound to the outer surface of the cell, or in the periplasm of Gram-negative bacteria (61, 62). A circular phylogenetic tree showing the relationship between the 9 Family 1 extracellular solute binding proteins whose genes were regulated during these studies is shown in Figure 9. While all of the SBP proteins showed the most homology to those from other *Alicyclobacillus* species, only non-Alicyclobacilli have been included in the tree to provide a better visual representation of the relationship of the CUT1 associated SBPs to those from other bacteria. These extracellular SBPs from *A. acidocaldarius* strain

DSM446 share 30-45% similarity to SBPs found in other Gram-positive bacteria. In some cases (Aaci_0913 and Aaci_2873), the SBPs primarily reside in thermophilic bacteria.

Further analysis of these proteins showed that the proposed proteins contain SBP bacterial Family 1 (SBP_bac_1) domains. Protein sequence analysis and classification through InterPro indicates that these proteins are involved in high affinity transport, and that these SBPs are membrane- anchored lipoproteins thought to bind oligosaccharides. SBPs with these domains have been found to bind both soluble and non-soluble oligosaccharides in *Caldicellulosiruptor bescii* (63). Likewise, SBP_bac_1 domain proteins have been implicated in the transport of sugars and xylo-oligosaccharides, including those with side groups such as D-glucuronic acid, which would likely be present when WAX is depolymerized (64). While the protein products from these genes have been annotated in the same Family of SBPs, the proposed proteins are different enough to indicate likely differences in the saccharide that is bound by the protein.

ABC-Type Transporters – CUT2 Family.

A second set of ABC-type transporters for carbohydrates was also regulated during the experiments. CUT2 Family transporters, which transport monosaccharides, were down-regulated when xylose was added (Figure 10). When glucose was added, similar down-regulation was noted, with the exception of the transporter encoded by genes Aaci_0259 through Aaci_0261, which ended up being one of the more highly up-regulated sets of genes observed during the experiments. The gene encoding the ATP-binding protein (Aaci_0260) was up-regulated to greater than double the up-regulation seen for the two other components suggesting that these genes may not be co-transcribed

or that Aaci_0260 has a separate promoter. Genes for this transporter were also the least down-regulated of the four sets of genes when xylose was added. Keeping with trends of regulation noted for other *A. acidocaldarius* strain DSM446 genes, down-regulation of CUT2 Family transporters was greater when xylose was added. One interesting trend noted during the experiments was that the SBP-encoding gene associated with each proposed transporter was not down-regulated to the extent of genes encoding the ATP-binding protein, or the integral membrane components. This indicates that the SBP gene was either not being expressed to the same level as genes for the other transporter components, or that expression of the SBP genes continued even when the monosaccharide was added. The SBP associated with the transporter encoded by genes Aaci_0259 to Aaci_0261 did not hold to this trend, as the SBP gene was regulated to a level similar to genes for the other components.

Figure 11 shows that genes for the ATP-binding proteins are located adjacent to genes for the other CUT2 transporter components, unlike gene regions encoding CUT1 Family transporters. Three of the ATP-binding proteins (Aaci_0708, Aaci_2279 and Aaci_2904) contain fused nucleotide binding domains, a trait common for the ATPase subunit of CUT2 transporters; however Aaci_0260 encodes a protein that contains only a single nucleotide binding domain, and is only 260 aa, as opposed to proteins encoded by the other ATP-binding protein genes, which are approximately 500 aa (56). Integral membrane proteins of CUT2 Family transporters are usually formed from two copies of the gene product forming a homodimer, which appears to be the case here, judging by the number of integral membrane components for three of the proposed transporters; however the CUT2 transporter encoded by genes Aaci_0708 through Aaci_0711 has two

separate genes encoding integral membrane components. Figure 9 shows that these genes were equally down-regulated by either sugar. Comparison of Gene Ortholog Neighborhoods within JGI-IMG shows similar gene structure for CUT2 transporters from A. acidocaldarius DSM446 to neighborhoods in a number of *Bacillus* species.

A group of genes spanning from Aaci_2276 through Aaci_2281, containing one of the CUT2 transporters, appears to have similar structure to the *rbs* operon for ribose utilization found in many bacteria (65). Each gene and the proposed function of the encoded protein, the levels of regulation by glucose and xylose in this experiment, and closest relative are shown in Table 3. Down-regulation of all genes from the operon, with the exception of the gene encoding the integral membrane and ATP-binding protein, is equal, and this trend is true regardless of which sugar was added. While the components of the CUT2 transporter are most closely related to those found in *Desulfosporosinus acidophilus*, proteins encoded by the accessory loci are more closely related to those of other bacterial species. However, when comparing gene ortholog neighborhoods, gene organization within this proposed operon is the same between *A. acidocaldarius* strain DSM446 and *D. acidophilus*. Within the Alicyclobacilli, the proposed *rbs* operon and even the associated transporter components, appear to be absent from *A. acidocaldarius* strain Tc-4-1.

A final CUT2 ABC-type transporter is encoded by genes Aaci_2902 to Aaci_2904, located in a region of the genome that contains genes for multiple glycoside hydrolases, most notably, α-arabinofuranosidase (Aaci_2894). Likewise, genes between Aaci_2894 and Aaci_2902 encode other transporters and enzymes involved in processing pentose sugars. These results indicate that this section of the genome may be involved in the

hydrolysis and transport of pentose sugars such as arabinose, which would be released through the activity of the enzyme encoded by Aaci_2894.

ABC-Type Transporters – Opp/Dpp Transporters

One final family of ABC-type transporters that will be considered as potential transporters of saccharides by *A. acidocaldarius* strain DSM446 are those that have been annotated as Opp transporters for oligopeptides or Dpp transporters for dipeptides. Figure 12 shows that 22 genes annotated as Opp/Dpp components were regulated during these experiments. Of these, there are three co-located sets of genes that appear to be operons (Aaci_1896 to Aaci_1900, Aaci_2258 to Aaci_2263 and Aaci_2470 to Aaci_2474), and then six additional genes that encode Family 5 extracellular SBPs (Aaci_0111, Aaci_0403, Aaci_2510, Aaci_2491, Aaci_2492 and Aaci_2889). Two of the sets of genes were some of the most highly down-regulated genes observed during the experiments.

Since the specificity of ABC-type transporters is determined by the SBP, phylogenetic comparison of the SBPs from these Opp/Dpp transporters to other bacteria is shown in Figure 13. As with most of the other transporters in *A. acidocaldarius* strain DSM446, the Opp/Dpp transporter SBPs are most closely related to those of other species of Alicyclobacilli; however, 7 of ten also showed good homology (>60%) to proteins from various species of *Sulfobacillus*. Since Alicyclobacilli and Sulfobacilli are closely related, genetically and phenotypically, these levels of homology may indicate that, at least in some cases, these transporter components have been conserved between these two genera. Typically, *Sulfobacillus* species are chemolithotrophic and gain energy from the oxidation of iron and sulfur; however, some species are mixotrophic or heterotrophic

(66-69). In contrast, most *Alicyclobacillus* species are heterotrophic, but there are examples of Alicyclobacilli that grow autotrophically, gaining energy from inorganic sources (66, 70-72). Likewise, comparison of species from the two Genera have led to reclassification from *Sulfobacillus* to *Alicyclobacillus* and vice versa (67). Another possible indicator of the relationship of Alicyclobacilli and Sulfobacilli is that both groups are characterized by cell walls that contain ω-cyclic fatty acids; these conserved transporters may contain lipid anchors specific for this type of membrane component (73-75).

The additional three SBPs (Aaci_2889, Aaci_2474 and Aaci_0111) are more closely related to SBPs found in various species of *Thermotoga*. This is interesting because numerous oligopeptide transporters in *Thermotoga maritima* have been proven to transport mono-, di-, and oligosaccharides (60). This relationship may support the function of *A. acidocaldarius* strain DSM446 Opp/Dpp transporters in the transport of saccharides released during the hydrolysis of WAX.

The presence of multiple copies (i.e., more copies than proposed transporters) of the SBPs is common for many ABC-type transporters found in Gram-positive and Gram-negative bacteria, as well as Archaea (61). Different types of ABC transporters have been shown to have 1, 2, or 4 SBPs, depending on the function and specific type of the transporter. Multiple SBPs allow for higher rates of substrate transport because of increased binding in proximity to the inner membrane component, where transport across the membrane actually takes place. In addition, multiple SBPs may broaden the specificity of the transporter for additional substrates (62, 76). Analysis of the proposed Opp/Dpp SBPs found in *A. acidocaldarius* strain DSM446 predicts that the proteins

encoded by Aaci_2889 and Aaci_2474 are approximately 100 aa longer than the other 8 SBPs that were regulated during the experiments. Protein functional analysis of the SBPs using InterProScan 5, provided by the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI), indicates that all but one of the translated proteins contains a signal peptide, necessary for transport of these proteins through the cell wall (77). The lipophilic nature of the signal peptide also acts as the lipoprotein anchor, keeping the SBP in proximity to the other transporter components. The protein encoded by Aaci_2492 did not have a signal peptide, so while the gene was down-regulated during the experiments, and shows SBP homology, the protein would likely not be localized to the external surface of the cell. Aaci_2491 is adjacent to Aaci_2492 and does encode an SBP with a signal peptide. Both of these annotated extracellular SBPs were only down-regulated when xylose, and not glucose, was added . The Opp/Dpp transporter encoded by Aaci_2258 through Aaci_2263 is the only of the three proposed Opp/Dpp transporters that has multiple copies of the SBP encoded in the same genome region.

Proximity of two of the Opp/Dpp transporters to glycoside hydrolase genes provides more evidence that these transporters may transport the WAX hydrolysis products produced by the catalytic activity of the glycoside hydrolase genes. Order of the genes encoding the different transporter components and the glycoside hydrolase genes is shown in Figure 14. For each transporter, the inner membrane component genes and the ATP-binding cassette genes are located next to one another. Although the Opp/Dpp transporter encoded by Aaci_2258 through Aaci_2263 is not in proximity to any carbohydrate processing genes, this transporter may still be used for transport of

saccharides. Alternately, this Opp/Dpp transporter may maintain the function of transporting oligo- and dipeptides during cell wall recycling.

While the function of the oligopeptide transporters in *A. acidocaldarius* is not known at this time, additional insight can be obtained from Opp/Dpp transporters found in *Thermotoga maritima* (60, 78). Transcription of components for two distinct Opp/Dpp family transporters was high in the presence of xylose and xylan polysaccharides. The primary difference between *T. maritima* and *A. acidocaldarius* is that the Opp/Dpp transporter in *T. maritima* is located in an apparent xylan-utilization cluster with a Family 10 xylanase gene transversely transcribed from the transporter gene, while in *A. acidocaldarius* neither transporter gene is in proximity to xylanase genes. Comparing gene ortholog neighborhoods, the *A. acidocaldarius* group of genes is more similar to another Opp/Dpp in *Thermotoga* that is adjacent to endoglucanase enzymes. The primary difference noted between these clusters of genes is that in *A. acidocaldarius*, glycoside hydrolase genes are located just upstream (Aaci_2475) and downstream (Aaci_1895) of the transporter genes, but are oriented in the same direction as the transporter genes.

While experiments to look at the specificity of these different Opp/Dpp transporters have not been performed, comparing gene expression during growth on xylose and WAX may provide insight on the substrate of each transporter. In a separate set of chemostat experiments looking at genes expressed by *A. acidocaldarius* during growth on xylose, genes Aaci_2471 through Aaci_2474 were down-regulated when a second sugar was added (results reported elsewhere). The other two proposed Opp/Dpp operon genes were not regulated during the experiments, indicating that the transporter encoded by

Aaci_2471 through Aaci_2474 is responsible for xylose transport, while the other two proposed Opp/Dpp transporters likely transport di- or oligosaccharides generated during hydrolysis of WAX.

Phosphotransferase System Transporters

Finally, components from two phosphoenolpyruvate: phosphotransferase type transporters, those typically associated with carbon catabolite repression in bacteria were regulated during the experiments. Two genes regulated are annotated to encode components for Enzyme IIA (EIIA) and Enzyme IIBC (EIIBC) components for fructose (Aaci_0889 and Aaci_0892, respectively) and a second set encoding EIIA and EIIBC components for a mannitol transport (Aaci_0220 and Aaci_0222 respectively). The EIIBC component of PTS transporters represents a fused protein that contains the transmembrane domain and the substrate binding site, and the sugar phosphorylation site, while EIIA is a cytosolic protein that also contains a phosphorylase domain that triggers catabolite repression in some bacteria (40, 79). Aaci_0220 was up-regulated 2.5-fold when xylose was added and was not regulated when glucose was added. Aaci_0222 was up-regulated (1.7-fold) when xylose was added, but down-regulated (2.0-fold) with glucose. For the fructose-specific transporter components, Aaci_0889 was up-regulated 2.7-fold when xylose was added and 1.7-fold when glucose was added. The EIIBC component (Aaci_0892) was up-regulated (1.6-fold) on glucose and down-regulated (4.4-fold) on xylose. Since neither mannitol nor fructose was tested, the response of these genes to these sugars is not known. Other genes adjacent to Aaci_0222 were similarly regulated, and may be part of an operon, but may not be functional in metabolism of WAX, xylose or glucose. These genes included those for mannitol metabolism:

Aaci_0220, which encodes an EIIC component and Aaci_0223, which encodes a mannitol dehydrogenase domain protein. In addition, a transcriptional anti-terminator related to BglG (Aaci_0221) and genes encoding two phosphotransferase system proteins-- histidine protein (HPr) (Aaci_0224) and a phosphotransferase phosphocarrier protein (Aaci_0225)-- were also regulated similarly for each sugar added. Also, genes surrounding Aaci_0892 were regulated in the same direction, but when xylose was added, down-regulation was more than double for Aaci_0892. Interestingly, the other genes were also related to fructose metabolism; one gene encodes a transcriptional regulator for fructose (Aaci_0890) and the other encodes 1-phosphofructokinase (Aaci_0891).

CONCLUSIONS

A. acidocaldarius was grown in batch chemostat studies where WAX was the sole carbon source for growth. When either xylose or glucose was added, the transcription level changed for a number of genes annotated to encode carbohydrate transporters . In all, 30 proposed sugar transporters are thought to be involved in uptake of WAX hydrolysis products. Carbohydrate transport by *A. acidocaldarius* appears to be facilitated primarily by a number of MFS-type and ABC-type transporters. Since *A. acidocaldarius* is a Gram-positive bacterium, prevalence of PTS-type transporters for saccharide uptake in a manner similar to other *Firmicutes* would be expected (80). However, only two PTS-type transporters were regulated during the study: one related to mannitol and a second for fructose. The final transporter type regulated by addition of xylose or glucose was a sodium solute symporter, which functions in a manner similar to MFS-type transporters.

Eleven total MFS-type transporters were regulated during the experiments: 1) six annotated as COG2814, or arabinose efflux permease proteins; 2) three annotated as COG2271, proposed to transport sugar phosphates; and 3) two proteins annotated for general function only.

ATP-type carbohydrate transporters were the most prevalent type of transporters regulated by the addition of xylose or glucose during growth of *A. acidocaldarius* on WAX; 16 ATP-type transporters were regulated during the experiments. These transporters can be further subdivided into three types: 1) CUT1, which are involved in uptake of di- and oligosaccharides; 2) CUT2, which are responsible for uptake of monosaccharides; and 3) proposed Opp/Dpp transporters thought to transport carbohydrates. One unusual aspect of the CUT1 ABC-type transporters of *A. acidocaldarius* is that only one gene annotated to be a CUT1 ATPase was regulated during the experiments, suggesting that all nine CUT1 transporters share this ATPase. Functionality of this set of transporters indicates that larger WAX hydrolysis products are transported by the CUT1 transporters. There are four sets of annotated CUT2 ATP-type transporters that were regulated during the experiments. All components (i.e., substrate binding protein, inter-membrane components, and ATPases) for these transporters, which would be involved in uptake of sugar monomers released during WAX hydrolysis, were present in apparent operons.

Finally, three ATP-type transporters annotated as oligo-/di-peptide transporters may actually be involved in carbohydrate uptake by *A. acidocaldarius*. Similar transport phenomena have been noted in *Thermotoga* species, in which Opp/Dpp transporters were shown to be involved in carbohydrate uptake (60). As with the CUT2 transporters, each

Opp/Dpp transporter has all components for a complete transporter in apparent operons. One interesting phenomenon for the proposed Opp/Dpp transporters is the presence of accessory substrate binding proteins located away from the primary operon for each specific transporter. These additional SBPs may broaden the substrate range of the respective transporter, but also increase rates of transport because more substrate would be accessible to inner membrane components.

Results from these experiments indicate that *A. acidocaldarius* possesses an arsenal of diverse transporters to facilitate uptake of carbohydrates during growth on polysaccharides such as WAX. As indicated previously, only a couple of PTS-type transporters commonly found in other Gram-positive bacteria were regulated during growth of *A. acidocaldarius*. The prevalence of MFS-type and ATP-type transporters in *A. acidocaldarius* reflect the harsh environment in which the bacterium has been found. Other Bacteria and Archaea found in environments with thermal and acidic conditions have also shown to capitalize on these types of transporters, especially ABC-type transporters (81, 82). These types of transporters are thought to be advantageous in oligotrophic environments due to the high affinity afforded to them by SBPs.

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Table 1. Families of carbohydrate transporters found in the *A. acidocaldarius* strain DSM 446 genome and the associated genome loci.

Table 2. Group of genes in *A. acidocaldarius* genome that appear to encode possible arabinose operon genes. List includes genes for metabolic enzymes and regulator that are associated with MFS-type and ABC-type transporters that are associated with arabinose uptake.

Table 3. Group of genes in *A. acidocaldarius* genome that appears to encode proteins that have similar structure to transporter components and metabolism genes the *rbs* operon found in many bacteria. Each gene and the proposed function, as well as levels of regulation and closest relative are shown.

Figure 1. Schematic showing families of bacterial transporters typically associated with uptake of saccharides.

Figure 2. Regulation of genes annotated as MFS-type transporters in *A. acidocaldarius* when grown on WAX alone compared to regulation when growing on WAX and either glucose or xylose.

Figure 3. Phylogenetic tree showing phylogenetic distance of 12 proposed MFS transporters in *A. acidocaldarius* genome to proteins from other Alicyclobacilli, as well as Bacteria and Archaea. Collapsed sections of tree shows number of *Alicyclobacillus* species with homology to the *A. acidocaldarius* DSM446 protein. Each protein listed in the tree is listed by Accession number and bacterium.

Figure 4. Schematic showing proximity of two MFS-type transporter genes to glycoside hydrolase genes and conservation of these genes among various *Alicyclobacillus* species. (A) MFS-type transporter gene divergently transcribed from glycoside hydrolase and CUT2 type ABC-transporter genes and comparison to similar arrangements in *A. acidocaldarius* strain Tc-4-1 and *A. hesperidum* strain URH17-3-68 genomes. (B) A hydrolase gene for a β-lactamase separating MFS-type and CUT1 type ABC transporter genes, which appears to only be conserved in species of *A. acidocaldarius*. Size of arrow is not proportional to actual gene size.

Figure 5. Schematic showing arrangement of MFS-type transporters in various related *Alicyclobacillus* species. (A) proximity of putative MFS-type transporter for arabinose in *A. acidocaldarius* strain DSM446 genome to α-arabinofuranosidase gene (Aaci_2894) and other genes annotated as arabinose metabolism genes and regulatory components. (B) arrangement of same genes in *A. acidocaldarius* strain Tc-4-1 (C) and *A. hesperidum* URH17-3-68 (C). Key: GH – glycoside hydrolase gene; REG – regulatory gene; MET – metabolic enzyme gene; MFS – multi-facilitator type transporter gene; and PUF – protein of unknown function. Size of arrow is not proportional to actual gene size.

Figure 6. Regulation of genes annotated to encode CUT1 ABC-type transporter components by glucose or xylose. Either sugar was added to *A. acidocaldarius* cultures growing on WAX as described. Labels for x-axis include genome locus and transporter component. Key: SBP – substrate binding protein gene; IMC – inner-membrane component gene; ATP – ATPase gene.

Figure 7. Gene organization of typical CUT1 transporters. Genes for ATPase component are part of operon and all of the annotated CUT1 ABC-type transporters in *A. acidocaldarius* strain DSM446 where gene for ATPase component appears to be at separate genome locus. Key: SBP – substrate binding protein gene; IMC – inner-membrane component gene; ATP – ATPase gene. Size of arrow is not proportional to actual gene size.

Figure 8. A phylogenetic tree showing homology of the product from gene Aaci_2139 with ATP-binding proteins from other bacteria. Each protein listed in the tree is listed by Accession number and bacterium.

Figure 9. A phylogenetic tree showing the relationship between Family 1 extracellular solute binding proteins encoded by genes regulated during these studies. While homology was greatest for other Alicyclobacilli, these species have been omitted from the comparison to show relationship to other bacterial species. Each protein listed in the tree is listed by Accession number and bacterium.

Figure 10. Regulation of CUT2 ATP-type transporters, when xylose or glucose was added to the *A. acidocaldarius* culture growing only on WAX. Labels for x-axis include genome locus and transporter component. Key: SBP – substrate binding protein gene; IMC – inner-membrane component gene; ATP – ATPase gene.

Figure 11. Order of genes for components of CUT2 ABC-type transporters. Key: SBP – gene for substrate binding protein; IMC – gene for inner-membrane component; and ATP – gene for ATPase component. Size of arrow is not proportional to actual gene size.

Figure 12. Regulation of genes annotated as Opp/Dpp transporter components. Labels for x-axis include genome locus and transporter component. Key: SBP – substrate binding protein gene; IMC – inner-membrane component gene; ATP – ATPase gene.

Figure 13. Phylogenetic tree showing comparison of the Family 5 SBPs from Opp/Dpp transporters encoded in the *A. acidocaldarius* genome to Family 5 SBPs from other bacteria. Each protein listed in the tree is listed by Accession number and bacterium.

Figure 14. Schematic showing proximity of genes for Opp/Dpp transporters to glycoside hydrolase genes in the *A. acidocaldarius* genome. A and B are Opp/Dpp transporters adjacent to glycoside hydrolase genes. The Opp/Dpp transporter encoded by genes shown in C are not in close proximity to other carbohydrate processing genes. Key: GH – glycoside hydrolase gene; REG – regulatory gene; MET – metabolic enzyme gene; and MFS – multi-facilitator type transporter gene. Size of arrow is not proportional to actual gene size.

Table 1.

Transporter Family	Genome Location
MFS Superfamily	
	Aaci_0257
	Aaci_0335
	Aaci_0792
	Aaci_1578
	Aaci_2045
	Aaci_2181
	Aaci_2497
	Aaci_2515
	Aaci_2527
	Aaci_2622
	Aaci_2665
	Aaci_2900
Sodium Solute Superfamily	
	Aaci_2204
ABC-Type – CUT1	
	Aaci_0075-Aaci_0077
	Aaci_0768-Aaci_0770
	Aaci_0794-Aaci_0796
	Aaci_0913-Aaci_0915
	Aaci_1215-Aaci_1217
	Aaci_2047-Aaci_2049
	Aaci_2139
	Aaci_2235-Aaci_2237
	Aaci_2306
	Aaci_2458-Aaci_2460
	Aaci_2871-Aaci_2873
ABC-Type – CUT2	
	Aaci_0259-Aaci_0261
	Aaci_0708-Aaci_0711
	Aaci_2277-Aaci_2279
	Aaci_2902-Aaci_2904
ABC-Type – Opp/Dpp	
	Aaci_0111
	Aaci_0403
	Aaci_1896-Aaci_1900
	Aaci_2258-Aaci_2263
	Aaci_2470-Aaci_2474
	Aaci_2491
	Aaci_2492
	Aaci_2510
	Aaci_2889

PTS-Type
Aaci_0220
Aaci_0222
Aaci_0892

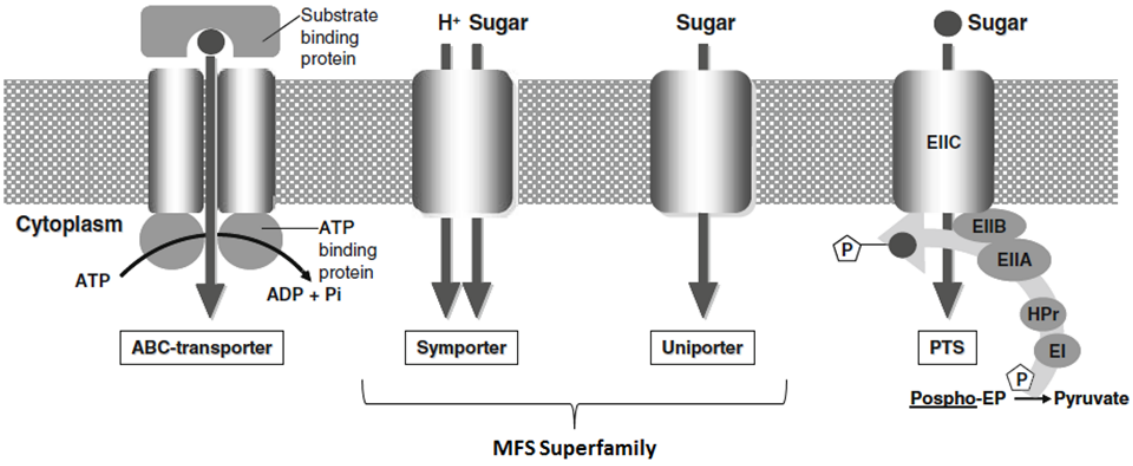
Table 2.

Gene Locus	*ara* operon gene	KEGG Orthology	Glucose/ Xylose	Closest Relative
Aaci_2905	*araR*	Transcriptional regulator, GntR Family	-2.4/-2.4	*Bacillus niacini* (2e-167)
Aaci_2901	*araR*	Transcriptional regulator, GntR Family	-2.8/-3.5	*Bacillus niacini* (3e-169)
Aaci_2899	*araB*	L-Ribulokinase	-3.3/-1.7	*Bacillus* sp. UNC41MFS5 (0)
Aaci_2898	*araD*	L-ribulose-5-phosphate 4-epimerase	-3.2/-2.2	*Bacillus* sp. UNC41MFS5 (2e-118)
Aaci_2897	*araA*	L-arabinose isomerase	-3.2/-2.3	*Anoxybacillus flavithermus* (0)
Aaci_2896	*araM*	Glycerol-1-phosphate dehydrogenase	-4.7/-2.5	*Bacillus* sp. UNC41MFS5 (4e-136)
Aaci_2895	*arsR*	Transcriptional regulator, MarR Family	-1.7/-2.9	*Paenibacillus sp.* Br (2e-131)
Aaci_2890	*araC*	Transcriptional regulator, AraC Family	-/-3.5	*Paenibacillus* sp. A9 *(5e-109)*

Table 3.

Gene Locus	rbs operon gene	Putative Function	Glucose/ Xylose	Closest Relative
Aaci_2276	*rbsK*	Ribokinase	-1.9/-2.2	*Cohnella thermotolerans* (1e-66)
Aaci_2277	*rbsB*	SBP	-1.8/-2.6	*Desulfosporosinus acidophilus (5e-150)*
Aaci_2278	*rbsC*	Inner membrane component	-3.0/-7.4	*Desulfosporosinus acidophilus (4e-152)*
Aaci_2279	*rbsA*	ATP-binding protein	-2.6/-6.8	*Desulfosporosinus acidophilus (0)*
Aaci_2280	*rbsD*	Ribose mutarotase	-1.7/-2.9	*Laribacter hongkongensis (1e-37)*
Aaci_2281	*rbsR*	Transcriptional regulator, LacI family	-2.1/-2.6	*Desulfotomaculum carboxydivorans (5e-109)*

Figure 1.


Substrate binding protein
H+ Sugar
Sugar
Sugar
EIIC
Cytoplasm
ATP
ATP binding protein
ADP + Pi
EIIB
EIIA
HPr
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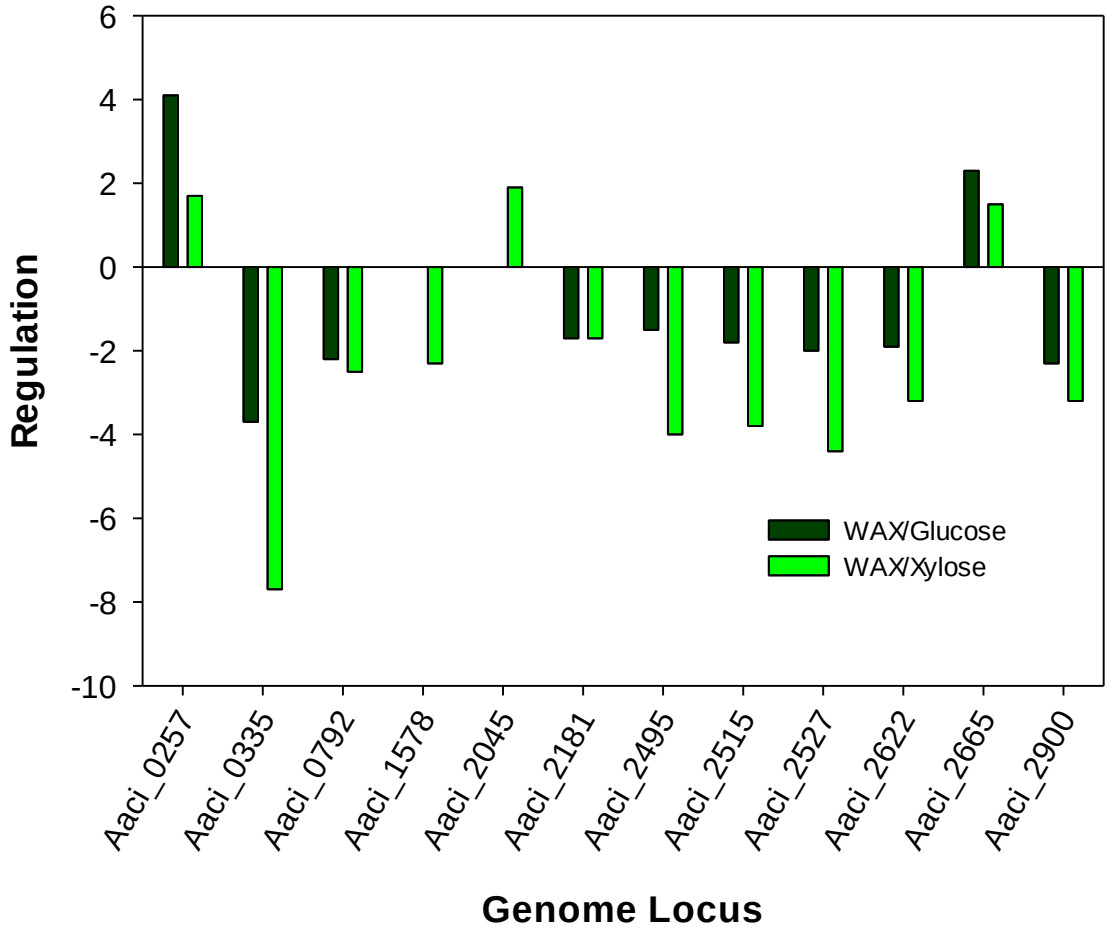
P
P
ABC-transporter
Symporter
Uniporter
PTS
Pospho-EP
Pyruvate
MFS Superfamily

Figure 2.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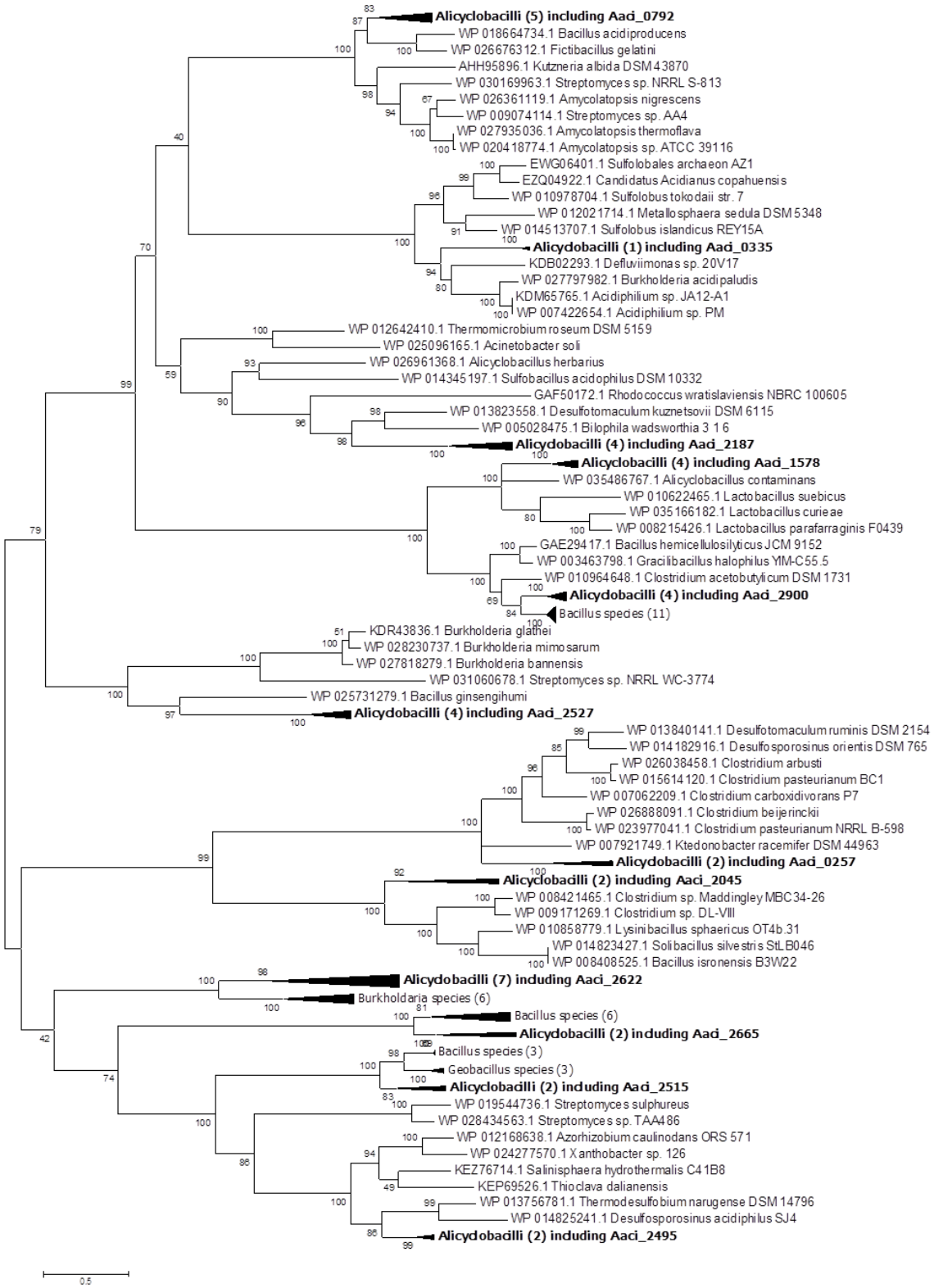
Aaci_2665
Aaci_2900
Genome Locus

Figure 3.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WP 009074114.1 Streptomyces sp. AA4
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EZQ04922.1 Candidatus Acidianus copahuensis
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WP 014513707.1 Sulfolobus islandicus REY15A
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WP 007422654.1 Acidiphilium sp. PM
WP 012642410.1 Thermomicrobium roseum DSM 5159
WP 025096165.1 Acinetobacter soli
WP 026961368.1 Alicyclobacillus herbarius
WP 014345197.1 Sulfobacillus acidophilus DSM 10332
GAF50172.1 Rhodococcus wratislaviensis NBRC 100605
WP 013823558.1 Desulfotomaculum kuznetsovii DSM 6115
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Alicyclobacilli (4) including Aad_1578
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WP 010622465.1 Lactobacillus suebicus
WP 035166182.1 Lactobacillus curieae
WP 008215426.1 Lactobacillus parafarraginis F0439
GAE29417.1 Bacillus hemicellulosilyticus JCM 9152
WP 003463798.1 Gracilibacillus halophilus YIM-C55.5
WP 010964648.1 Clostridium acetobutylicum DSM 1731
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Bacillus species (11)
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WP 028230737.1 Burkholderia mimosarum
WP 027818279.1 Burkholderia bannensis
WP 031060678.1 Streptomyces sp. NRRL WC-3774
WP 025731279.1 Bacillus ginsengihumi
Alicydobacilli (4) including Aad_2527
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WP 014182916.1 Desulfosporosinus orientis DSM 765
WP 026038458.1 Clostridium arbusti
WP 015614120.1 Clostridium pasteurianum BC1
WP 007062209.1 Clostridium carboxidivorans P7
WP 026888091.1 Clostridium beijerinckii
WP 023977041.1 Clostridium pasteurianum NRRL B-598
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Bacillus species (3)
Geobacillus species (3)
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WP 024277570.1 Xanthobacter sp. 126
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KEP69526.1 Thioclava dalianensis
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Figure 4.

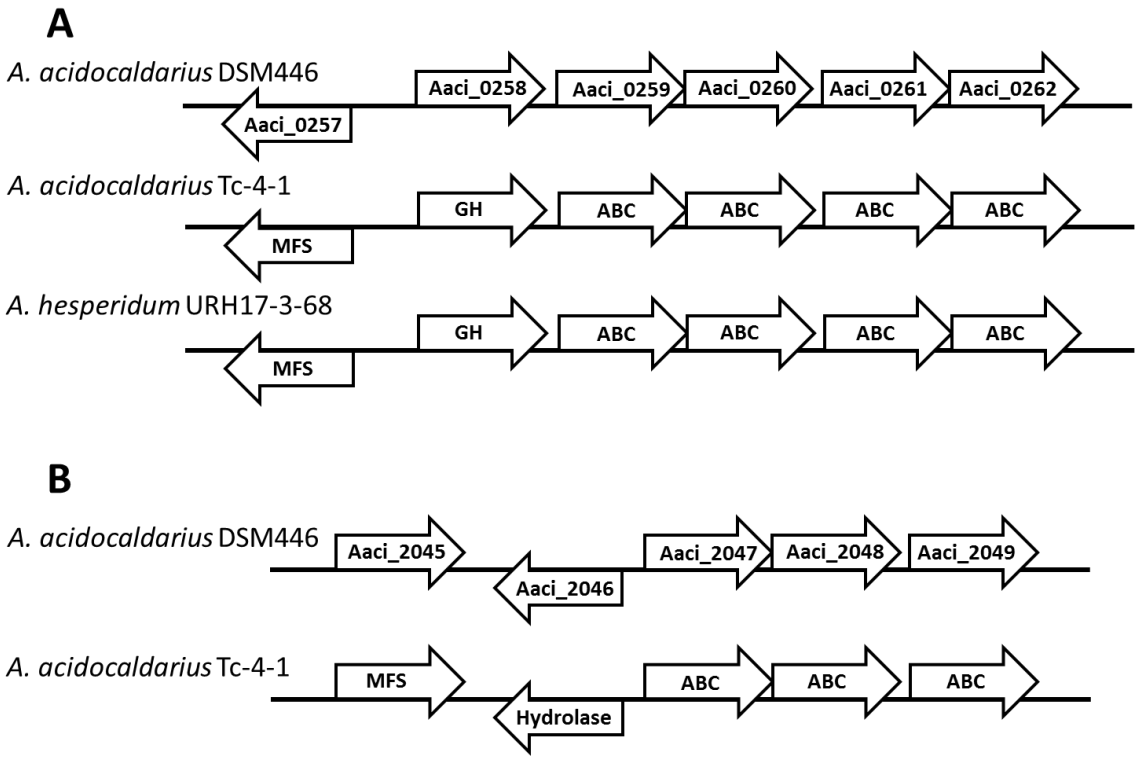
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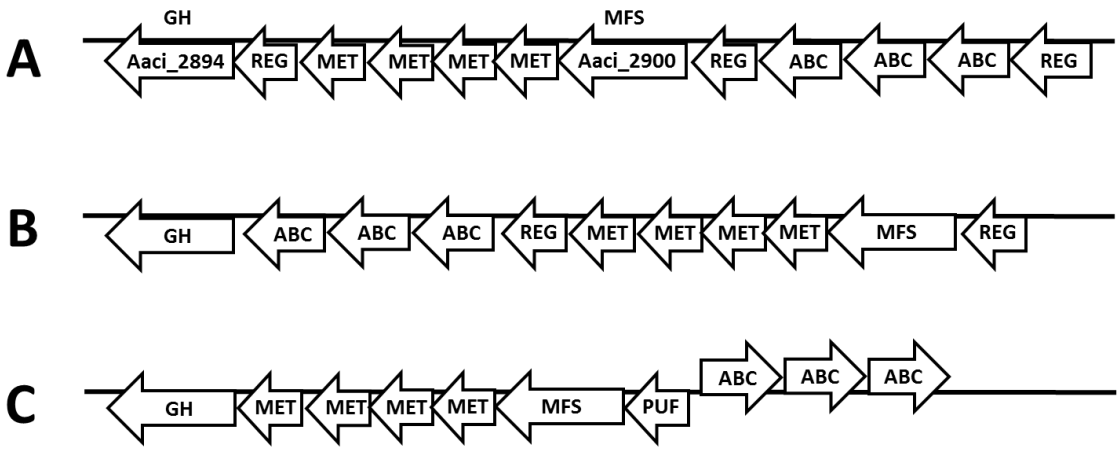
Figure 5.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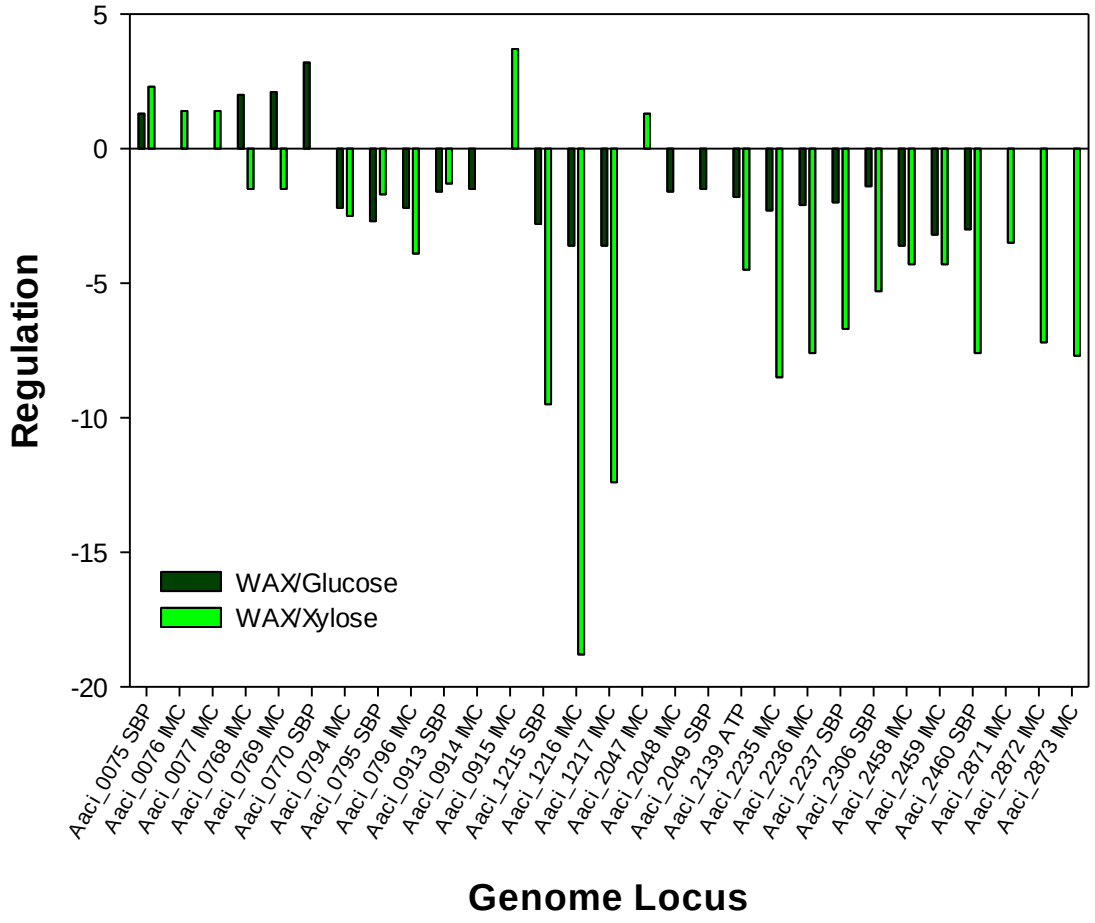
ABC
ABC
REG
B
GH
ABC
ABC
ABC
REG
MET
MET
MET
MET
MFS
REG
C
GH
MET
MET
MET
MET
MFS
PUF
ABC
ABC
ABC

Figure 6.

▪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-10
-15
-20
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Aaci_0077 IMC
Aaci_0768 IMC
Aaci_0769 IMC
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Aaci_0796 IMC
Aaci_0913 SBP
Aaci_0914 IMC
Aaci_0915 IMC
Aaci_1215 SBP
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Aaci_1217 IMC
Aaci_2047 IMC
Aaci_2048 IMC
Aaci_2049 SBP
Aaci_2139 ATP
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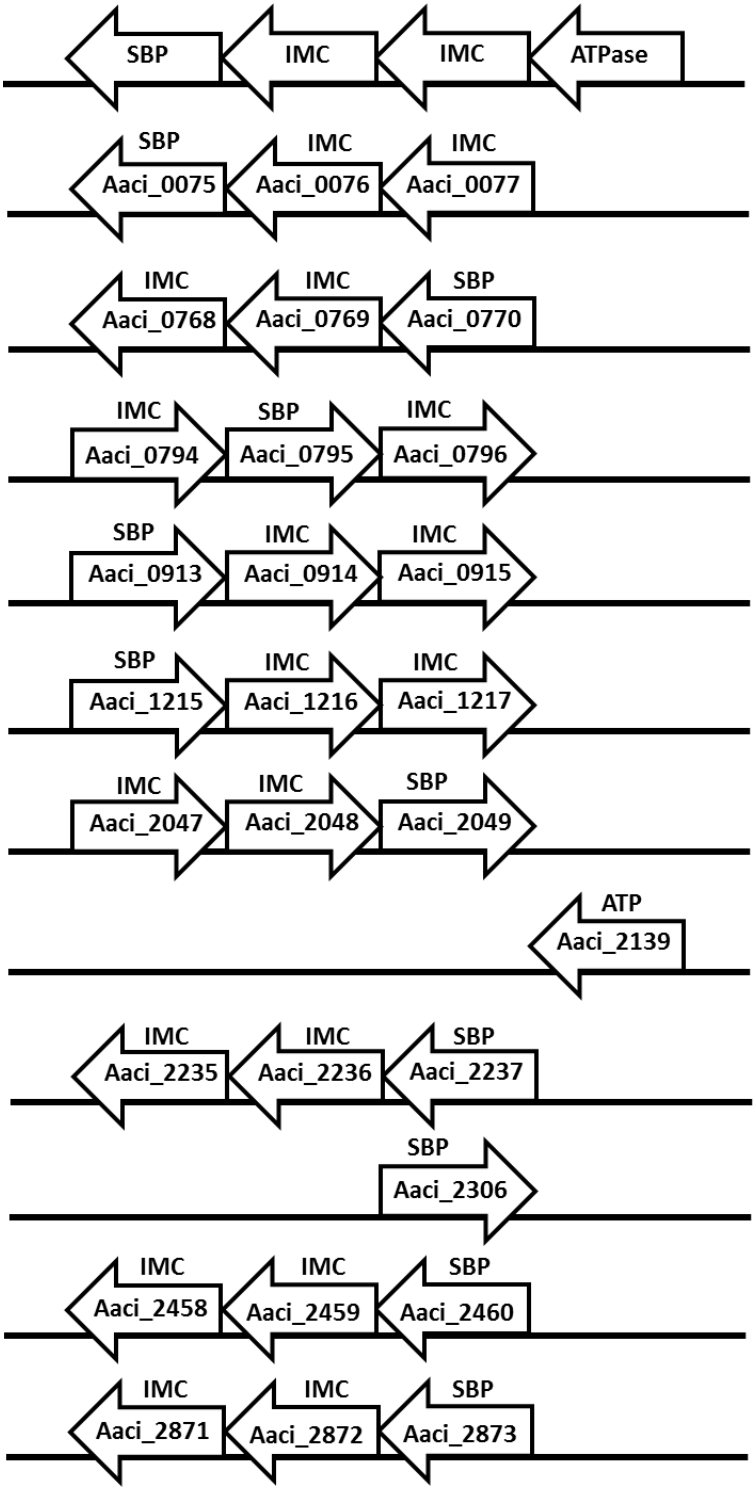
Aaci_2236 IMC
Aaci_2237 SBP
Aaci_2306 SBP
Aaci_2458 IMC
Aaci_2459 IMC
Aaci_2460 SBP
Aaci_2871 IMC
Aaci_2872 IMC
Aaci_2873 IMC
Genome Locus

Figure 7.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IMC
IMC
ATPase
SBP
Aaci_0075
IMC
Aaci_0076
IMC
Aaci_0077
IMC
Aaci_0768
IMC
Aaci_0769
SBP
Aaci_0770
IMC
Aaci_0794
SBP
Aaci_0795
IMC
Aaci_0796
SBP
Aaci_0913
IMC
Aaci_0914
IMC
Aaci_0915
SBP
Aaci_1215
IMC
Aaci_1216
IMC
Aaci_1217
IMC
Aaci_2047
IMC
Aaci_2048
SBP
Aaci_2049
ATP
Aaci_2139
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Aaci_2235
IMC
Aaci_2236
SBP
Aaci_2237
SBP
Aaci_2306
IMC
Aaci_2458
IMC
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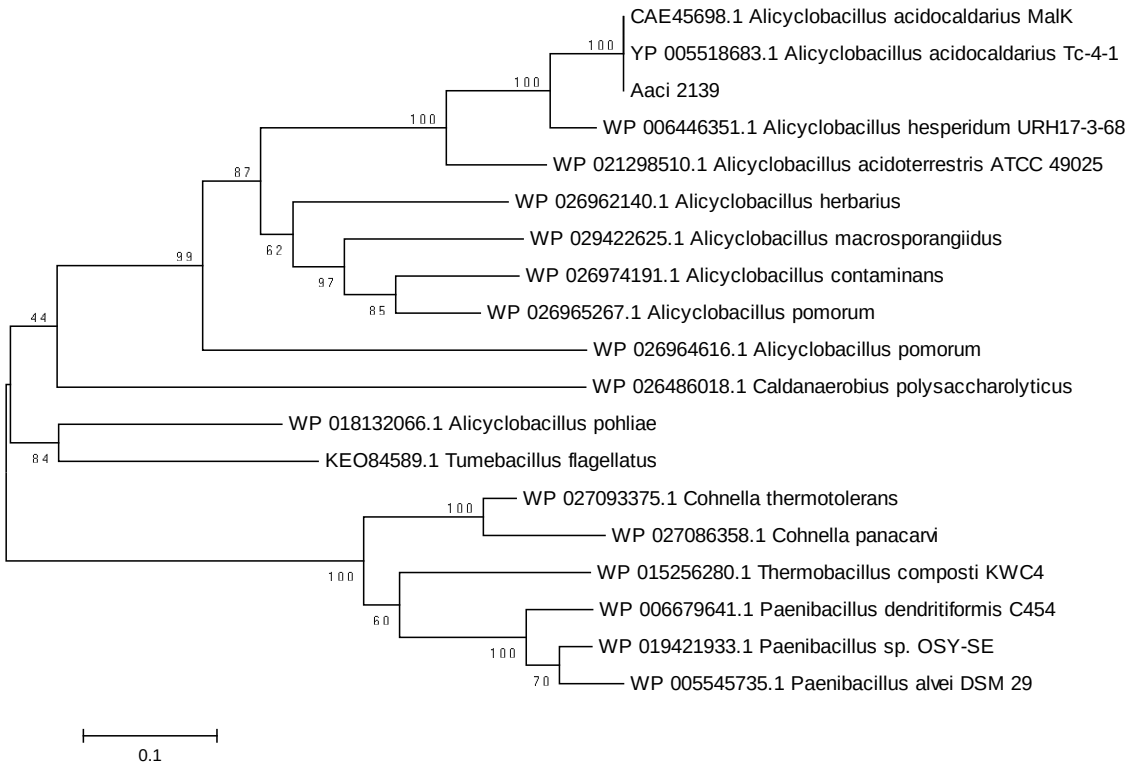
IMC
Aaci_2871
IMC
Aaci_2872
SBP
Aaci_2873

Figure 8.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WP 026962140.1 Alicyclobacillus herbarius
WP 029422625.1 Alicyclobacillus macrosporangiidus
WP 026974191.1 Alicyclobacillus contaminans
WP 026965267.1 Alicyclobacillus pomorum
WP 026964616.1 Alicyclobacillus pomorum
WP 026486018.1 Caldanaerobius polysaccharolyticus
WP 018132066.1 Alicyclobacillus pohliae
KEO84589.1 Tumebacillus flagellatus
WP 027093375.1 Cohnella thermotolerans
WP 027086358.1 Cohnella panacarvi
WP 015256280.1 Thermobacillus composti KWC4
WP 006679641.1 Paenibacillus dendritiformis C454
WP 019421933.1 Paenibacillus sp. OSY-SE
WP 005545735.1 Paenibacillus alvei DSM 29
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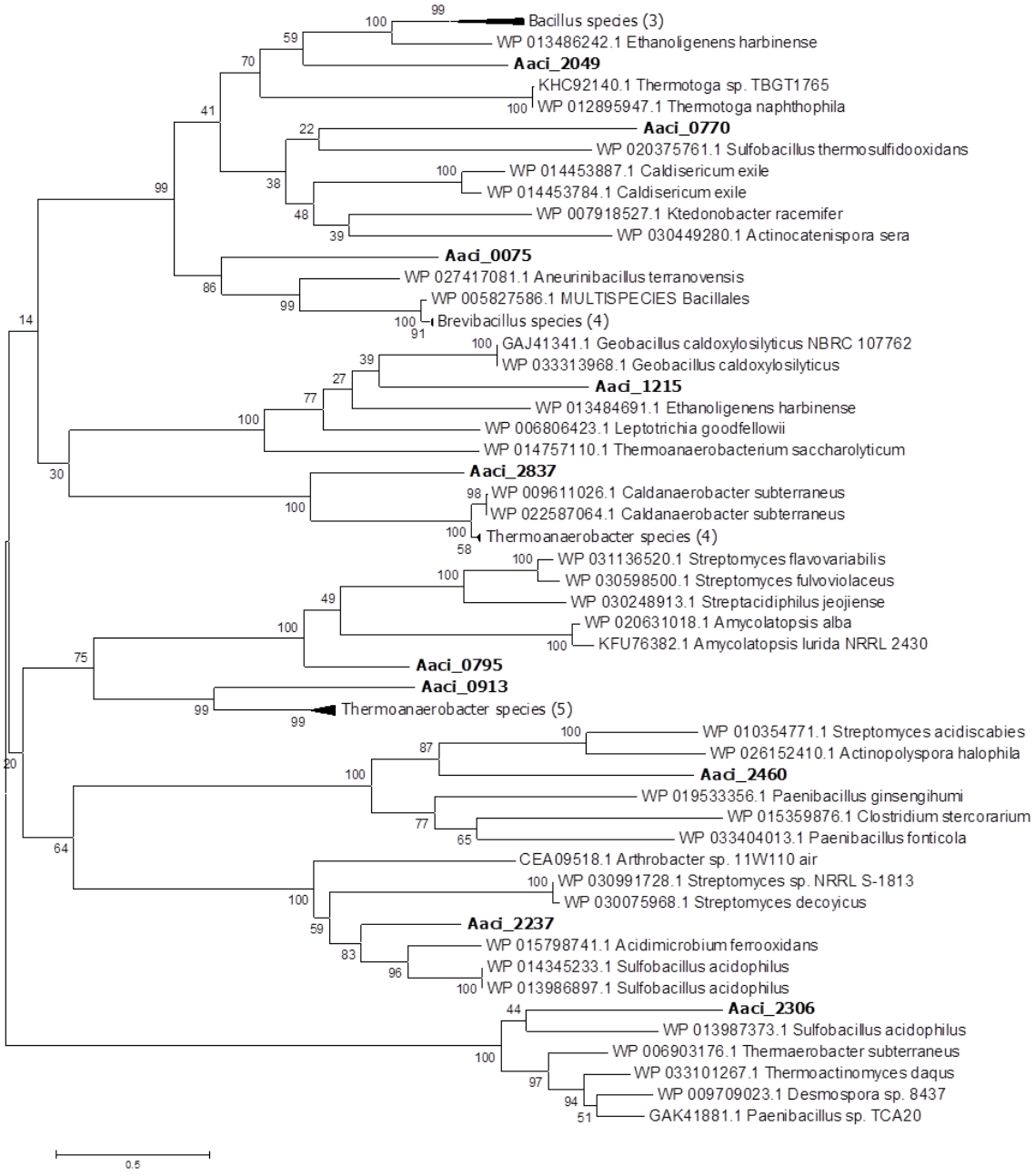
100
87
62
97
85
99
44
84
100
100
60
100
70
0.1

Figure 9.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WP 014453887.1 Caldisericum exile
WP 014453784.1 Caldisericum exile
WP 007918527.1 Ktedonobacter racemifer
WP 030449280.1 Actinocatenispora sera
Aaci_0075
WP 027417081.1 Aneurinibacillus terranovensis
WP 005827586.1 MULTISPECIES Bacillales
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WP 033313968.1 Geobacillus caldoxylosilyticus
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WP 006806423.1 Leptotrichia goodfellowii
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WP 020631018.1 Amycolatopsis alba
KFU76382.1 Amycolatopsis lurida NRRL 2430
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Aaci_0913
Thermoanaerobacter species (5)
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WP 026152410.1 Actinopolyspora halophila
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WP 033404013.1 Paenibacillus fonticola
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WP 030991728.1 Streptomyces sp. NRRL S-1813
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WP 015798741.1 Acidimicrobium ferrooxidans
WP 014345233.1 Sulfobacillus acidophilus
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Aaci_2306
WP 013987373.1 Sulfobacillus acidophilus
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WP 009709023.1 Desmospora sp. 8437
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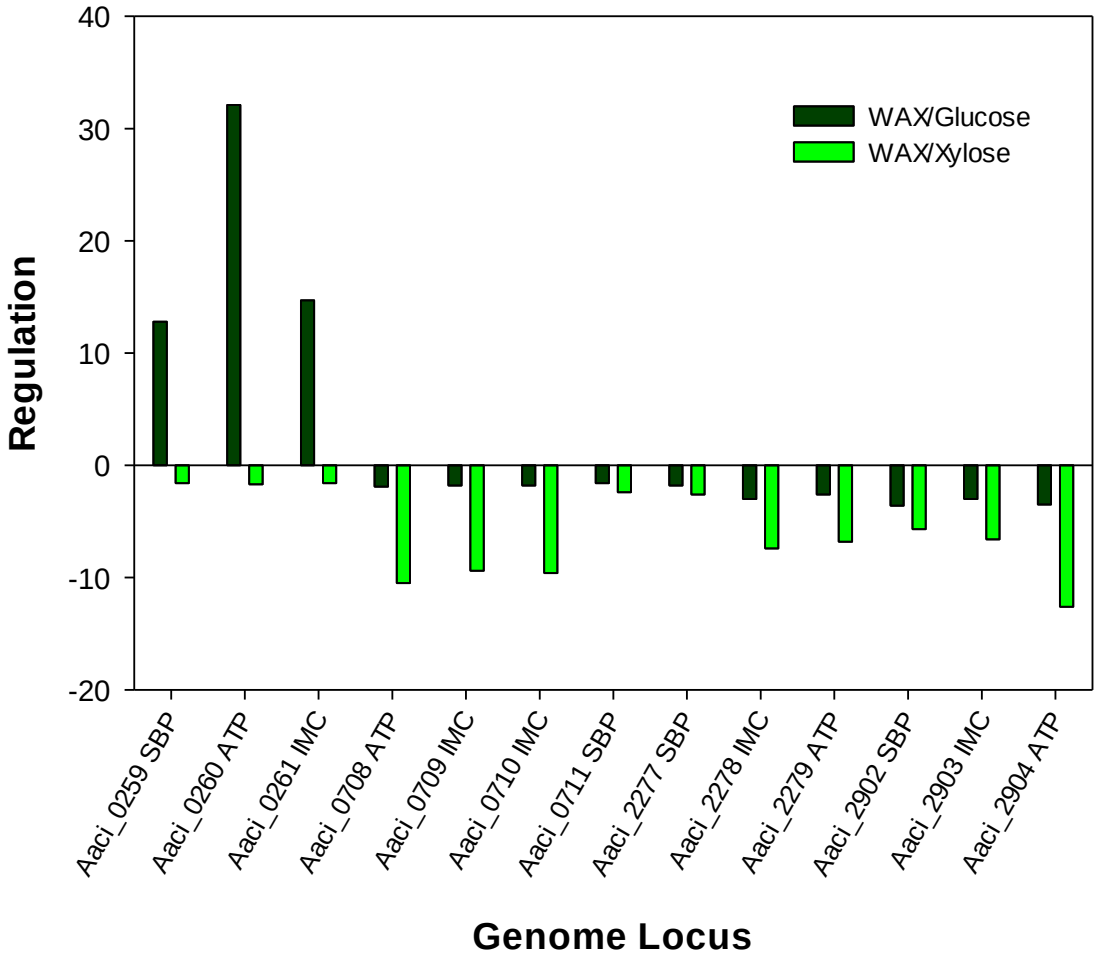
Figure 10.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30
20
10
0
-10
-20
Regulation
WAX/Glucose
WAX/Xylose
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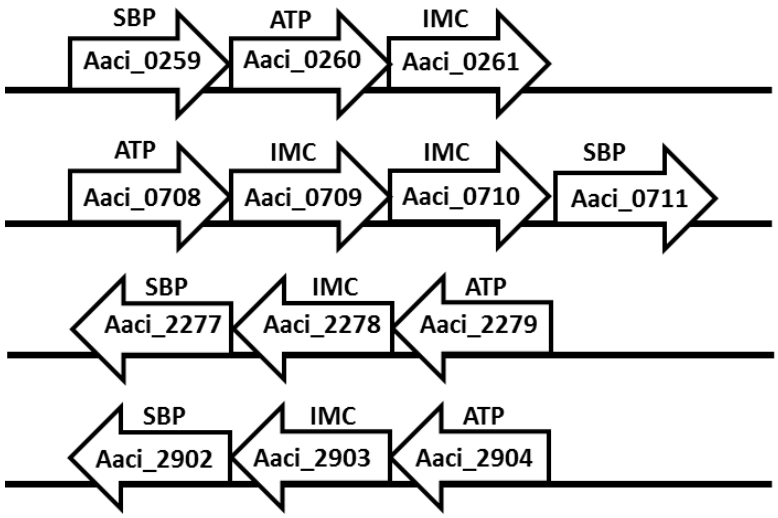
Aaci_0260 ATP
Aaci_0261 IMC
Aaci_0708 ATP
Aaci_0709 IMC
Aaci_0710 IMC
Aaci_0711 SBP
Aaci_2277 SBP
Aaci_2278 IMC
Aaci_2279 ATP
Aaci_2902 SBP
Aaci_2903 IMC
Aaci_2904 ATP
Genome Locus

Figure 11.


SBP
Aaci_0259
ATP
Aaci_0260
IMC
Aaci_0261
ATP
Aaci_0708
IMC
Aaci_0709
IMC
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SBP
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SBP
Aaci_2277
IMC
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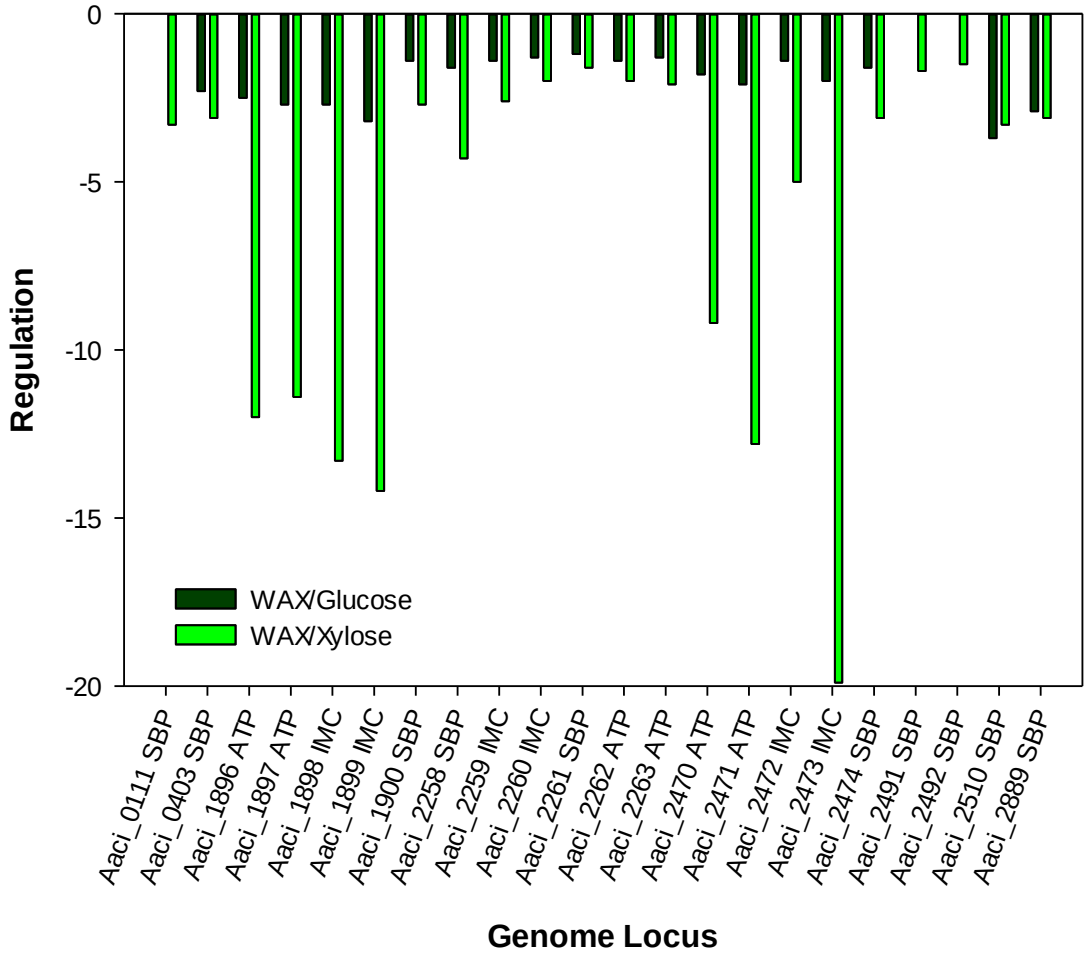
ATP
Aaci_2279
SBP
Aaci_2902
IMC
Aaci_2903
ATP
Aaci_2904

Figure 12.

▪


0
-5
-10
-15
-20
Regulation
WAX/Glucose
WAX/Xylose
Aaci_0111 SBP
Aaci_0403 SBP
Aaci_1896 ATP
Aaci_1897 ATP
Aaci_1898 IMC
Aaci_1899 IMC
Aaci_1900 SBP
Aaci_2258 SBP
Aaci_2259 IMC
Aaci_2260 IMC
Aaci_2261 SBP
Aaci_2262 ATP
Aaci_2263 ATP
Aaci_2470 ATP
Aaci_2471 ATP
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Aaci_2473 IMC
Aaci_2474 SBP
Aaci_2491 SBP
Aaci_2492 SBP
Aaci_2510 SBP
Aaci_2889 SBP
Genome Locus

Figure 13.

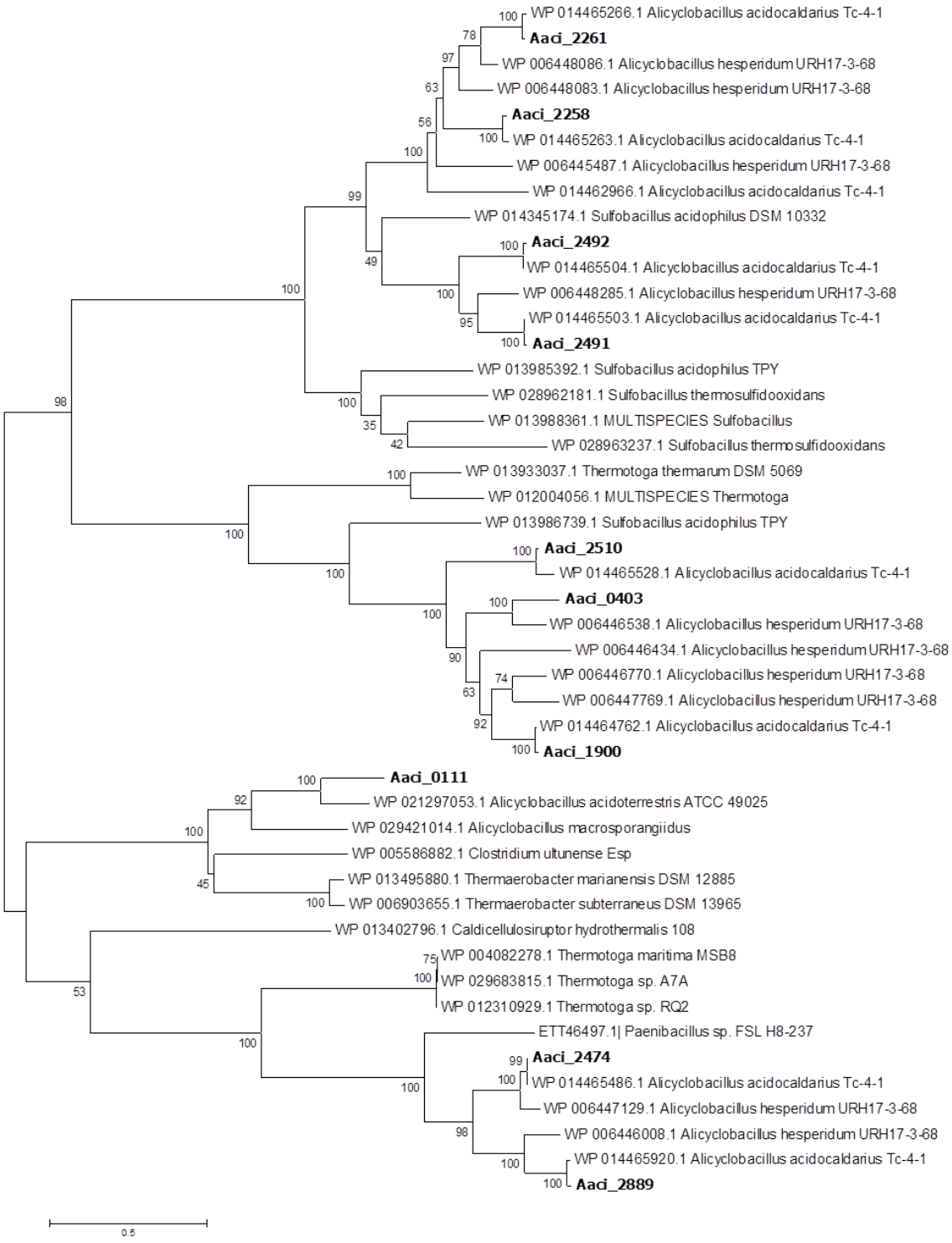
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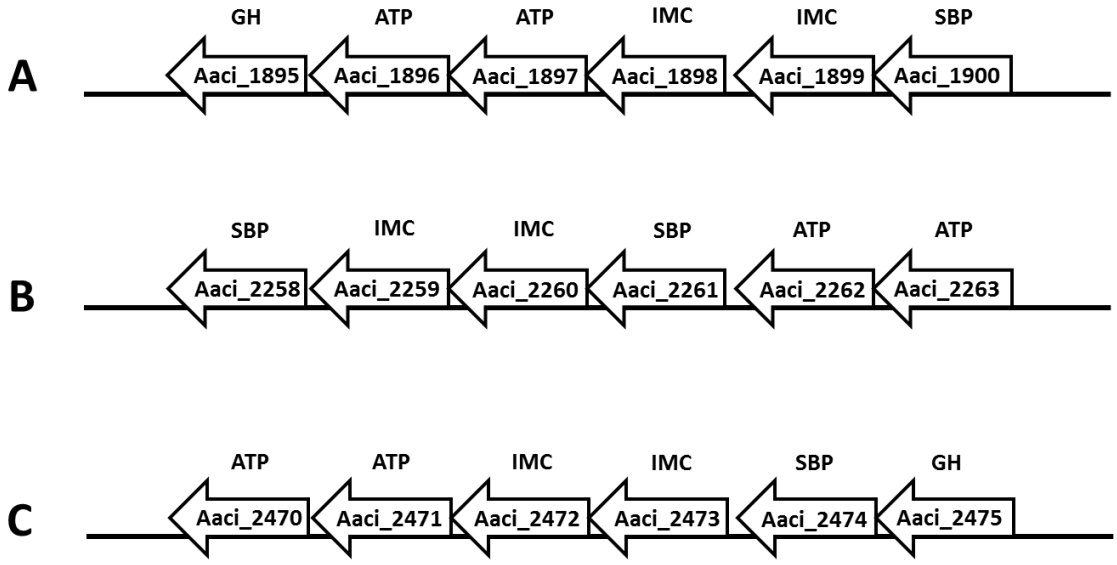
Figure 14.


A
GH
ATP
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IMC
IMC
SBP
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SBP
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Aaci_2261
Aaci_2262
Aaci_2263
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ATP
ATP
IMC
IMC
SBP
GH
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Aaci_2471
Aaci_2472
Aaci_2473
Aaci_2474
Aaci_2475

CHAPTER V: SUMMARY

Discussion of Research Findings

Alicyclobacillus acidocaldarius is a Gram-positive, heterotrophic, thermoacidophile originally isolated from Nymph Creek in Yellowstone National Park. This bacterium exhibits a wide substrate range that includes monosaccharides, disaccharides, as well as oligo- and poly-saccharides including cellulose and hemicellulose. The genome of *A. acidocaldarius* encodes a number of glycosyl hydrolase enzymes allowing depolymerization of lignocellulose derived polysaccharides. In addition, the genome contains genes that encode components of the Gram-positive carbon catabolite repression system (CCR), including catabolite control protein A (CcpA), histidine protein (HPr), HPr kinase/phosphorylase, Crh, and cis-acting catabolite responsive elements (*cre*). For this reason, *A. acidocaldarius* should preferentially use glucose over other saccharides present in the growth environment.

Based on this hypothesis, a number of chemostat experiments were performed using sugar monomers or the polysaccharide wheat arabinoxylan (WAX) to determine which genes were regulated using monosaccharide and complex polysaccharide substrates. In an initial set of experiments, *A. acidocaldarius* was grown on either a pentose sugar (xylose) or a hexose sugar (fructose), and glucose was added to induce CCR. Likewise, two pentose sugars (xylose/arabinose) were also tested. Results indicated that *A. acidocaldarius* was able to use xylose/glucose, xylose/arabinose and fructose/glucose simultaneously and suggested that CCR was not the primary regulator of carbon metabolism during the conditions tested. Xylose, arabinose and glucose at concentrations of 2 g/L were completely metabolized, while fructose was only partially metabolized. In

each experiment, the addition of the second sugar yielded twice as much carbon, which resulted in nearly doubling of the cell density of the culture as determined by optical density.

A common theme between all three experiments was that many of the same genes were regulated regardless of the sugar being use for growth or the sugar added. Interestingly, with the exception of two transporters, most genes were down-regulated during transition from steady-state growth on the first monosaccharides to non-steady state with two monosaccharides. During transition from non-steady-state growth on two sugars to steady-state growth on two sugars, the same genes were up-regulated to nearly the same extent; however there were a few cases where up-regulation was several fold higher than the original down-regulation. Gene categories that were down-regulated included transcriptional regulators (mostly transcriptional activators), a variety of transporters, and enzymes related to metabolism of cellular components, such as cell walls lipids and amino acids, as well as carbon overflow. Some central metabolism genes were also down-regulated during growth on fructose and glucose, including genes for respiration. In general, these results indicate that through a yet-to-be-determined signal transduction mechanism, transport, growth and carbon overflow were temporarily halted when the inducing sugar was added, and then the transcriptional state of the cell returned to a similar level when growing on both sugars. Probably the most important result from the experiments is that *A. acidocaldarius*, unlike many Gram-positive bacteria, is able to simultaneously use pentose and hexose sugars, indicating that this bacterium would be useful for breaking down plant biomass which contains both types of sugars. Parallel utilization of sugars appears to be a function of the types of transporters encoded in the

genome, and a possibly defective HPr component of the CCR system, which may inhibit binding of CcpA to promoter regions which is the key step in CCR.

A second set of batch chemostat experiments was performed to understand the effect of adding monomer sugars to *A. acidocaldarius* exponentially growing on WAX. These experiments showed the utility of this thermoacidophile for depolymerizing the hemicellulose fraction of lignocellulosic biomass. Molecular analysis was carried out using high density oligonucleotide microarray studies. If genes were down-regulated when the inducing sugar was added, the assumption was made that the protein product of the gene was being expressed during growth on WAX. Conversely, if genes were up-regulated when the monosaccharide was added, the assumption was that the gene product was induced by the sugar. Comparison of xylan metabolism in *A. acidocaldarius* to hemicellulose metabolism in other Bacteria and Archaea showed similar mechanisms and may indicate gene transfer from other Prokaryotes allowing growth on these complex carbohydrates. In general, *A. acidocaldarius* strain DSM 446 appears to have enzymes necessary for the complete depolymerization of WAX. Likewise, these enzymes were expressed when *A. acidocaldarius* strain DSM 446 was grown on WAX. A number of the enzymes also appear to have a broad substrate range, allowing for hydrolysis of various bonds present in WAX even though the annotated activity of the enzyme would not predict this hydrolysis. Results from the second set of experiments provided additional support of a non-standard Gram-positive CCR system. While gene transcription was regulated when *A. acidocaldarius* growing on WAX was challenged with glucose or xylose, levels of regulation appeared to be greater when xylose was

added to the culture. Under normal scenarios for regulation, glucose would be expected to exert higher levels of regulation for gene transcription.

During CCR, phosphoenolpyruvate phosphotransferase system (PTS) transporters are expected to facilitate carbohydrate transport in Gram-positive bacteria. In all, 30 proposed sugar transporters are thought to be involved in uptake of WAX hydrolysis products by *A. acidocaldarius*. Carbohydrate transport by *A. acidocaldarius* appears to be facilitated primarily by a number of MFS-type and ABC-type transporters. Only two PTS-type transporters were regulated during the study: one related to mannitol and a second for fructose.

ATP-type carbohydrate transporters were the most prevalent type of transporters regulated by the addition of xylose or glucose during growth of *A. acidocaldarius* on WAX; 16 ATP-type transporters were regulated during the experiments. These transporters can be further subdivided into three types: 1) CUT1, which are involved in uptake of di- and oligosaccharides; 2) CUT2, which are responsible for uptake of monosaccharides; and 3) proposed Opp/Dpp transporters thought to transport carbohydrates. One unusual aspect of the CUT1 ABC-type transporters of *A. acidocaldarius* is that only one gene annotated to be a CUT1 ATPase was regulated during the experiments, suggesting that all nine CUT1 transporters share this ATPase. Functionality of this set of transporters indicates that larger WAX hydrolysis products are transported by the CUT1 transporters. There are four sets of annotated CUT2 ATP-type transporters that were regulated during the experiments. All components (i.e., substrate binding protein, inter-membrane components, and ATPases) for these transporters, which

would be involved in uptake of sugar monomers released during WAX hydrolysis, were present in apparent operons.

Finally, three ATP-type transporters annotated as oligo-/di-peptide transporters may actually be involved in carbohydrate uptake by *A. acidocaldarius*. As with the CUT2 transporters, each Opp/Dpp transporter has all components for a complete transporter in apparent operons. One interesting phenomenon for the proposed Opp/Dpp transporters is the presence of accessory substrate binding proteins located away from the primary operon for each specific transporter. These additional SBPs may broaden the substrate range of the respective transporter, but also increase rates of transport because more substrate would be accessible to inner membrane components.

Results from these experiments indicated that *A. acidocaldarius* possesses an arsenal of diverse transporters to facilitate uptake of carbohydrates during growth on polysaccharides such as WAX. As indicated previously, only a couple of PTS-type transporters commonly found in other Gram-positive bacteria were regulated during growth of *A. acidocaldarius*. The prevalence of MFS-type and ATP-type transporters in *A. acidocaldarius* may reflect the harsh environment in which the bacterium has been found. Other Bacteria and Archaea found in environments with thermal and acidic conditions have also shown to capitalize on these types of transporters, especially ABC-type transporters. These types of transporters are thought to be advantageous in oligotrophic environments due to the high affinity afforded to them by SBPs.

Questions for Future Inquiry

Research performed during execution of this dissertation only scratched the surface related to understanding the physiology and associated gene regulation in *A. acidocaldarius*.

While research described in this dissertation was initially designed to understand CCR in *A. acidocaldarius*, results indicated that other regulatory mechanisms may be more important than CCR. Characterization of the HPr protein found in *A. acidocaldarius* through phosphorylation assays and association with CcpA would help to ascertain whether the additional amino acids before the regulatory histidine are the cause of the relaxed CCR. HPr kinase/phosphorylase could be used to phosphorylate HPr and then protein interaction experiments with CcpA could be performed. Likewise, binding of this complex to promoters of certain genes and operons could be performed.

Additional chemostat experiments to characterize gene expression when *A. acidocaldarius* is grown on other mono-, di-, oligo- and polysaccharides would allow for better characterization of all aspects of gene expression by this bacterium. Growth rates and metabolism of the different compounds could then be used to help define biomass components in development of metabolic models for *A. acidocaldarius*. A genome-enabled metabolic model for *A. acidocaldarius* was created in an unrelated project, using elementary flux mode analysis. Additional data related to growth on different carbon sources would help to refine this model and make it broadly applicable for a range of conditions.

Experiments with oligo- and polysaccharides would allow for determination of the battery of glycoside hydrolase enzymes expressed in response to other carbohydrates associated with lignocellulose, as well as other compounds. Glycoside hydrolase enzymes found in *A. acidocaldarius* have been expressed in *E. coli* and *Pichia pastoris* and characterized for activity on a number of substrates, so this important aspect of this bacterium's physiology has been characterized. Chemostat experiments would allow determination of how these enzymes coordinate for complete depolymerization of different carbohydrates.

Additional characterization of many of the carbohydrate transporters regulated during the experiment would help to specify which transporters are used for specific mono-, di-, oligo- and polysaccharides. These studies would include expression of transporter components followed by exposure to different sugars. Sugar specificity for each of the transporters would be determined through this approach. This would be especially useful for some of the substrate binding proteins that were regulated during the experiment. In addition, experiments to understand carbohydrate components transported by the different oligopeptide/dipeptide transporters would provide information on specificity, as well as information to possibly reclassify these transporters.

APPENDIX I: Xylose/Glucose Chemostat Experiments – Gene Regulation

JGI Seq ID	SEQ_ID	GENE_INFO	Fold change
Aaci_0135	RAAC00201	Hypothetical protein	5.418 down
Aaci_0136	RAAC00200	PE-PGRS family protein	6.230 down
Aaci_0137	RAAC00199	Hypothetical protein	4.635 down
Aaci_0138	RAAC00198	Hypothetical protein	3.579 down
Aaci_0139	RAAC00197	GAF modulated transcriptional regulator, LuxR family	5.616 down
Aaci_0159	RAAC01420	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	4.876 down
Aaci_0160	RAAC01419	MaoC domain-containing protein dehydratase	4.175 down
Aaci_0161	RAAC01418	MaoC domain-containing protein dehydratase	3.272 down
Aaci_0334	RAAC03003	Flavin reductase domain-containing FMN-binding protein	5.518 down
Aaci_0335	RAAC03004	Major Facilitator Superfamily MFS-1; General substrate (sugar) transporter	4.839 down
Aaci_0340	RAAC03009	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	3.849 down
Aaci_0341	RAAC03010	Dihydrodipicolinate synthase (EC 4.2.1.52)	4.183 down
Aaci_0403	RAAC02483	Extracellular solute-binding protein family 5	3.305 down
Aaci_0427	RAAC02457	Hypothetical protein	4.828 down
Aaci_0441	RAAC02442	Iron-containing alcohol dehydrogenase	3.904 down
Aaci_0442	RAAC02441	Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	5.811 down
Aaci_0443	RAAC02440	Major facilitator superfamily MFS-1; General substrate (sugar) transporter	7.344 down
Aaci_0444	RAAC02439	PAS Modulated Fis Family Sigma-54-dependent transcriptional activator	7.270 down
Aaci_0811	RAAC01643	Phenylacetate-coenzyme A ligase (EC 6.2.1.30)	3.323 down
Aaci_0876	RAAC01715	Helix-turn-helix, AraC family	12.229 down
Aaci_0902	RAAC01745	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	3.850 down
Aaci_0904	RAAC01747	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	4.583 down
Aaci_1057	RAAC01900	Aldehyde dehydrogenase (EC 1.2.1.3)	3.428 down

Aaci_1211	RAAC02609	2-HYDROXYCYCLOHEXANE-1-CARBOXYL-COA DEHYDROGENASE (EC 1.3.-.-), short-chain dehydrogenase/reductase SDR	3.199 down
Aaci_1215	RAAC02613	Extracellular solute-binding protein family 1, Lactose-binding protein	3.489 down
Aaci_1216	RAAC02614	Lactose transport system permease protein lacF	3.059 down
Aaci_1253	RAAC00078	Transcriptional regulator, GPR1/FUN34/yaaH family protein	14.589 down
Aaci_1254	RAAC00079	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Acetate/CoA ligase	6.194 down
Aaci_1452	RAAC02922	Acyl-CoA dehydrogenase (EC 1.3.99.-)	9.770 down
Aaci_1454	RAAC02924	Malonate-semialdehyde dehydrogenase (acetylating) (EC 1.2.1.18) / Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	5.800 down
Aaci_1455	RAAC02925	3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4), Enoyl-CoA hydratase/isomerase	5.130 down
Aaci_1456	RAAC02926	Acetyl-coenzyme A synthetase (EC 6.2.1.1)/AMP-(fatty) acid ligase	3.872 down
Aaci_1457	RAAC02927	3-ketoacyl-CoA thiolase (EC 2.3.1.16), acetyl-CoA acetyltransferase	4.078 down
Aaci_1470	RAAC02923	CDP-diacylglycerol/glycerol-3-phosphate 3-phosphatidyltransferase (2.7.8.5)	7.217 down
Aaci_1853	RAAC02037	unassigned membrane protein, GPR1/FUN34/yaaH family	12.494 down
Aaci_2035	RAAC00839	AMP-(fatty)acid ligases	4.200 down
Aaci_2057	RAAC00815	Acetyl-coenzyme A synthetase (EC 6.2.1.1)/Psuedogene?	5.264 down
Aaci_2058	RAAC00814	Acetyl-CoA acetyltransferase (EC 2.3.1.9)	4.691 down
Aaci_2059	RAAC00813	3-oxoacid CoA-transferase subunit B (2.8.3.5)	7.077 down
Aaci_2060	RAAC00812	3-oxoacid CoA-transferase subunit A (2.8.3.5)	7.553 down
Aaci_2471	RAAC01290	Oligopeptide transport ATP-binding protein oppD	4.988 down
Aaci_2472	RAAC01291	Oligopeptide transport system permease protein oppC	4.667 down
Aaci_2473	RAAC01292	Oligopeptide transport system permease protein oppB	5.637 down
Aaci_2474	RAAC01293	Oligopeptide-binding protein oppA, family 5 extracellular solute-binding protein	6.618 down
Aaci_2504	RAAC01327	Aldehyde dehydrogenase (EC 1.2.1.3)	9.002 down
Aaci_2505	RAAC01381	Putative, collagen triple helix repeat-containing protein	3.001 down

Aaci_2510	RAAC01376	Extracellular solute-binding protein family 5	6.890 down
Aaci_2515	RAAC01371	Hypothetical protein/Glucarate/galactarate transporter	3.903 down
Aaci_2529	RAAC01355	L-lactate permease	4.884 down
Aaci_2622	RAAC00299	Major Facilitator Superfamily, MFS-1 Transporter	3.248 down
Aaci_2889	RAAC00596	Oligopeptide-binding protein oppA, family 5 extracellular solute-binding protein	4.591 down
Aaci_2902	RAAC02733	Periplasmic binding protein/LacI transcriptional regulator	3.013 down
	RAAC01420	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	4.876 down
Upregulated Genes			
Aaci_2203	RAAC00663	Hypothetical protein	3.429 up
Aaci_2204	RAAC00662	Sodium solute transporter superfamily	3.843 up
Aaci_2842	RAAC00538	Sulfate transport system permease protein cysW	3.403 up
Aaci_2843	RAAC00539	Sulfate transport system permease protein cysT	3.068 up

APPENDIX II: Xylose/Arabinose Chemostat Experiments – Gene Regulation

JGI Seq ID	SEQ_ID	GENE_INFO	Fold change
Aaci_0135	RAAC00201	Hypothetical protein	4.172 down
Aaci_0136	RAAC00200	PE-PGRS family protein	4.220 down
Aaci_0137	RAAC00199	Hypothetical protein	3.134 down
Aaci_0139	RAAC00197	GAF modulated transcriptional regulator, LuxR family	4.215 down
Aaci_0150	RAAC02277	ABC-1 domain-containing protein, 2-octaprenylphenol hydroxylase (EC 1.14.13.-)	3.146 down
Aaci_0151	RAAC02276	Hypothetical Protein	3.339 down
Aaci_0159	RAAC01420	AMP-dependent synthetase and ligase, Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	3.575 down
Aaci_0160	RAAC01419	MaoC domain-containing protein dehydratase	3.280 down
Aaci_0259	RAAC04053	D-xylose-binding protein, XylF	9.301 down
Aaci_0260	RAAC04054	D-xylose transport ATP-binding protein xylG	9.534 down
Aaci_0261	RAAC04055	Xylose transport system permease protein xylH	7.240 down
Aaci_0334	RAAC03003	Flavin reductase domain-containing FMN-binding protein	5.528 down
Aaci_0335	RAAC03004	Major Facilitator Superfamily MFS-1; General substrate (sugar) transporter	5.442 down
Aaci_0339	RAAC03008	4-hydroxyphenylacetate 3-monooxygenase oxygenase subunit (5.3.3.3)	3.107 down
Aaci_0340	RAAC03009	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	4.185 down
Aaci_0341	RAAC03010	Dihydrodipicolinate synthase (EC 4.2.1.52)	3.623 down
Aaci_0403	RAAC02483	Extracellular solute-binding protein family 5	11.517 down
Aaci_0427	RAAC02457	Hypothetical Protein	3.977 down
Aaci_0441	RAAC02442	Iron-containing alcohol dehydrogenase	3.854 down
Aaci_0442	RAAC02441	Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	5.847 down
Aaci_0443	RAAC02440	Major facilitator superfamily MFS-1; General substrate (sugar) transporter	7.192 down
Aaci_0444	RAAC02439	PAS Modulated Fis Family Sigma-54-dependent transcriptional activator	6.862 down
Aaci_0810	RAAC01642	phenylacetate-CoA oxygenase subunit PaaJ	3.337 down

Aaci_0811	RAAC01643	Phenylacetate-coenzyme A ligase (EC 6.2.1.30)	5.681 down
Aaci_0825	RAAC01657	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	3.019 down
Aaci_0876	RAAC01715	Helix-turn-helix, AraC family	8.841 down
Aaci_0902	RAAC01745	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	3.138 down
Aaci_0904	RAAC01747	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	3.876 down
Aaci_1253	RAAC00078	Transcriptional regulator, GPR1/FUN34/yaaH family protein	10.993 down
Aaci_1254	RAAC00079	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Acetate/CoA ligase	6.048 down
Aaci_1452	RAAC02922	Acyl-CoA dehydrogenase (EC 1.3.99.-)	11.384 down
Aaci_1454	RAAC02924	Malonate-semialdehyde dehydrogenase (acetylating) (EC 1.2.1.18) / Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	6.743 down
Aaci_1455	RAAC02925	3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4), Enoyl-CoA hydratase/isomerase	7.639 down
Aaci_1456	RAAC02926	Acetyl-coenzyme A synthetase (EC 6.2.1.1)/AMP-(fatty) acid ligase	5.105 down
Aaci_1457	RAAC02927	3-ketoacyl-CoA thiolase (EC 2.3.1.16), acetyl-CoA acetyltransferase	6.835 down
Aaci_1458	RAAC02928	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	3.075 down
Aaci_1470	RAAC02923	3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	7.630 down
Aaci_1853	RAAC02037	Unassigned membrane protein, GPR1/FUN34/yaaH family	9.763 down
Aaci_2035	RAAC00839	AMP-(fatty)acid ligases	3.197 down
Aaci_2057	RAAC00815	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Psuedogene?	4.633 down
Aaci_2058	RAAC00814	Acetyl-CoA acetyltransferase (EC 2.3.1.9)	4.043 down
Aaci_2059	RAAC00813	3-oxoacid CoA-transferase subunit B (2.8.3.5)	6.122 down
Aaci_2060	RAAC00812	3-oxoacid CoA-transferase subunit A (2.8.3.5)	6.794 down
Aaci_2070	RAAC00803	LmbE family protein, GlcNAc-PI de-N-acetylase family protein	3.093 down
Aaci_2179	RAAC00690	Xylulose kinase (EC 2.7.1.17)	3.891 down
Aaci_2180	RAAC00689	Xylose isomerase (EC 5.3.1.5)	3.623 down
Aaci_2471	RAAC01290	Oligopeptide transport ATP-binding protein oppD	3.584 down

Aaci_2472	RAAC01291	Oligopeptide transport system permease protein oppC	4.616 down
Aaci_2473	RAAC01292	Oligopeptide transport system permease protein oppB	3.842 down
Aaci_2474	RAAC01293	Oligopeptide-binding protein oppA	6.580 down
Aaci_2504	RAAC01327	Aldehyde dehydrogenase (EC 1.2.1.3)	4.957 down
Aaci_2510	RAAC01376	Extracellular solute-binding protein family 5	4.450 down
Aaci_2515	RAAC01371	Hypothetical protein/Glucarate/galactarate transporter	4.925 down
Aaci_2516	RAAC01370	Hypothetical cytosolic protein, xylose isomerase domain-containing protein	3.213 down
Aaci_2529	RAAC01355	L-lactate permease	3.795 down
Aaci_2622	RAAC00299	Major Facilitator Superfamily Transporter	4.557 down
Aaci_2889	RAAC00596	Oligopeptide-binding protein oppA, family 5 extracellular solute-binding protein	6.308 down
Aaci_2902	RAAC02733	periplasmic binding protein/LacI transcriptional regulator	3.393 down
Aaci_2904	RAAC02735	ABC transporter related, ATP-binding protein	3.091 down
	RAAC02484	unassigned	3.703 down
Upregulated Genes			
Aaci_2203	RAAC00663	Hypothetical protein	7.496 up
Aaci_2204	RAAC00662	Sodium solute transporter superfamily	9.128 up
Aaci_2842	RAAC00538	Sulfate transport system permease protein cysW	3.777 up
Aaci_2843	RAAC00539	Sulfate transport system permease protein cysT	3.376 up
	RAAC00537	Sulfate transport ATP-binding protein cysA	3.698 up

APPENDIX III: Fructose/Glucose Chemostat Experiments – Gene Regulation

JGI Seq ID	SEQ_ID	GENE_INFO	Fold change
Aaci_0055	RAAC02666	Glycerol kinase (EC 2.7.1.30)	3.527 down
Aaci_0133	RAAC00203	Ribonucleoside-diphosphate reductase beta chain (EC 1.17.4.1)	3.129 down
Aaci_0135	RAAC00201	Hypothetical protein	10.430 down
Aaci_0136	RAAC00200	PE-PGRS family protein	10.989 down
Aaci_0137	RAAC00199	Hypothetical protein	4.907 down
Aaci_0139	RAAC00197	GAF modulated transcriptional regulator, LuxR family	5.878 down
Aaci_0150	RAAC02277	ABC-1 domain-containing protein, 2-octaprenylphenol hydroxylase (EC 1.14.13.-)	6.284 down
Aaci_0151	RAAC02276	Hypothetical Protein	5.217 down
Aaci_0152	RAAC02275	Aldehyde dehydrogenase (EC 1.2.1.3)	4.261 down
Aaci_0153	RAAC02274	Iron-containing alcohol dehydrogenase II (EC 1.1.1.1)	4.205 down
Aaci_0159	RAAC01420	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	5.164 down
Aaci_0160	RAAC01419	MaoC domain-containing protein dehydratase	4.625 down
Aaci_0161	RAAC01418	MaoC domain-containing protein dehydratase	3.536 down
Aaci_0334	RAAC03003	Flavin reductase domain-containing FMN-binding protein	8.570 down
Aaci_0335	RAAC03004	Major Facilitator Superfamily MFS-1; General substrate (sugar) transporter	6.888 down
Aaci_0339	RAAC03008	4-hydroxyphenylacetate 3-monooxygenase oxygenase subunit (5.3.3.3)	3.308 down
Aaci_0340	RAAC03009	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	6.018 down
Aaci_0341	RAAC03010	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase/ Dihydrodipicolinate synthase (EC 4.2.1.52)	6.433 down
Aaci_0427	RAAC02457	Hypothetical Protein	5.313 down
Aaci_0444	RAAC02439	PAS Modulated Fis Family Sigma-54-dependent transcriptional activator	9.513 down
Aaci_0504	RAAC04363	HesB/YadR/YfhF-family protein	3.235 down
Aaci_0510	RAAC00976	Hypothetical protein, Cytochrome oxidase assembly protein	10.048 down
Aaci_0511	RAAC00977	Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	15.568 down

Aaci_0512	RAAC00978	Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	7.817 down
Aaci_0513	RAAC00979	Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	8.711 down
Aaci_0514	RAAC04082	Cytochrome c oxidase polypeptide IV (EC 1.9.3.1)	5.155 down
Aaci_0810	RAAC01642	phenylacetate-CoA oxygenase subunit PaaJ	4.218 down
Aaci_0811	RAAC01643	Phenylacetate-coenzyme A ligase (EC 6.2.1.30)	10.575 down
Aaci_0825	RAAC01657	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	4.704 down
Aaci_0826	RAAC01658	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	4.498 down
Aaci_0827	RAAC01659	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	3.211 down
Aaci_0876	RAAC01715	Transcriptional regulator, AraC family	11.090 down
Aaci_0904	RAAC01747	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	3.168 down
Aaci_1055	RAAC01897	Esterase (EC 3.1.1.-), alpha/beta hydrolase	3.630 down
Aaci_1214	RAAC02612	LacI family transcriptional regulator, Ribose operon repressor	3.114 down
Aaci_1215	RAAC02613	Extracellular solute-binding protein family 1, Lactose-binding protein	4.805 down
Aaci_1253	RAAC00078	Transcriptional regulator, GPR1/FUN34/yaaH family protein	19.119 down
Aaci_1254	RAAC00079	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Acetate/CoA ligase	7.537 down
Aaci_1374	RAAC02842	Hypothetical protein	3.071 down
Aaci_1452	RAAC02922	Acyl-CoA dehydrogenase (EC 1.3.99.-)	27.291 down
Aaci_1453	RAAC02923	3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	21.754 down
Aaci_1454	RAAC02924	Malonate-semialdehyde dehydrogenase (acetylating) (EC 1.2.1.18) / Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	11.741 down
Aaci_1455	RAAC02925	3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4), Enoyl-CoA hydratase/isomerase	12.579 down
Aaci_1456	RAAC02926	Acetyl-coenzyme A synthetase (EC 6.2.1.1)/AMP-(fatty) acid ligase	5.326 down
Aaci_1457	RAAC02927	3-ketoacyl-CoA thiolase (EC 2.3.1.16), acetyl-CoA acetyltransferase	7.503 down
Aaci_1458	RAAC02928	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	3.806 down
Aaci_1608	RAAC01041	Pyruvate,phosphate dikinase (EC 2.7.9.1)	5.150 down

Aaci_1609	RAAC01040	Fructose-1,6-bisphosphatase (EC 3.1.3.11)	7.510 down
Aaci_1714	RAAC03277	Hypothetical protein	3.264 down
Aaci_1853	RAAC02037	Hypothetical protein, GPR1/FUN34/yaaH family	6.901 down
Aaci_2035	RAAC00839	AMP-(fatty)acid ligases	3.747 down
Aaci_2057	RAAC00815	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Psuedogene?	8.729 down
Aaci_2058	RAAC00814	Acetyl-CoA acetyltransferase (EC 2.3.1.9)	4.379 down
Aaci_2059	RAAC00813	3-oxoacid CoA-transferase, B subunit	7.565 down
Aaci_2060	RAAC00812	3-oxoacid CoA-transferase, A subunit	14.051 down
Aaci_2157	RAAC00713	Xylose isomerase domain-containing protein	3.200 down
Aaci_2421	RAAC01389	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3), Cobalamin (Vitamin B12) biosynthesis CbiX protein	3.761 down
Aaci_2474	RAAC01293	Oligopeptide-binding protein oppA	3.223 down
Aaci_2504	RAAC01327	Aldehyde dehydrogenase (EC 1.2.1.3)	9.593 down
Aaci_2510	RAAC01376	Extracellular solute-binding protein family 5	6.623 down
Aaci_2515	RAAC01371	Hypothetical protein/Glucarate/galactarate transporter	8.269 down
Aaci_2516	RAAC01370	Xylose isomerase domain protein TIM barrel	3.318 down
Aaci_2529	RAAC01355	L-lactate permease	3.738 down
Aaci_2750	RAAC00443	Stage III sporulation protein D	3.013 down
Aaci_2890	RAAC00598	Transcriptional regulator, AraC family	3.081 down
Aaci_2901	RAAC00001	Transcriptional regulator, GntR Family	3.153 down
Aaci_2902	RAAC02733	periplasmic binding protein/LacI transcriptional regulator	6.429 down
Aaci_2904	RAAC02735	ABC transporter related, ATP-binding protein	4.277 down
Aaci_2906	RAAC00003	Major facilitator superfamily, MFS_1	4.048 down
Upregulated Genes			
Aaci_2204	RAAC00662	Sodium-glucose/galactose cotransporter	3.736 up

Aaci_2879	RAAC04764	General Substrate Transporter, Major Facilitator Superfamily MFS-1	3.878 up

APPENDIX IV: WAX/Glucose Chemostat Experiments – Gene Regulation

JGI Seq ID	SEQ_ID	GENE_INFO	Fold change
Aaci_2901	RAAC00001	Transcriptional regulator, GntR Family	2.779 down
Aaci_2907	RAAC00004	Hypothetical cytosolic protein	2.306 down
Aaci_1240	RAAC00064	Hypothetical protein	2.483 down
Aaci_1241	RAAC00065	Hypothetical protein	2.576 down
Aaci_1242	RAAC00066	Hypothetical protein	2.201 down
Aaci_1249	RAAC00074	Hypothetical protein	2.474 down
Aaci_1253	RAAC00078	Transcriptional regulator, GPR1/FUN34/yaaH family protein	2.773 down
Aaci_1254	RAAC00079	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Acetate/CoA ligase	2.335 down
Aaci_0139	RAAC00197	GAF modulated transcriptional regulator, LuxR family	2.064 down
Aaci_0138	RAAC00198	Hypothetical protein	2.140 down
Aaci_0137	RAAC00199	Hypothetical protein	2.132 down
Aaci_0136	RAAC00200	PE-PGRS family protein	2.502 down
Aaci_0135	RAAC00201	Hypothetical protein	2.383 down
Aaci_0295	RAAC00249	Hypothetical protein	2.980 down
Aaci_2631	RAAC00309	PA-phosphatase like phosphoesterase	2.075 down
Aaci_2633	RAAC00311	putative transmembrane anti-sigma factor	2.155 down
Aaci_2634	RAAC00312	RNA polymerase, sigma-24 subunit, ECF subfamily	2.640 down
Aaci_2668	RAAC00355	aminotransferase class-III	2.029 down
Aaci_2851	RAAC00549	Transcriptional regulator, MerR family	2.687 down
Aaci_2888	RAAC00595	UspA domain protein	2.408 down
Aaci_2889	RAAC00596	extracellular solute-binding protein, Family 5	2.904 down
Aaci_2896	RAAC00604	3-dehydroquinate synthase, Arabinose operon protein araM	4.717 down

Aaci_2897	RAAC00605	L-arabinose isomerase (EC 5.3.1.4)	3.175 down
Aaci_2898	RAAC00606	class II aldolase/adducin family protein; L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4)	3.202 down
Aaci_2899	RAAC00607	L-ribulokinase (EC 2.7.1.16)	3.301 down
Aaci_2900	RAAC00608	sugar transporter; Arabinose/Xylose/Galactose permease; proton symporter	2.271 down
Aaci_2238	RAAC00625	transcriptional regulator, LacI family; Catabolite control protein A	2.363 down
Aaci_2237	RAAC00626	extracellular solute-binding protein family 1; Alpha-glucoside-binding protein	2.048 down
Aaci_2236	RAAC00627	binding-protein-dependent transport systems inner membrane component	2.110 down
Aaci_2235	RAAC00628	binding-protein-dependent transport systems inner membrane component	2.262 down
Aaci_2200	RAAC00667	aldo/keto reductase	2.189 down
Aaci_2180	RAAC00689	Xylose isomerase (EC 5.3.1.5)	2.285 down
Aaci_2179	RAAC00690	Xylulose kinase (EC 2.7.1.17)	2.118 down
Aaci_2177	RAAC00692	peptidase M3A and M3B thimet/oligopeptidase F	2.012 down
Aaci_2112	RAAC00758	RNA polymerase, sigma 28 subunit, SigI	2.134 down
Aaci_2070	RAAC00803	LmbE family protein	3.076 down
Aaci_2069	RAAC00804	major facilitator superfamily MFS_1; SUGAR/SODIUM SYMPORTER	2.741 down
Aaci_2060	RAAC00812	3-oxoacid CoA-transferase subunit A (2.8.3.5)	2.798 down
Aaci_2059	RAAC00813	3-oxoacid CoA-transferase subunit B (2.8.3.5)	3.420 down
Aaci_2058	RAAC00814	Acetyl-CoA acetyltransferase (EC 2.3.1.9)	3.471 down
Aaci_2057	RAAC00815	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Psuedogene?	3.267 down
Aaci_2035	RAAC00839	AMP-(fatty)acid ligases	4.506 down
Aaci_0537	RAAC00840	arsenate reductase (thioredoxin)	2.970 down
Aaci_0536	RAAC00841	Arsenical pump membrane protein	2.117 down
Aaci_1622	RAAC01027	Betaine-aldehyde dehydrogenase; 2-aminomuconate 6-semialdehyde dehydrogenase (EC 1.2.1.32)	2.272 down
Aaci_1593	RAAC01058	protein of unknown function DUF224 cysteine-rich region domain protein	2.160 down

Aaci_0104	RAAC01151	signal transduction histidine kinase, nitrogen specific, NtrB	2.344 down
Aaci_2445	RAAC01263	purine or other phosphorylase family 1	2.105 down
Aaci_2452	RAAC01270	AMP-dependent synthetase and ligase; Acyl-acyl carrier protein synthetase (EC 6.2.1.20)	2.035 down
Aaci_2458	RAAC01276	binding-protein-dependent transport systems inner membrane component	3.606 down
Aaci_2459	RAAC01277	binding-protein-dependent transport systems inner membrane component	3.180 down
Aaci_2460	RAAC01278	extracellular solute-binding protein family 1	3.020 down
Aaci_2461	RAAC01279	hypothetical protein	3.001 down
Aaci_2462	RAAC01280	oxidoreductase domain protein	2.816 down
Aaci_2471	RAAC01290	Oligopeptide transport ATP-binding protein oppD	2.085 down
Aaci_2473	RAAC01292	Oligopeptide transport system permease protein oppB	2.003 down
Aaci_2475	RAAC01294	glycoside hydrolase family 9; Endoglucanase C (EC 3.2.1.4)	2.788 down
Aaci_2504	RAAC01327	Aldehyde dehydrogenase (EC 1.2.1.3)	2.273 down
Aaci_2534	RAAC01350	FAD linked oxidase domain protein; (S)-2-hydroxy-acid oxidase subunit GlcE (EC 1.1.3.15)	2.015 down
Aaci_2533	RAAC01351	protein of unknown function DUF224 cysteine-rich region domain protein; (S)-2-hydroxy-acid oxidase subunit GlcF (EC 1.1.3.15)	2.737 down
Aaci_2532	RAAC01352	FAD linked oxidase domain protein; (S)-2-hydroxy-acid oxidase chain D (EC 1.1.3.15)	2.213 down
Aaci_2529	RAAC01355	L-lactate permease	7.002 down
	RAAC01356		3.577 down
Aaci_2528	RAAC01357	alpha/beta hydrolase fold protein; Lysophospholipase L2 (EC 3.1.1.5)	2.540 down
Aaci_2527	RAAC01359	major facilitator superfamily MFS_1	2.015 down
Aaci_2516	RAAC01370	Xylose isomerase domain protein TIM barrel	2.101 down
Aaci_2510	RAAC01376	extracellular solute-binding protein family 5	3.685 down
Aaci_2554	RAAC01383	sigma 54 modulation protein/ribosomal protein S30EA	2.139 down
Aaci_2403	RAAC01406	hypothetical protein	2.095 down
Aaci_0164	RAAC01415	aminoglycoside phosphotransferase	2.609 down

Aaci_0163	RAAC01416	Hypothetical cytosolic protein	2.526 down
Aaci_0161	RAAC01418	MaoC domain-containing protein dehydratase	2.107 down
Aaci_0160	RAAC01419	MaoC domain-containing protein dehydratase	2.209 down
Aaci_0159	RAAC01420	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	2.556 down
Aaci_0157	RAAC01422	short-chain dehydrogenase/reductase SDR	2.087 down
Aaci_2278	RAAC01502	inner-membrane translocator; Ribose transport system permease protein rbsC	2.972 down
Aaci_2279	RAAC01503	ABC transporter related; Ribose transport ATP-binding protein rbsA	2.646 down
Aaci_2281	RAAC01505	transcriptional regulator, LacI family	2.147 down
Aaci_2290	RAAC01515	2-dehydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase	2.007 down
Aaci_0776	RAAC01605	N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase-like protein exopolysaccharide biosynthesis protein	2.261 down
Aaci_0792	RAAC01622	major facilitator superfamily MFS_1	2.204 down
Aaci_0793	RAAC01624	transcriptional regulator, LacI family	2.479 down
Aaci_0794	RAAC01625	binding-protein-dependent transport systems inner membrane component	2.750 down
Aaci_0795	RAAC01626	extracellular solute-binding protein family 1	2.181 down
Aaci_0796	RAAC01627	binding-protein-dependent transport systems inner membrane component	2.420 down
Aaci_0810	RAAC01642	phenylacetate-CoA oxygenase subunit PaaJ	2.227 down
Aaci_0811	RAAC01643	Phenylacetate-coenzyme A ligase (EC 6.2.1.30)	2.068 down
Aaci_0824	RAAC01656	Histidinol-phosphate aminotransferase (EC 2.6.1.9)	2.785 down
Aaci_0825	RAAC01657	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	3.208 down
Aaci_0826	RAAC01658	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	3.556 down
Aaci_0827	RAAC01659	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	2.177 down
Aaci_0861	RAAC01699	YceI family protein	2.385 down
Aaci_0876	RAAC01715	Helix-turn-helix, AraC family	2.971 down
Aaci_0897	RAAC01740	peptidase S9 prolyl oligopeptidase active site domain protein	2.292 down

	RAAC01744	unassigned	3.067 down
Aaci_0902	RAAC01745	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	4.931 down
Aaci_0903	RAAC01746	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	4.620 down
Aaci_0904	RAAC01747	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	6.187 down
Aaci_0905	RAAC01748	Lactate 2-monooxygenase (EC 1.13.12.4)	3.335 down
Aaci_1899	RAAC01990	binding-protein-dependent transport systems inner membrane component	3.190 down
Aaci_1898	RAAC01991	binding-protein-dependent transport systems inner membrane component	2.740 down
Aaci_1897	RAAC01992	oligopeptide/dipeptide ABC transporter, ATPase subunit	2.655 down
Aaci_1896	RAAC01993	oligopeptide/dipeptide ABC transporter, ATPase subunit	2.464 down
Aaci_1895	RAAC01994	beta-galactosidase/Beta-glucosidase (EC 3.2.1.21)	2.343 down
Aaci_1894	RAAC01995	Hypothetical protein	2.100 down
Aaci_1893	RAAC01996	Diaminopimelate epimerase (EC 5.1.1.7)	2.327 down
	RAAC02036	unassigned	10.717 down
Aaci_1853	RAAC02037	unassigned membrane protein, GPR1/FUN34/yaaH family	8.118 down
Aaci_1810	RAAC02084	Type II secretion system F domain protein	2.003 down
Aaci_1811	RAAC02085	type II secretion system protein E	2.109 down
Aaci_1746	RAAC02144	Anti-sigma F factor antagonist	2.159 down
Aaci_1745	RAAC02145	anti-sigma regulatory factor, serine/threonine protein kinase	2.282 down
Aaci_1744	RAAC02146	RNA polymerase, sigma 28 subunit, SigF	2.319 down
Aaci_0649	RAAC02241	Transcriptional regulator, MarR family	2.281 down
Aaci_0622	RAAC02268	transcriptional regulator, AbrB family	2.269 down
	RAAC02273	unassigned	3.097 down
Aaci_0153	RAAC02274	iron-containing alcohol dehydrogenase	2.935 down
Aaci_0152	RAAC02275	Aldehyde dehydrogenase (EC 1.2.1.3)	3.226 down
Aaci_0151	RAAC02276	hypothetical protein	3.231 down

Aaci_0150	RAAC02277	ABC-1 domain protein; Ubiquinone biosynthesis monooxygenase UbiB	2.934 down
Aaci_3008	RAAC02345	Hypothetical protein	2.078 down
	RAAC02374	unassigned	2.201 down
Aaci_0445	RAAC02438	AMP-dependent synthetase and ligase; Acetyl-coenzyme A synthetase (EC 6.2.1.1)	2.838 down
Aaci_0444	RAAC02439	PAS Modulated Fis Family Sigma-54-dependent transcriptional activator	3.364 down
Aaci_0443	RAAC02440	Major facilitator superfamily MFS-1; General substrate (sugar) transporter	6.015 down
Aaci_0442	RAAC02441	Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	5.317 down
Aaci_0441	RAAC02442	Iron-containing alcohol dehydrogenase	5.166 down
Aaci_0427	RAAC02457	Hypothetical protein	2.860 down
Aaci_0403	RAAC02483	Extracellular solute-binding protein family 5	2.282 down
Aaci_1156	RAAC02558	ParB domain protein nuclease	3.207 down
Aaci_1157	RAAC02559	Hypothetical protein	3.123 down
Aaci_1158	RAAC02560	Hypothetical protein	2.829 down
Aaci_1209	RAAC02607	acyl-CoA dehydrogenase domain protein; Cyclohexanecarboxyl-CoA dehydrogenase (EC 1.3.99.-)	2.246 down
Aaci_1210	RAAC02608	Naphthoate synthase (EC 4.1.3.36)	3.294 down
Aaci_1211	RAAC02609	2-HYDROXYCYCLOHEXANE-1-CARBOXYL-COA DEHYDROGENASE (EC 1.3.-.-), short-chain dehydrogenase/reductase SDR	2.727 down
Aaci_1212	RAAC02610	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	2.153 down
Aaci_1214	RAAC02612	transcriptional regulator, LacI family	2.754 down
Aaci_1215	RAAC02613	extracellular solute-binding protein family 1	2.825 down
Aaci_1216	RAAC02614	binding-protein-dependent transport systems inner membrane component	3.642 down
Aaci_1217	RAAC02615	binding-protein-dependent transport systems inner membrane component	3.604 down
Aaci_0060	RAAC02661	Alpha-glucuronidase; Xylan alpha-1,2-glucuronosidase (EC 3.2.1.131)	2.513 down
Aaci_0059	RAAC02662	short-chain dehydrogenase/reductase SDR; 2-deoxy-D-gluconate 3-dehydrogenase (EC 1.1.1.125)	2.805 down

Aaci_0058	RAAC02663	Transcriptional regulator, GntR family	2.987 down
Aaci_0055	RAAC02666	Glycerol kinase (EC 2.7.1.30)	2.250 down
Aaci_0048	RAAC02676	Alpha-L-arabinofuranosidase-like protein; XX	2.810 down
Aaci_2902	RAAC02733	periplasmic binding protein/LacI transcriptional regulator	3.595 down
Aaci_2903	RAAC02734	inner-membrane translocator	2.982 down
Aaci_2904	RAAC02735	ABC transporter related, ATP-binding protein	3.490 down
Aaci_1452	RAAC02922	Acyl-CoA dehydrogenase (EC 1.3.99.-)	3.690 down
Aaci_1470	RAAC02923	3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	3.746 down
Aaci_1454	RAAC02924	Malonate-semialdehyde dehydrogenase (acetylating) (EC 1.2.1.18) / Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	3.889 down
Aaci_1455	RAAC02925	3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4), Enoyl-CoA hydratase/isomerase	3.836 down
Aaci_1456	RAAC02926	Acetyl-coenzyme A synthetase (EC 6.2.1.1)/AMP-(fatty) acid ligase	4.193 down
Aaci_1457	RAAC02927	3-ketoacyl-CoA thiolase (EC 2.3.1.16), acetyl-CoA acetyltransferase	4.069 down
Aaci_1458	RAAC02928	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	3.401 down
Aaci_0310	RAAC02980	twitching motility protein	2.087 down
Aaci_0311	RAAC02981	Peptidase family A24	2.807 down
Aaci_0312	RAAC02982	DEAD/DEAH box helicase domain protein	2.276 down
Aaci_0318	RAAC02988	transglutaminase domain protein	2.293 down
Aaci_0319	RAAC02989	glycosidase PH1107-related	2.487 down
Aaci_0334	RAAC03003	Flavin reductase domain-containing FMN-binding protein	3.383 down
Aaci_0335	RAAC03004	Major Facilitator Superfamily MFS-1; General substrate (sugar) transporter	3.717 down
Aaci_0339	RAAC03008	4-hydroxyphenylacetate 3-monooxygenase oxygenase subunit (5.3.3.3)	2.258 down
Aaci_0340	RAAC03009	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	2.333 down
Aaci_0341	RAAC03010	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase/ Dihydrodipicolinate synthase (EC 4.2.1.52)	2.460 down
Aaci_0381	RAAC03049	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	2.650 down

	RAAC03123	unassigned	2.179 down
Aaci_0600	RAAC03124	hypothetical protein	2.245 down
Aaci_3142	RAAC03139	hypothetical protein	2.345 down
Aaci_3076	RAAC03196	Diguanylate cyclase/phosphodiesterase domain 2 (EAL)	3.163 down
	RAAC03198	unassigned	3.013 down
Aaci_3077	RAAC03199	Predicted polymerase, most proteins contain PALM domain, HD hydrolase domain and Zn-ribbon domain	2.471 down
Aaci_3078	RAAC03200	response regulator receiver protein	2.138 down
	RAAC03210	Hypothetical protein	2.041 down
	RAAC03211	Hypothetical protein	2.155 down
	RAAC03212	Hypothetical protein	2.344 down
Aaci_1714	RAAC03277	Hypothetical protein	2.468 down
Aaci_1155	RAAC03503	Hypothetical protein	4.217 down
	RAAC04133	unassigned	4.590 down
	RAAC04226	unassigned	2.146 down
	RAAC04271	unassigned	2.068 down
Aaci_3106	RAAC04273	Hypothetical protein	2.158 down
Aaci_1065	RAAC04486	transcriptional regulator, XRE family	2.089 down
	RAAC04505	unassigned	2.303 down
Aaci_0241	RAAC04536	peptidase S15; X-Pro dipeptidyl-peptidase (S15) family protein	2.028 down
Aaci_0234	RAAC04544	diguanylate cyclase/phosphodiesterase	2.255 down
Aaci_0220	RAAC04557	PTS system, mannitol-specific IIBC component (EC 2.7.1.69)	2.039 down
Aaci_3109	RAAC04635	Hypothetical protein	2.095 down
	RAAC04651	unassigned	2.026 down
Aaci_1213	RAAC04671	Protein of unknown function DUF2029	2.824 down

	RAAC04698	Transposase	2.056 down
	RAAC04770	unassigned	2.441 down
	RAAC04829	unassigned	2.244 down
	RAAC04887	unassigned	2.326 down
	RAAC04888	unassigned	3.155 down
	RAAC04918	unassigned	2.371 down
	RAAC04944	unassigned	4.284 down
	Upregulated Genes		
	SEQ_ID	**GENE_INFO**	**Fold change**
Aaci_2928	RAAC00026	SSU ribosomal protein S18P	2.475 up
Aaci_2929	RAAC00027	Single-strand DNA binding protein	2.604 up
Aaci_2930	RAAC00028	SSU ribosomal protein S6P	2.276 up
Aaci_1304	RAAC00134	Uracil phosphoribosyltransferase (EC 2.4.2.9) / Pyrimidine operon regulatory protein pyrR	9.245 up
Aaci_1305	RAAC00135	Aspartate carbamoyltransferase (EC 2.1.3.2)	14.368 up
Aaci_1306	RAAC00136	Dihydroorotase (EC 3.5.2.3)	10.763 up
Aaci_1307	RAAC00137	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	13.414 up
Aaci_1308	RAAC00138	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	8.713 up
Aaci_1309	RAAC00139	Orotidine 5"-phosphate decarboxylase (EC 4.1.1.23)	7.267 up
Aaci_1310	RAAC00140	Orotate phosphoribosyltransferase (EC 2.4.2.10)	12.533 up
Aaci_1311	RAAC00141	FAD-dependent oxidoreductase (EC 1.-.-.-)	5.258 up
Aaci_1312	RAAC00142	Major facilitator superfamily MFS_1	2.001 up
Aaci_2596	RAAC00273	cytochrome c biogenesis protein transmembrane region	2.288 up
Aaci_2617	RAAC00293	thioesterase superfamily protein; Acyl-CoA hydrolase (EC 3.1.2.20)	2.132 up
Aaci_2624	RAAC00301	Copper resistance protein, CopC	3.712 up
Aaci_2625	RAAC00302	Hypothetical membrane associated protein; nuclear export factor GLE1	3.243 up
Aaci_2645	RAAC00323	Hypothetical membrane spanning protein; protein of unknown function DUF81	2.187 up

Aaci_2665	RAAC00351	major facilitator superfamily MFS_1	2.275 up
Aaci_2679	RAAC00367	SSU ribosomal protein S9P	2.927 up
Aaci_2680	RAAC00368	LSU ribosomal protein L13P	2.792 up
Aaci_2681	RAAC00369	tRNA pseudouridine synthase A (EC 4.2.1.70)	2.773 up
Aaci_2682	RAAC00370	LSU ribosomal protein L17P	2.248 up
Aaci_2683	RAAC00371	DNA-directed RNA polymerase alpha chain (EC 2.7.7.6)	2.053 up
Aaci_2684	RAAC00372	SSU ribosomal protein S4P	2.608 up
Aaci_2685	RAAC00373	SSU ribosomal protein S11P	2.825 up
Aaci_2686	RAAC00374	SSU ribosomal protein S13P	2.700 up
Aaci_2687	RAAC00376	LSU ribosomal protein L36P	2.286 up
Aaci_2689	RAAC00378	Hypothetical protein	2.865 up
Aaci_2690	RAAC00379	Adenylate kinase (EC 2.7.4.3) / Nucleoside-diphosphate kinase (EC 2.7.4.6)	3.419 up
Aaci_2691	RAAC00380	Protein translocase subunit secY	2.500 up
Aaci_2692	RAAC00381	LSU ribosomal protein L15P	3.317 up
Aaci_2693	RAAC00382	LSU ribosomal protein L30P	2.892 up
Aaci_2694	RAAC00383	SSU ribosomal protein S5P	2.706 up
Aaci_2695	RAAC00384	LSU ribosomal protein L18P	2.389 up
Aaci_2696	RAAC00385	LSU ribosomal protein L6P	2.247 up
Aaci_2697	RAAC00386	SSU ribosomal protein S8P	2.551 up
Aaci_2698	RAAC00387	SSU ribosomal protein S14P	3.139 up
Aaci_2699	RAAC00388	LSU ribosomal protein L5P	2.185 up
Aaci_2700	RAAC00389	LSU ribosomal protein L24P	2.122 up
Aaci_2701	RAAC00390	LSU ribosomal protein L14P	3.023 up
Aaci_2702	RAAC00391	SSU ribosomal protein S17P	2.550 up
Aaci_2703	RAAC00392	LSU ribosomal protein L29P	2.086 up
Aaci_2705	RAAC00394	SSU ribosomal protein S3P	2.700 up
Aaci_2706	RAAC00395	LSU ribosomal protein L22P	2.692 up
Aaci_2707	RAAC00396	SSU ribosomal protein S19P	2.301 up
Aaci_2708	RAAC00397	LSU ribosomal protein L2P	2.865 up
Aaci_2709	RAAC00398	LSU ribosomal protein L23P	2.555 up
Aaci_2710	RAAC00399	LSU ribosomal protein L1E (= L4P)	2.148 up
Aaci_2711	RAAC00400	LSU ribosomal protein L3P	2.295 up
Aaci_2712	RAAC00401	SSU ribosomal protein S10P	2.803 up
Aaci_2715	RAAC00404	SSU ribosomal protein S7P	2.193 up
Aaci_2017	RAAC00406	hypothetical protein; LSU ribosomal protein L7AE	2.122 up
Aaci_2721	RAAC00411	LSU ribosomal protein L12P (L7/L12)	2.826 up
Aaci_2722	RAAC00412	LSU ribosomal protein L10P	4.210 up
Aaci_2723	RAAC00413	LSU ribosomal protein L1P	2.068 up
Aaci_2724	RAAC00414	LSU ribosomal protein L11P	2.041 up
Aaci_2725	RAAC00415	Transcription antitermination protein nusG	2.400 up

Aaci_2756	RAAC00449	ATP synthase epsilon chain (EC 3.6.3.14)	3.159 up
Aaci_2757	RAAC00450	ATP synthase beta chain (EC 3.6.3.14)	2.908 up
Aaci_2758	RAAC00451	ATP synthase gamma chain (EC 3.6.3.14)	2.722 up
Aaci_2759	RAAC00452	ATP synthase alpha chain (EC 3.6.3.14)	2.733 up
Aaci_2760	RAAC00453	ATP synthase delta chain (EC 3.6.3.14)	2.609 up
Aaci_2761	RAAC00454	ATP synthase B chain (EC 3.6.3.14)	2.461 up
Aaci_2673	RAAC00456	ATP synthase A chain (EC 3.6.3.14)	2.211 up
Aaci_2765	RAAC00458	hypothetical protein	2.389 up
Aaci_2779	RAAC00470	protein-(glutamine-N5) methyltransferase, release factor-specific	2.421 up
Aaci_2780	RAAC00472	Bacterial Peptide Chain Release Factor 1 (RF-1)	2.311 up
Aaci_2814	RAAC00507	O-acetyl-L-homoserine sulfhydrolase (EC 2.5.1.49) / O-acetyl-L-serine sulfhydrolase (EC 2.5.1.47)	7.081 up
Aaci_2815	RAAC00508	Uncharacterized protein family UPF0324	6.781 up
Aaci_2827	RAAC00521	Cyanate lyase (EC 4.2.1.104)	2.715 up
Aaci_2840	RAAC00536	Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) / Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)	3.378 up
Aaci_2841	RAAC00537	Sulfate transport ATP-binding protein cysA	16.984 up
Aaci_2842	RAAC00538	Sulfate transport system permease protein cysW	19.443 up
Aaci_2843	RAAC00539	Sulfate transport system permease protein cysT	13.713 up
Aaci_2844	RAAC00540	Sulfate-binding protein	16.533 up
Aaci_2845	RAAC00543	ABC transporter related	2.645 up
Aaci_2846	RAAC00544	binding-protein-dependent transport systems inner membrane component	5.119 up
Aaci_2847	RAAC00545	alkanesulfonate monooxygenase, FMNH(2)-dependent	3.382 up
Aaci_2848	RAAC00546	aliphatic sulfonates family ABC transporter, periplsmic ligand-binding protein	3.734 up
Aaci_2849	RAAC00547	FMN reductase (EC 1.5.1.29)	6.810 up
Aaci_2850	RAAC00548	Hypothetical protein; Protein of unknown function DUF2292	7.732 up
Aaci_2892	RAAC00600	Ammonium transporter	6.654 up
Aaci_2893	RAAC00601	Nitrogen regulatory protein P-II	2.573 up
Aaci_2173	RAAC00697	protein of unknown function DUF395 YeeE/YedE	4.047 up
Aaci_2159	RAAC00711	hypothetical protein	2.229 up
Aaci_2144	RAAC00727	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	2.328 up
Aaci_2130	RAAC00740	Phenylalanyl-tRNA synthetase alpha chain (EC 6.1.1.20)	2.031 up
Aaci_2110	RAAC00760	S-adenosylmethionine:tRNA ribosyltransferase-isomerase (EC 5.-.-.-)	2.213 up

Aaci_2109	RAAC00761	Queuine tRNA-ribosyltransferase (EC 2.4.2.29)	2.163 up
	RAAC00777	unassigned	6.203 up
Aaci_2092	RAAC00778	Hypothetical protein	2.026 up
Aaci_2091	RAAC00779	Protoporphyrinogen oxidase (EC 1.3.3.4)	2.286 up
Aaci_2090	RAAC00780	Ferrochelatase (EC 4.99.1.1)	2.888 up
Aaci_2089	RAAC00781	Uroporphyrinogen decarboxylase (EC 4.1.1.37)	3.359 up
Aaci_2038	RAAC00836	Histidyl-tRNA synthetase (EC 6.1.1.21)	2.442 up
Aaci_2037	RAAC00837	Aspartyl-tRNA synthetase (EC 6.1.1.12)	2.242 up
Aaci_2027	RAAC00847	protein of unknown function UPF0118; Hypothetical membrane spanning protein	2.782 up
Aaci_2026	RAAC00849	Alanyl-tRNA synthetase (EC 6.1.1.7)	2.095 up
Aaci_1964	RAAC00910	Spore germination protein	2.020 up
Aaci_1958	RAAC00916	Phosphate transporter	3.015 up
Aaci_1957	RAAC00917	Hypothetical protein	2.412 up
Aaci_1955	RAAC00920	Hypothetical protein	2.857 up
Aaci_1954	RAAC00921	Prolipoprotein diacylglyceryl transferase (EC 2.4.99.-)	2.026 up
Aaci_1938	RAAC00938	Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	5.020 up
Aaci_1937	RAAC00939	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	4.546 up
Aaci_1935	RAAC00941	ABC transporter, CydDC cysteine exporter (CydDC- E) family, permease/ATP-binding protein CydC	2.325 up
Aaci_1906	RAAC00968	phosphate uptake regulator, PhoU	2.424 up
Aaci_1905	RAAC00969	phosphate ABC transporter, ATPase subunit; Phosphate transport ATP-binding protein pstB	3.734 up
Aaci_0515	RAAC00980	PAS/PAC sensor signal transduction histidine kinase	2.065 up
Aaci_0516	RAAC00981	two component transcriptional regulator, LuxR family	2.455 up
Aaci_1628	RAAC01020	Glutamine synthetase (EC 6.3.1.2)	6.918 up
Aaci_1557	RAAC01095	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	2.748 up
Aaci_0072	RAAC01117	FAD dependent oxidoreductase	3.759 up
Aaci_2422	RAAC01238	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)	10.653 up
Aaci_2423	RAAC01239	Sulfite reductase [NADPH] hemoprotein beta-component (EC 1.8.1.2)	13.944 up
Aaci_2424	RAAC01240	adenylylsulfate reductase, thioredoxin dependent; Phosphoadenosine phosphosulfate reductase (EC 1.8.4.8)	14.796 up
Aaci_2465	RAAC01284	Exonuclease RNase T and DNA polymerase III	3.421 up
Aaci_2466	RAAC01285	Domain of unknown function DUF294, putative nucleotidyltransferase substrate-binding	11.497 up
Aaci_2467	RAAC01286	Glutamine synthetase (EC 6.3.1.2)	9.453 up
Aaci_2468	RAAC01287	Ammonium transporter / Methylammonium	13.233

		transporter	up
Aaci_2421	RAAC01389	cobalamin (vitamin B12) biosynthesis CbiX protein	18.937 up
Aaci_2420	RAAC01390	Hypothetical protein	2.329 up
Aaci_2406	RAAC01403	Na(+)/H(+) antiporter/exchanger	2.583 up
Aaci_2270	RAAC01494	Adenylosuccinate synthetase (EC 6.3.4.4)	3.447 up
Aaci_2271	RAAC01495	Exodeoxyribonuclease III (EC 3.1.11.2)	2.136 up
Aaci_2272	RAAC01496	Peptidase S41	2.893 up
Aaci_2273	RAAC01497	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	5.749 up
Aaci_2274	RAAC01498	transcriptional regulator, DeoR family; Central glycolytic genes regulator	5.693 up
Aaci_2309	RAAC01534	Formamidase (EC 3.5.1.49)	2.309 up
Aaci_2310	RAAC01535	integral membrane sensor signal transduction histidine kinase	2.173 up
Aaci_0742	RAAC01567	Triosephosphate isomerase (EC 5.3.1.1)	2.510 up
Aaci_0743	RAAC01568	Phosphoglycerate mutase (EC 5.4.2.1)	2.391 up
Aaci_0744	RAAC01569	Enolase (EC 4.2.1.11)	2.237 up
Aaci_0746	RAAC01571	major facilitator superfamily MFS_1	2.942 up
Aaci_0753	RAAC01582	hypothetical protein	2.467 up
Aaci_0754	RAAC01583	Glycosyltransferase (EC 2.4.1.-)	3.066 up
Aaci_0755	RAAC01584	hypothetical protein	3.658 up
Aaci_0756	RAAC01585	hypothetical protein	4.197 up
Aaci_0757	RAAC01586	Hypothetical protein	4.268 up
Aaci_0763	RAAC01593	amino acid permease-associated region	7.381 up
Aaci_0764	RAAC01594	Urea carboxylase-associated protein 2	3.951 up
Aaci_0765	RAAC01595	Urea carboxylase-associated protein 1	6.974 up
Aaci_0766	RAAC01596	Urea carboxylase (EC 6.3.4.6)	2.433 up
Aaci_0767	RAAC01597	Allophanate hydrolase (EC 3.5.1.54)	2.181 up
Aaci_0768	RAAC01599	binding-protein-dependent transport systems inner membrane component	2.090 up
Aaci_0769	RAAC01600	binding-protein-dependent transport systems inner membrane component	3.155 up
Aaci_0770	RAAC01601	extracellular solute-binding protein family 1	2.076 up
Aaci_0852	RAAC01689	Iron permease FTR1	3.234 up
Aaci_0864	RAAC01703	Sulfate adenylyltransferase (EC 2.7.7.4)	4.868 up
Aaci_0865	RAAC01704	transcriptional regulator, MarR family	4.036 up
Aaci_0866	RAAC01705	Protein of unknown function DUF2071	5.626 up
Aaci_0890	RAAC01731	transcriptional regulator, DeoR family; Fructose repressor	2.276 up
Aaci_0891	RAAC01732	1-phosphofructokinase (EC 2.7.1.56)	2.047 up
Aaci_0918	RAAC01761	Protoheme IX farnesyltransferase (EC 2.5.1.-)	3.330 up
Aaci_0924	RAAC01767	ATP phosphoribosyltransferase (EC 2.4.2.17)	2.060 up
Aaci_0926	RAAC01769	Imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19)	2.004 up

Aaci_0927	RAAC01770	Imidazole glycerol phosphate synthase, glutamine amidotransferase subunit (EC 2.4.2.-)	2.096 up
Aaci_0928	RAAC01771	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	2.267 up
Aaci_0929	RAAC01772	imidazoleglycerol phosphate synthase, cyclase subunit	2.133 up
Aaci_0930	RAAC01773	phosphoribosyl-ATP diphosphatase	2.027 up
Aaci_0947	RAAC01790	Transcriptional regulators, LysR family	2.196 up
Aaci_0979	RAAC01823	Superoxide dismutase (EC 1.15.1.1)	2.597 up
Aaci_0988	RAAC01832	Cysteine desulfurase (EC 2.8.1.7) / Selenocysteine lyase (EC 4.4.1.16)	2.025 up
Aaci_0991	RAAC01835	Rieske (2Fe-2S) iron-sulphur domain protein	2.119 up
Aaci_1004	RAAC01848	Hypothetical protein	2.338 up
Aaci_1034	RAAC01876	cell cycle protein; Rod shape-determining protein rodA	3.450 up
Aaci_1035	RAAC01877	Aminotransferase	2.072 up
Aaci_1036	RAAC01878	hypothetical protein	2.041 up
Aaci_1038	RAAC01880	Hypothetical protein	3.689 up
Aaci_1049	RAAC01891	3D domain protein	2.721 up
Aaci_1054	RAAC01898	putative permease	2.099 up
Aaci_2341	RAAC01933	3H domain protein; Transcriptional repressor for NAD biosynthesis in gram-positives	2.216 up
Aaci_2378	RAAC01972	GTP-sensing transcriptional pleiotropic repressor CodY	2.131 up
Aaci_2381	RAAC01975	Multicopper oxidase type 3	3.563 up
Aaci_2382	RAAC01976	Hemerythrin HHE cation binding domain protein	2.209 up
Aaci_1858	RAAC02030	major facilitator superfamily MFS_1, drug resistance transporter	2.687 up
Aaci_1857	RAAC02031	Transcriptional regulator, GntR family	3.499 up
Aaci_1856	RAAC02032	Radical SAM superfamily protein	3.160 up
Aaci_1824	RAAC02069	cell shape determining protein, MreB/Mrl family	2.106 up
Aaci_1823	RAAC02070	Rod shape-determining protein mreC	2.164 up
Aaci_1822	RAAC02071	Rod shape-determining protein mreD	2.379 up
Aaci_0678	RAAC02209	short-chain dehydrogenase/reductase SDR	2.102 up
Aaci_0662	RAAC02228	cytochrome oxidase assembly	2.129 up
Aaci_0661	RAAC02229	Cytochrome c oxidase polypeptide IV (EC 1.9.3.1)	2.217 up
Aaci_0661	RAAC02230	Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	2.107 up
Aaci_0628	RAAC02261	hypothetical protein	2.808 up
Aaci_1165	RAAC02566	Prophage antirepressor protein	2.618 up
Aaci_1229	RAAC02627	Argininosuccinate lyase (EC 4.3.2.1)	2.621 up
Aaci_1230	RAAC02629	Aspartokinase (EC 2.7.2.4)	2.438 up
Aaci_1231	RAAC02630	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	2.179 up
Aaci_1232	RAAC02631	Phosphoserine aminotransferase (EC 2.6.1.52)	2.209 up

Aaci_1233	RAAC02632	Haloacid dehalogenase domain protein hydrolase	2.169 up
Aaci_1357	RAAC02824	Para-aminobenzoate synthetase component I (EC 2.6.1.85) / 4-amino-4-deoxychorismate lyase (EC 4.1.3.38)	2.119 up
Aaci_1419	RAAC02890	SSU ribosomal protein S2P	2.029 up
Aaci_1431	RAAC02901	Hypothetical cytosolic protein; protein of unknown function DUF150	2.369 up
Aaci_1432	RAAC02902	NusA antitermination factor	2.316 up
Aaci_1433	RAAC02903	protein of unknown function DUF448	2.253 up
Aaci_1439	RAAC02909	Riboflavin kinase (EC 2.7.1.26) / FMN adenylyltransferase (EC 2.7.7.2); riboflavin biosynthesis protein RibF	2.122 up
Aaci_1442	RAAC02912	Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8) / Polynucleotide adenylyltransferase (EC 2.7.7.19)	2.126 up
Aaci_1443	RAAC02913	Polysaccharide deacetylase (EC 3.5.1.-)	2.035 up
Aaci_1444	RAAC02914	Processing peptidase, M16 family	2.234 up
Aaci_1445	RAAC02915	Deoxyuridine 5"-triphosphate nucleotidohydrolase (EC 3.6.1.23)	2.023 up
Aaci_1487	RAAC02961	DNA mismatch repair protein MutL	2.366 up
Aaci_0343	RAAC03013	MMPL domain protein	2.752 up
Aaci_0344	RAAC03014	protein of unknown function DUF1453; Cytochrome c biosynthesis protein, CcdC	2.064 up
Aaci_0345	RAAC03015	peptidase S11 D-alanyl-D-alanine carboxypeptidase 1	2.252 up
Aaci_0346	RAAC03016	Peptidoglycan glycosyltransferase	2.150 up
Aaci_0393	RAAC03064	Adenylosuccinate lyase (EC 4.3.2.2)	3.522 up
Aaci_0394	RAAC03065	Phosphoribosylamidoimidazole-succinocarboxamide synthase (EC 6.3.2.6)	3.322 up
Aaci_0395	RAAC03066	Phosphoribosylformylglycinamidine synthase, purS component (EC 6.3.5.3)	3.136 up
Aaci_0396	RAAC03067	Phosphoribosylformylglycinamidine synthase I(EC 6.3.5.3)	2.978 up
Aaci_0397	RAAC03068	Phosphoribosylformylglycinamidine synthase II(EC 6.3.5.3)	3.330 up
Aaci_0398	RAAC03069	Amidophosphoribosyltransferase (EC 2.4.2.14)	2.906 up
Aaci_0399	RAAC03070	Phosphoribosylformylglycinamidine cyclo-ligase (EC 6.3.3.1)	3.738 up
Aaci_0400	RAAC03071	Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3) / IMP cyclohydrolase (EC 3.5.4.10)	3.336 up
Aaci_0401	RAAC03072	Phosphoribosylamine--glycine ligase (EC 6.3.4.13)	3.182 up
Aaci_0402	RAAC03073	protein of unknown function DUF6 transmembrane; Transporter, drug/metabolite exporter family	2.115 up
Aaci_0884	RAAC03688	hypothetical protein	5.399 up
Aaci_0879	RAAC03693	protein of unknown function DUF985	2.830 up

Aaci_0257	RAAC04051	major facilitator superfamily MFS_1	4.054 up
Aaci_0259	RAAC04053	D-xylose-binding protein	12.842 up
Aaci_0260	RAAC04054	D-xylose transport ATP-binding protein xylG	32.091 up
Aaci_0261	RAAC04055	Xylose transport system permease protein xylH	14.747 up
Aaci_2781	RAAC04115	LSU ribosomal protein L31P	2.143 up
Aaci_1691	RAAC04326	Nucleoside diphosphate kinase (EC 2.7.4.6)	2.250 up
Aaci_1690	RAAC04327	Patatin	2.250 up
Aaci_0213	RAAC04566	Major facilitator superfamily MFS_1;	2.054 up

APPENDIX V: WAX/Xylose Chemostat Experiments – Gene Regulation

	Downregulated Genes		
JGI Seq ID	**SEQ_ID**	**GENE_INFO**	**Fold change 2**
Aaci_2901	RAAC00001	Transcriptional regulator, GntR Family	3.468 down
Aaci_2907	RAAC00004	Hypothetical cytosolic protein	3.358 down
Aaci_0009	RAAC00060	Cytidine deaminase (EC 3.5.4.5)	2.094 down
Aaci_0010	RAAC00061	Hypothetical protein	2.042 down
Aaci_1240	RAAC00064	Hypothetical protein	3.831 down
Aaci_1241	RAAC00065	Hypothetical protein	3.131 down
Aaci_1242	RAAC00066	Hypothetical protein	2.721 down
Aaci_1249	RAAC00074	Hypothetical protein	3.383 down
Aaci_1250	RAAC00075	3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	2.179 down
Aaci_1253	RAAC00078	Transcriptional regulator, GPR1/FUN34/yaaH family protein	3.729 down
Aaci_1254	RAAC00079	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Acetate/CoA ligase	23.928 down
Aaci_0147	RAAC00189	acetyl-CoA acetyltransferase; 3-ketoacyl-CoA thiolase (EC 2.3.1.16)	2.761 down
Aaci_0146	RAAC00190	short-chain dehydrogenase/reductase SDR; 3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	2.629 down
Aaci_0145	RAAC00191	Enoyl-CoA hydratase/isomerase (EC 4.2.1.17)	2.571 down
Aaci_0144	RAAC00192	acyl-CoA dehydrogenase domain protein	2.435 down
Aaci_0143	RAAC00193	acyl-CoA dehydrogenase domain protein	2.283 down
Aaci_0140	RAAC00196	Malate dehydrogenase (oxaloacetate-decarboxylating)	11.580 down
Aaci_0139	RAAC00197	GAF modulated transcriptional regulator, LuxR family	29.557 down
Aaci_0138	RAAC00198	Hypothetical protein	6.531 down
Aaci_0137	RAAC00199	Hypothetical protein	10.198 down
Aaci_0136	RAAC00200	PE-PGRS family protein	10.368

			down
Aaci_0135	RAAC00201	Hypothetical protein	7.324 down
Aaci_0113	RAAC00228	binding-protein-dependent transport systems inner membrane component	2.116 down
Aaci_0295	RAAC00249	Hypothetical protein	3.141 down
Aaci_0286	RAAC00257	AMP-dependent synthetase and ligase; Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	2.959 down
Aaci_0280	RAAC00266	hypothetical protein	3.616 down
Aaci_2594	RAAC00269	Transcriptional regulator, LacI family	2.252 down
Aaci_2622	RAAC00299	major facilitator superfamily MFS_1	3.153 down
Aaci_2630	RAAC00307	glycoside hydrolase family 3 domain protein; Beta-glucosidase (EC 3.2.1.21) / Beta-xylosidase (EC 3.2.1.37)	4.151 down
Aaci_2636	RAAC00314	Hypothetical protein	3.093 down
Aaci_2637	RAAC00315	Hypothetical protein	4.792 down
	RAAC00328	unassigned	2.105 down
Aaci_2650	RAAC00329	CRISPR-associated protein Cas2	2.592 down
Aaci_2787	RAAC00481	Enoyl-CoA hydratase/isomerase; 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55)	2.406 down
Aaci_2795	RAAC00488	Acyl-CoA dehydrogenase domain protein (EC 1.3.99.-)	2.978 down
Aaci_2807	RAAC00501	hypothetical protein; Alpha/beta hydrolase	4.253 down
Aaci_2824	RAAC00517	Glycosyl transferase, family 2	3.224 down
Aaci_2825	RAAC00519	hypothetical protein	7.330 down
Aaci_2828	RAAC00523	hypothetical protein	8.916 down
Aaci_2829	RAAC00524	Aminoglycoside phosphotransferase (EC 2.7.1.-)	2.498 down
Aaci_2851	RAAC00549	Transcriptional regulator, MerR family	12.611 down
Aaci_2852	RAAC00550	Acyl-CoA dehydrogenase domain protein(EC 1.3.99.3)	2.930 down
Aaci_2853	RAAC00551	Hypothetical protein	3.178 down
Aaci_2871	RAAC00571	binding-protein-dependent transport systems inner membrane component	3.511 down
Aaci_2872	RAAC00572	binding-protein-dependent transport systems inner membrane component	7.228 down

Aaci_2873	RAAC00573	extracellular solute-binding protein family 1; Maltose/maltodextrin-binding protein	7.691 down
Aaci_2874	RAAC00574	alpha amylase catalytic region	2.426 down
Aaci_2881	RAAC00583	protein of unknown function DUF969	2.305 down
Aaci_2889	RAAC00596	extracellular solute-binding protein, Family 5	3.099 down
Aaci_2890	RAAC00598	Transcriptional regulator, AraC family	3.543 down
Aaci_2893	RAAC00601	Nitrogen regulatory protein P-II	3.319 down
Aaci_2894	RAAC00602	Alpha-L-arabinofuranosidase (EC 3.2.1.55)	3.435 down
Aaci_2896	RAAC00604	3-dehydroquinate synthase, Arabinose operon protein araM	2.460 down
Aaci_2897	RAAC00605	L-arabinose isomerase (EC 5.3.1.4)	2.298 down
Aaci_2898	RAAC00606	class II aldolase/adducin family protein; L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4)	2.156 down
Aaci_2900	RAAC00608	sugar transporter; Arabinose/Xylose/Galactose permease; proton symporter	3.185 down
Aaci_2238	RAAC00625	transcriptional regulator, LacI family; Catabolite control protein A	7.394 down
Aaci_2237	RAAC00626	extracellular solute-binding protein family 1; Alpha-glucoside-binding protein	6.659 down
Aaci_2236	RAAC00627	binding-protein-dependent transport systems inner membrane component	7.623 down
Aaci_2235	RAAC00628	binding-protein-dependent transport systems inner membrane component	8.469 down
Aaci_2234	RAAC00629	PfkB domain protein; Fructokinase (EC 2.7.1.4)	2.833 down
Aaci_2233	RAAC00630	Homoserine dehydrogenase (EC 1.1.1.3)	2.988 down
Aaci_2232	RAAC00632	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	6.483 down
Aaci_2230	RAAC00633	Dihydroxy-acid dehydratase (EC 4.2.1.9)	3.976 down
Aaci_2229	RAAC00634	Acetolactate synthase large subunit (EC 2.2.1.6)	3.668 down
Aaci_2228	RAAC00635	Acetolactate synthase small subunit (EC 2.2.1.6)	3.515 down
Aaci_2227	RAAC00636	Ketol-acid reductoisomerase (EC 1.1.1.86) / 2-dehydropantoate 2-reductase (EC 1.1.1.169)	3.286 down
Aaci_2226	RAAC00637	3-isopropylmalate dehydrogenase (EC 1.1.1.85)	3.100 down
Aaci_2225	RAAC00638	Isopropylmalate/citramalate isomerase large subunit (EC 4.2.1.33) / Isopropylmalate/citramalate isomerase large subunit (EC 4.2.1.35)	2.460 down
Aaci_2224	RAAC00639	3-isopropylmalate dehydratase small subunit (EC 4.2.1.33)	2.058 down

Aaci_2209	RAAC00655	NUDIX hydrolase	2.081 down
Aaci_2208	RAAC00656	transcriptional coactivator/pterin dehydratase	2.781 down
Aaci_2207	RAAC00657	Hypothetical protein	2.905 down
Aaci_2206	RAAC00659	Xylose isomerase domain protein TIM barrel	5.033 down
Aaci_2205	RAAC00660	aldo/keto reductase	4.327 down
Aaci_2180	RAAC00689	Xylose isomerase (EC 5.3.1.5)	2.206 down
Aaci_2179	RAAC00690	Xylulose kinase (EC 2.7.1.17)	2.161 down
Aaci_2157	RAAC00713	Xylose isomerase domain protein TIM barrel	3.601 down
Aaci_2156	RAAC00714	Xylose isomerase domain protein TIM barrel	3.763 down
Aaci_2155	RAAC00715	Xylose isomerase domain protein TIM barrel	3.534 down
Aaci_2154	RAAC00716	oxidoreductase domain protein	3.005 down
Aaci_2153	RAAC00717	oxidoreductase domain protein	3.183 down
Aaci_2139	RAAC00732	ABC transporter related	4.488 down
Aaci_2138	RAAC00733	Phosphotransferase system, phosphocarrier protein HPr; Catabolite repression protein crh	2.243 down
Aaci_2137	RAAC00734	methyl-accepting chemotaxis sensory transducer	2.519 down
Aaci_2079	RAAC00792	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	2.146 down
Aaci_2070	RAAC00803	LmbE family protein	8.321 down
Aaci_2069	RAAC00804	major facilitator superfamily MFS_1; SUGAR/SODIUM SYMPORTER	6.976 down
Aaci_2067	RAAC00806	short-chain dehydrogenase/reductase SDR; 3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	2.486 down
Aaci_2060	RAAC00812	3-oxoacid CoA-transferase subunit A (2.8.3.5)	18.950 down
Aaci_2059	RAAC00813	3-oxoacid CoA-transferase subunit B (2.8.3.5)	21.919 down
Aaci_2058	RAAC00814	Acetyl-CoA acetyltransferase (EC 2.3.1.9)	11.723 down
Aaci_2057	RAAC00815	Acetyl-coenzyme A synthetase (EC 6.2.1.1), Psuedogene?	13.153 down
Aaci_2056	RAAC00816	Acyl-CoA dehydrogenase domain protein	2.605 down
Aaci_2055	RAAC00817	Propionyl-CoA carboxylase biotin-containing subunit (EC 6.4.1.3)	3.848 down

Aaci_2054	RAAC00818	biotin/lipoyl attachment domain-containing protein	7.157 down
Aaci_2054	RAAC00819	Biotin carboxyl carrier protein	4.808 down
Aaci_2053	RAAC00820	pyruvate carboxyltransferase; Hydroxymethylglutaryl-CoA lyase (EC 4.1.3.4)	3.187 down
Aaci_2052	RAAC00821	Propionyl-CoA carboxylase beta chain (EC 6.4.1.3)	2.807 down
Aaci_2035	RAAC00839	AMP-(fatty)acid ligases	19.420 down
Aaci_0537	RAAC00840	arsenate reductase (thioredoxin)	4.961 down
Aaci_0536	RAAC00841	Arsenical pump membrane protein	3.878 down
Aaci_0527	RAAC00985	methyl-accepting chemotaxis sensory transducer	2.177 down
Aaci_0526	RAAC00986	diguanylate cyclase and metal dependent phosphohydrolase	2.145 down
Aaci_1625	RAAC01024	4-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2)	5.149 down
Aaci_1623	RAAC01026	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	5.820 down
Aaci_1622	RAAC01027	Betaine-aldehyde dehydrogenase; 2-aminomuconate 6-semialdehyde dehydrogenase (EC 1.2.1.32)	5.292 down
Aaci_1621	RAAC01028	2-oxopent-4-enoate hydratase (EC 4.2.1.80)	4.537 down
Aaci_1620	RAAC01029	Acetaldehyde dehydrogenase (EC 1.2.1.10)	4.674 down
Aaci_1619	RAAC01030	4-hydroxy-2-oxovalerate aldolase (EC 4.1.3.39)	3.448 down
Aaci_1618	RAAC01031	4-oxalocrotonate decarboxylase (EC 4.1.1.77)	3.134 down
Aaci_1617	RAAC01032	4-oxalocrotonate tautomerase (EC 5.3.2.-)	2.403 down
Aaci_1608	RAAC01041	Pyruvate,phosphate dikinase (EC 2.7.9.1)	3.511 down
Aaci_1600	RAAC01050	Ribonucleoside-diphosphate reductase beta chain (EC 1.17.4.1)	2.963 down
Aaci_1599	RAAC01051	Ribonucleoside-diphosphate reductase alpha chain (EC 1.17.4.1)	3.352 down
Aaci_1596	RAAC01054	acyl-CoA dehydrogenase domain protein	3.358 down
Aaci_1583	RAAC01068	5-methyltetrahydrofolate--homocysteine methyltransferase (EC 2.1.1.13) homocysteine-binding subunit / Methylenetetrahydrofolate reductase (EC 1.5.1.20)	2.157 down
Aaci_1578	RAAC01073	sugar transporter; Arabinose/Xylose/Galactose permease	2.325 down
Aaci_1576	RAAC01075	Myo-inositol catabolism IolB domain protein	2.129 down

Aaci_1573	RAAC01079	Glutamine synthetase (EC 6.3.1.2)	2.043 down
Aaci_0062	RAAC01107	Na+/solute symporter	5.315 down
Aaci_0063	RAAC01108	Hypothetical protein	6.900 down
Aaci_0104	RAAC01151	signal transduction histidine kinase, nitrogen specific, NtrB	3.743 down
Aaci_0110	RAAC01158	Transcriptional regulator, GntR family	3.180 down
Aaci_0111	RAAC01159	extracellular solute-binding protein family 5	3.315 down
	RAAC01173	DNA primase	2.598 down
Aaci_2450	RAAC01268	Peptidase (EC 3.4.-.-) / Cell surface sugar-binding protein	6.474 down
Aaci_2451	RAAC01269	Acyltransferase family protein	4.827 down
Aaci_2452	RAAC01270	AMP-dependent synthetase and ligase; Acyl-acyl carrier protein synthetase (EC 6.2.1.20)	9.950 down
Aaci_2453	RAAC01271	Oligoendopeptidase F (EC 3.4.24.-)	2.309 down
Aaci_2458	RAAC01276	binding-protein-dependent transport systems inner membrane component	4.277 down
Aaci_2459	RAAC01277	binding-protein-dependent transport systems inner membrane component	4.343 down
Aaci_2460	RAAC01278	extracellular solute-binding protein family 1	7.605 down
Aaci_2461	RAAC01279	hypothetical protein	4.820 down
Aaci_2462	RAAC01280	oxidoreductase domain protein	3.723 down
Aaci_2469	RAAC01288	Phosphatase/phosphohexomutase family protein	2.418 down
Aaci_2470	RAAC01289	Oligopeptide transport ATP-binding protein oppF	9.160 down
Aaci_2471	RAAC01290	Oligopeptide transport ATP-binding protein oppD	12.826 down
Aaci_2472	RAAC01291	Oligopeptide transport system permease protein oppC	5.020 down
Aaci_2473	RAAC01292	Oligopeptide transport system permease protein oppB	19.872 down
Aaci_2474	RAAC01293	extracellular solute-binding protein family 5; Oligopeptide-binding protein oppA	3.085 down
Aaci_2475	RAAC01294	glycoside hydrolase family 9; Endoglucanase C (EC 3.2.1.4)	2.845 down
Aaci_2487	RAAC01306	drug resistance transporter, EmrB/QacA subfamily	2.064 down
	RAAC01308	Hypothetical cytosolic protein	2.121 down

Aaci_2495	RAAC01316	Glucarate/galactarate transporter	4.048 down
Aaci_2504	RAAC01327	Aldehyde dehydrogenase (EC 1.2.1.3)	2.044 down
Aaci_2534	RAAC01350	FAD linked oxidase domain protein; (S)-2-hydroxy-acid oxidase subunit GlcE (EC 1.1.3.15)	3.006 down
Aaci_2533	RAAC01351	protein of unknown function DUF224 cysteine-rich region domain protein; (S)-2-hydroxy-acid oxidase subunit GlcF (EC 1.1.3.15)	4.347 down
Aaci_2532	RAAC01352	FAD linked oxidase domain protein; (S)-2-hydroxy-acid oxidase chain D (EC 1.1.3.15)	2.988 down
Aaci_2530	RAAC01354	Malate synthase (EC 2.3.3.9)	2.275 down
Aaci_2529	RAAC01355	L-lactate permease	14.133 down
	RAAC01356	unassigned	7.360 down
Aaci_2528	RAAC01357	alpha/beta hydrolase fold protein; Lysophospholipase L2 (EC 3.1.1.5)	2.130 down
Aaci_2527	RAAC01358	major facilitator superfamily MFS_1, sugar transport related (SEED)	3.038 down
Aaci_2527	RAAC01359	major facilitator superfamily MFS_1	5.684 down
Aaci_2526	RAAC01360	(S)-2-hydroxy-acid oxidase chain D (EC 1.1.3.15)	5.692 down
Aaci_2525	RAAC01361	unassigned	6.031 down
Aaci_2524	RAAC01362	Proline dehydrogenase (EC 1.5.99.8)	8.814 down
Aaci_2523	RAAC01363	GABA-specific permease	5.623 down
Aaci_2522	RAAC01364	Amino acid permease	7.230 down
Aaci_2521	RAAC01365	Proline iminopeptidase (EC 3.4.11.5)	2.809 down
Aaci_2516	RAAC01370	Xylose isomerase domain protein TIM barrel	2.045 down
Aaci_2515	RAAC01371	Hypothetical protein; Major facilitator superfamily MFS_1	3.804 down
Aaci_2510	RAAC01376	extracellular solute-binding protein family 5	3.260 down
Aaci_0166	RAAC01413	Serine/threonine protein phosphatase (EC 3.1.3.16)	2.141 down
Aaci_0164	RAAC01415	aminoglycoside phosphotransferase	3.293 down
Aaci_0162	RAAC01417	Transcriptional regulator, IclR family	2.761 down
Aaci_0161	RAAC01418	MaoC domain-containing protein dehydratase	7.439 down
Aaci_0160	RAAC01419	MaoC domain-containing protein dehydratase	13.142 down

Aaci_0159	RAAC01420	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	19.684 down
Aaci_0158	RAAC01421	Acyl-CoA dehydrogenase (EC 1.3.99.-)	9.264 down
Aaci_0157	RAAC01422	short-chain dehydrogenase/reductase SDR	10.612 down
Aaci_0156	RAAC01423	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	8.343 down
Aaci_0155	RAAC01425	Acyl-CoA dehydrogenase domain protein; Acyl-CoA dehydrogenase, short-chain specific (EC 1.3.99.2)	11.295 down
Aaci_2241	RAAC01465	Transcription elongation factor greA	2.361 down
Aaci_2258	RAAC01482	Oligopeptide-binding protein oppA	4.346 down
Aaci_2259	RAAC01483	Oligopeptide transport system permease protein appB	2.576 down
Aaci_2260	RAAC01484	Oligopeptide transport system permease protein oppC	2.004 down
Aaci_2263	RAAC01487	Oligopeptide transport ATP-binding protein oppD	2.123 down
Aaci_2275	RAAC01499	Pantothenate kinase (EC 2.7.1.33)	2.186 down
Aaci_2276	RAAC01500	PfkB domain protein; Ribokinase (EC 2.7.1.15)	2.190 down
Aaci_2277	RAAC01501	Periplasmic binding protein/LacI transcriptional regulator; ATP-dependent ribose uptake	2.595 down
Aaci_2278	RAAC01502	inner-membrane translocator; Ribose transport system permease protein rbsC	7.407 down
Aaci_2279	RAAC01503	ABC transporter related; Ribose transport ATP-binding protein rbsA	6.843 down
Aaci_2280	RAAC01504	RbsD or FucU transport; ABC-type ribose transport system, auxiliary component; D-ribose mutarotase (EC 5.1.3.-)	2.896 down
Aaci_2281	RAAC01505	transcriptional regulator, LacI family	2.625 down
Aaci_2305	RAAC01531	putative methyl-accepting chemotaxis sensory transducer	3.835 down
Aaci_2306	RAAC01532	Extracellular solute-binding protein family 1; Predicted beta-glucoside-regulated ABC transport system	5.336 down
Aaci_2307	RAAC01533	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	3.405 down
	RAAC01575	Aldehyde dehydrogenase (EC 1.2.1.3)	2.798 down
Aaci_0785	RAAC01614	NAD-dependent epimerase/dehydratase; dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)	3.180 down
Aaci_0786	RAAC01615	Glycoside hydrolase clan GH-D; Alpha-galactosidase (EC 3.2.1.22)	2.757 down
Aaci_0787	RAAC01616	Oxidoreductase (EC 1.1.1.-)	2.174 down

Aaci_0788	RAAC01617	Carbohydrate kinase, FGGY-like protein	2.018 down
Aaci_0789	RAAC01618	Hypothetical protein; Glycoside hydrolase, catalytic domain	2.924 down
Aaci_0790	RAAC01619	Methylmalonyl CoA epimerase (EC 5.1.99.1)	3.424 down
Aaci_0792	RAAC01622	major facilitator superfamily MFS_1; Sugar transporter	2.479 down
Aaci_0794	RAAC01625	binding-protein-dependent transport systems inner membrane component	1.705 down
Aaci_0795	RAAC01626	extracellular solute-binding protein family 1	3.923 down
Aaci_0796	RAAC01627	binding-protein-dependent transport systems inner membrane component	4.177 down
Aaci_0797	RAAC01628	Glycoside hydrolase family 4; Alpha-galactosidase (EC 3.2.1.22)	2.946 down
Aaci_0798	RAAC01629	2-methylcitrate synthase (EC 2.3.3.5)	6.403 down
Aaci_0799	RAAC01630	2-methylcitrate dehydratase (EC 4.2.1.79) / 2-methylisocitrate dehydratase (EC 4.2.1.99)	4.631 down
Aaci_0800	RAAC01631	Methylisocitrate lyase (EC 4.1.3.30)	3.333 down
Aaci_0806	RAAC01638	Transcriptional regulator, TetR family	2.501 down
Aaci_0807	RAAC01639	Phenylacetic acid degradation protein paaA	3.470 down
Aaci_0808	RAAC01640	Phenylacetic acid degradation protein paaB	5.179 down
Aaci_0809	RAAC01641	Phenylacetic acid degradation protein paaC	3.930 down
Aaci_0810	RAAC01642	phenylacetate-CoA oxygenase subunit PaaJ	6.838 down
Aaci_0811	RAAC01643	Phenylacetate-coenzyme A ligase (EC 6.2.1.30)	6.950 down
Aaci_0812	RAAC01644	Hypothetical cytosolic protein; Ethyl tert-butyl ether degradation EthD	3.075 down
Aaci_0813	RAAC01645	Succinate-semialdehyde dehydrogenase [NADP+] (EC 1.2.1.16)	4.609 down
Aaci_0814	RAAC01646	Enoyl-CoA hydratase (EC 4.2.1.17)	4.484 down
Aaci_0822	RAAC01654	Hypothetical protein	2.789 down
Aaci_0823	RAAC01655	Transcriptional regulator	2.122 down
Aaci_0824	RAAC01656	Histidinol-phosphate aminotransferase (EC 2.6.1.9)	2.232 down
Aaci_0825	RAAC01657	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	2.858 down
Aaci_0826	RAAC01658	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	3.642 down

Aaci_0827	RAAC01659	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	2.725 down
Aaci_0838	RAAC01672	Multidrug/protein/lipid ABC transporter family, ATP-binding and permease protein	2.177 down
Aaci_0840	RAAC01674	Hypothetical protein	4.156 down
Aaci_0860	RAAC01698	ABC transporter ATP-binding protein	2.273 down
Aaci_0876	RAAC01715	Helix-turn-helix, AraC family	18.569 down
Aaci_0877	RAAC01716	Multidrug resistance protein B	2.209 down
Aaci_0891	RAAC01732	1-phosphofructokinase (EC 2.7.1.56)	2.024 down
Aaci_0892	RAAC01733	PTS system, fructose-specific IIBC component (EC 2.7.1.69)	4.390 down
Aaci_0901	RAAC01744	Hypothetical protein	5.100 down
Aaci_0902	RAAC01745	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	7.739 down
Aaci_0903	RAAC01746	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	7.366 down
Aaci_0904	RAAC01747	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	9.487 down
Aaci_0905	RAAC01748	Lactate 2-monooxygenase (EC 1.13.12.4)	6.545 down
Aaci_0906	RAAC01749	Septum formation protein Maf	2.195 down
Aaci_0943	RAAC01786	Hypothetical protein	2.551 down
Aaci_0998	RAAC01842	Hypothetical protein	2.276 down
	RAAC01843	unassigned	6.022 down
Aaci_1045	RAAC01887	Glutamyl aminopeptidase	2.364 down
Aaci_1055	RAAC01897	Alpha/beta hydrolase fold-3 domain protein; Esterase (EC 3.1.1.-)	6.051 down
Aaci_1899	RAAC01900	Aldehyde dehydrogenase (EC 1.2.1.3)	4.463 down
Aaci_2339	RAAC01930	Hypothetical protein	3.500 down
Aaci_2340	RAAC01931	Hypothetical protein	3.219 down
Aaci_1900	RAAC01989	Oligosaccharide-binding protein	2.714 down
Aaci_1899	RAAC01990	binding-protein-dependent transport systems inner membrane component	14.224 down
Aaci_1898	RAAC01991	binding-protein-dependent transport systems inner membrane component	13.344 down

Aaci_1897	RAAC01992	oligopeptide/dipeptide ABC transporter, ATPase subunit	11.370 down
Aaci_1896	RAAC01993	oligopeptide/dipeptide ABC transporter, ATPase subunit	11.993 down
Aaci_1895	RAAC01994	beta-galactosidase/Beta-glucosidase (EC 3.2.1.21)	7.880 down
Aaci_1894	RAAC01995	Hypothetical protein	4.814 down
Aaci_1893	RAAC01996	Diaminopimelate epimerase (EC 5.1.1.7)	3.398 down
	RAAC02036	unassigned	7.936 down
Aaci_1853	RAAC02037	unassigned membrane protein, GPR1/FUN34/yaaH family	7.257 down
Aaci_0711	RAAC02175	ABC transporter, solute-binding, sugar transport	2.425 down
Aaci_0710	RAAC02176	Inner-membrane translocator; Ribose/xylose/arabinose/galactoside ABC-type transport systems	9.583 down
Aaci_0709	RAAC02177	Inner-membrane translocator; Ribose/xylose/arabinose/galactoside ABC-type transport systems	9.378 down
Aaci_0708	RAAC02178	ABC transporter related; Ribose transport ATP-binding protein rbsA	10.528 down
Aaci_0707	RAAC02179	Hypothetical protein	7.268 down
Aaci_0706	RAAC02180	L-rhamnose isomerase (EC 5.3.1.14)	2.528 down
Aaci_0692	RAAC02194	Hypothetical protein	2.264 down
Aaci_0691	RAAC02195	Hypothetical protien	2.927 down
Aaci_0690	RAAC02196	TrmA family RNA methyltransferase	2.889 down
	RAAC02200	unassigned	2.962 down
Aaci_0685	RAAC02201	Hypothetical protein	7.704 down
	RAAC02204	unassigned	2.208 down
Aaci_0655	RAAC02235	LuxR family two component transcriptional regulator	2.016 down
Aaci_0653	RAAC02237	ABC transporter permease protein	2.015 down
Aaci_0652	RAAC02238	ABC transporter related protein; ABC transporter ATP-binding protein	3.007 down
	RAAC02273	unassigned	3.206 down
Aaci_0153	RAAC02274	iron-containing alcohol dehydrogenase	2.147 down
Aaci_0152	RAAC02275	Aldehyde dehydrogenase (EC 1.2.1.3)	2.394

			down
Aaci_0151	RAAC02276	hypothetical protein	2.743 down
Aaci_0150	RAAC02277	ABC-1 domain protein; Ubiquinone biosynthesis monooxygenase UbiB	2.615 down
Aaci_0148	RAAC02279	Hypothetical protein	3.081 down
Aaci_2981	RAAC02317	Hypothetical protein	2.307 down
Aaci_2982	RAAC02318	Transposase IS66	2.125 down
Aaci_2983	RAAC02319	Transposase	2.020 down
Aaci_2984	RAAC02320	Transposase	2.467 down
Aaci_2987	RAAC02321	phosphoribosylglycinamide synthetase	2.185 down
Aaci_2988	RAAC02322	major facilitator superfamily protein	4.454 down
Aaci_2989	RAAC02323	Methyltransferase (EC 2.1.1.-)	2.809 down
Aaci_2990	RAAC02324	Radical SAM domain protein; Fe-S OXIDOREDUCTASE (1.8.-.-)	3.138 down
	RAAC02325	Pollen-specific membrane integral protein	2.629 down
	RAAC02326	unassigned	2.963 down
Aaci_2991	RAAC02327	cobalamin synthesis protein P47K	3.319 down
Aaci_2993	RAAC02328	Hypothetical protein	2.924 down
	RAAC02329	unassigned	3.859 down
Aaci_2994	RAAC02332	N-acetyltransferase GCN5	2.843 down
Aaci_3039	RAAC02376	Hypothetical protein	2.399 down
	RAAC02378	unassigned	2.174 down
Aaci_2557	RAAC02379	family 2 glycosyl transferase	2.123 down
Aaci_0446	RAAC02437	Ribonuclease HI (EC 3.1.26.4)	3.518 down
Aaci_0445	RAAC02438	AMP-dependent synthetase and ligase; Acetyl-coenzyme A synthetase (EC 6.2.1.1)	13.334 down
Aaci_0444	RAAC02439	PAS Modulated Fis Family Sigma-54-dependent transcriptional activator	19.784 down
Aaci_0443	RAAC02440	Major facilitator superfamily MFS-1; General substrate (sugar) transporter	18.074 down
Aaci_0442	RAAC02441	Methylmalonate-semialdehyde dehydrogenase	18.717

		(acylating) (EC 1.2.1.27)	down
Aaci_0441	RAAC02442	Iron-containing alcohol dehydrogenase	18.526 down
Aaci_0427	RAAC02457	Hypothetical protein	11.366 down
Aaci_0419	RAAC02466	phosphotransferase system, phosphocarrier protein HPr	2.484 down
Aaci_0403	RAAC02483	Extracellular solute-binding protein family 5	3.146 down
	RAAC02484	unassigned	2.126 down
Aaci_1081	RAAC02486	FliA/WhiG subfamily RNA polymerase sigma-28 subunit	2.449 down
Aaci_0482	RAAC02490	Hypothetical protein	2.130 down
Aaci_1134	RAAC02542	Hypothetical protein	2.321 down
Aaci_1136	RAAC02543	DNA adenine methylase (EC 2.1.1.72)	2.163 down
Aaci_1209	RAAC02607	acyl-CoA dehydrogenase domain protein; Cyclohexanecarboxyl-CoA dehydrogenase (EC 1.3.99.-)	2.003 down
Aaci_1210	RAAC02608	Naphthoate synthase (EC 4.1.3.36)	5.469 down
Aaci_1211	RAAC02609	2-HYDROXYCYCLOHEXANE-1-CARBOXYL-COA DEHYDROGENASE (EC 1.3.-.-), short-chain dehydrogenase/reductase SDR	6.124 down
Aaci_1212	RAAC02610	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	4.056 down
Aaci_1214	RAAC02612	transcriptional regulator, LacI family	12.536 down
Aaci_1215	RAAC02613	extracellular solute-binding protein family 1	9.510 down
Aaci_1216	RAAC02614	binding-protein-dependent transport systems inner membrane component	18.792 down
Aaci_1217	RAAC02615	binding-protein-dependent transport systems inner membrane component	12.395 down
Aaci_1218	RAAC02616	Glycoside hydrolase family 2 TIM barrel; Beta-galactosidase (EC 3.2.1.23)	13.832 down
Aaci_0058	RAAC02663	Transcriptional regulator, GntR family	2.022 down
Aaci_0055	RAAC02666	Glycerol kinase (EC 2.7.1.30)	8.001 down
	RAAC02667	Putative pit accessory protein	2.956 down
	RAAC02668	Low-affinity inorganic phosphate transporter	2.261 down
Aaci_0048	RAAC02676	Alpha-L-arabinofuranosidase-like protein; XX	5.593 down
	RAAC02692	Nucleoside transport system permease protein	2.386

			down
	RAAC02702	Isocitrate lyase (EC 4.1.3.1)	4.652 down
Aaci_2902	RAAC02733	periplasmic binding protein/LacI transcriptional regulator	5.720 down
Aaci_2903	RAAC02734	inner-membrane translocator	6.553 down
Aaci_2904	RAAC02735	ABC transporter related, ATP-binding protein	12.566 down
	RAAC02737	6-phosphogluconate dehydrogenase (EC 1.1.1.44)	2.749 down
	RAAC02842	unassigned	3.482 down
Aaci_1452	RAAC02922	Acyl-CoA dehydrogenase (EC 1.3.99.-)	22.063 down
Aaci_1470	RAAC02923	3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	12.868 down
Aaci_1454	RAAC02924	Malonate-semialdehyde dehydrogenase (acetylating) (EC 1.2.1.18) / Methylmalonate-semialdehyde dehydrogenase (acylating) (EC 1.2.1.27)	10.915 down
Aaci_1455	RAAC02925	3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4), Enoyl-CoA hydratase/isomerase	8.483 down
Aaci_1456	RAAC02926	Acetyl-coenzyme A synthetase (EC 6.2.1.1)/AMP-(fatty) acid ligase	7.179 down
Aaci_1457	RAAC02927	3-ketoacyl-CoA thiolase (EC 2.3.1.16), acetyl-CoA acetyltransferase	6.714 down
Aaci_1458	RAAC02928	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	4.168 down
	RAAC02975	unassigned	2.065 down
	RAAC02976	unassigned	2.129 down
Aaci_0310	RAAC02980	twitching motility protein	2.638 down
Aaci_0311	RAAC02981	Peptidase family A24	2.905 down
Aaci_0318	RAAC02988	transglutaminase domain protein	2.410 down
Aaci_0319	RAAC02989	glycosidase PH1107-related	2.006 down
	RAAC02995	Arginine permease	2.962 down
Aaci_0334	RAAC03003	Flavin reductase domain-containing FMN-binding protein	7.843 down
Aaci_0335	RAAC03004	Major Facilitator Superfamily MFS-1; General substrate (sugar) transporter	7.692 down
	RAAC03005	Transcriptional regulator, GntR family	3.773 down
	RAAC03006	5-oxopent-3-ene-1,2,5-tricarboxylate decarboxylase	3.523

		(EC 4.1.1.68)	down
	RAAC03007	5-carboxymethyl-2-hydroxymuconic-semialdehyde dehydrogenase (EC 1.2.1.60)	4.388 down
Aaci_0339	RAAC03008	4-hydroxyphenylacetate 3-monooxygenase oxygenase subunit (5.3.3.3)	8.404 down
Aaci_0340	RAAC03009	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	8.329 down
Aaci_0341	RAAC03010	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase/ Dihydrodipicolinate synthase (EC 4.2.1.52)	7.667 down
Aaci_0381	RAAC03049	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	3.801 down
	RAAC03050	unassigned	2.760 down
	RAAC03104	unassigned	2.325 down
	RAAC03105	unassigned	3.408 down
	RAAC03106	unassigned	2.325 down
	RAAC03152	unassigned	2.278 down
	RAAC03157	Lactoylglutathione lyase (EC 4.4.1.5)	2.402 down
	RAAC03158	Arsenical pump membrane protein	2.543 down
Aaci_0537	RAAC03159	Arsenate reductase (EC 1.20.4.1)	3.139 down
	RAAC03160	Phosphinothricin N-acetyltransferase (EC 2.3.1.183)	2.336 down
	RAAC03184	Transcriptional regulator	2.647 down
	RAAC03185	unassigned	2.556 down
	RAAC03186	Branched-chain amino acid transport protein azlC	2.531 down
Aaci_3078	RAAC03200	response regulator receiver protein	2.329 down
	RAAC03256	unassigned	2.570 down
	RAAC03265	unassigned	2.278 down
Aaci_1714	RAAC03277	Hypothetical protein	7.068 down
	RAAC03325	Transposase	2.276 down
	RAAC03386	unassigned	2.265 down
	RAAC03442	unassigned	4.675 down
	RAAC03543	unassigned	2.122

			down
	RAAC03583	ADP-ribosylation/Crystallin J1	2.879 down
	RAAC03660	unassigned	4.328 down
	RAAC03693	unassigned	3.067 down
	RAAC03694	Protoporphyrinogen oxidase (EC 1.3.3.4)	2.178 down
	RAAC03776	unassigned	2.071 down
	RAAC03777	unassigned	2.398 down
	RAAC03783	unassigned	2.414 down
	RAAC03832	Transposase	2.632 down
	RAAC03988	unassigned	2.375 down
	RAAC04008	unassigned	28.673 down
	RAAC04160	unassigned	2.369 down
	RAAC04225	unassigned	2.180 down
	RAAC04393	unassigned	2.406 down
	RAAC04430	Transposase	2.289 down
	RAAC04532	unassigned	7.512 down
Aaci_0241	RAAC04536	peptidase S15; X-Pro dipeptidyl-peptidase (S15) family protein	3.572 down
	RAAC04537	D-beta-hydroxybutyrate dehydrogenase (EC 1.1.1.30)	2.049 down
	RAAC04548	unassigned	2.117 down
	RAAC04592	unassigned	2.035 down
	RAAC04599	unassigned	6.655 down
	RAAC04600	unassigned	3.344 down
	RAAC04617	unassigned	4.062 down
	RAAC04618	unassigned	6.336 down
	RAAC04658	unassigned	2.005 down
Aaci_1213	RAAC04671	Protein of unknown function DUF2029	3.258

			down
	RAAC04696	unassigned	2.332 down
	RAAC04760	unassigned	3.302 down
	RAAC04770	unassigned	4.717 down
	RAAC04774	unassigned	2.039 down
	RAAC04807	unassigned	2.474 down
	RAAC04850	unassigned	2.121 down
	RAAC04862	Transposase	2.009 down
	RAAC04875	unassigned	2.713 down
	RAAC04887	unassigned	2.039 down
	RAAC04888	unassigned	4.976 down
	RAAC04918	unassigned	2.357 down
	RAAC04921	unassigned	3.323 down
	RAAC04944	unassigned	5.134 down
	RAAC04948	unassigned	2.856 down
	RAAC04962	unassigned	2.208 down
	RAAC04964	unassigned	2.256 down
	Upregulated Genes		
	SEQ_ID	**GENE_INFO**	**Fold change 2**
Aaci_2908	RAAC00005	Nitroreductase family protein	2.183 up
Aaci_2909	RAAC00006	Multidrug resistance protein B	2.043 up
Aaci_2915	RAAC00013	Transcriptional regulators, LysR family	2.383 up
Aaci_1267	RAAC00095	Hypothetical Membrane Spanning Protein	2.106 up

Aaci_1280	RAAC00109	Thioredoxin reductase (EC 1.8.1.9)	2.287 up
Aaci_1281	RAAC00110	Hypothetical protein	3.130 up
Aaci_1282	RAAC00111	Site-specific recombinase	2.617 up
Aaci_1298	RAAC00128	Hypothetical protein	2.401 up
Aaci_1304	RAAC00134	Uracil phosphoribosyltransferase (EC 2.4.2.9) / Pyrimidine operon regulatory protein pyrR	14.835 up
Aaci_1305	RAAC00135	Aspartate carbamoyltransferase (EC 2.1.3.2)	18.158 up
Aaci_1306	RAAC00136	Dihydroorotase (EC 3.5.2.3)	12.349 up
Aaci_1307	RAAC00137	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	14.486 up
Aaci_1308	RAAC00138	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	7.621 up
Aaci_1309	RAAC00139	Orotidine 5"-phosphate decarboxylase (EC 4.1.1.23)	6.902 up
Aaci_1310	RAAC00140	Orotate phosphoribosyltransferase (EC 2.4.2.10)	9.235 up
Aaci_1311	RAAC00141	FAD-dependent oxidoreductase (EC 1.-.-.-)	5.115 up
Aaci_1312	RAAC00142	Major facilitator superfamily MFS_1	2.145 up
Aaci_0299	RAAC00244	Homoserine dehydrogenase (EC 1.1.1.3)	4.633 up
Aaci_2596	RAAC00273	cytochrome c biogenesis protein transmembrane region	2.248 up
	RAAC00274	Thiol:disulfide interchange protein tlpA	2.228 up
	RAAC00275	unassigned	2.291 up
	RAAC00277	unassigned	2.978 up
	RAAC00278	Iron-sulfur cluster assembly/repair protein ApbE	2.179 up
	RAAC00285	unassigned	2.198 up
	RAAC00286	Tetracycline resistance protein	2.221 up
	RAAC00287	Transporter	3.799 up
	RAAC00288	Zinc-specific metalloregulatory protein	2.210 up
Aaci_2617	RAAC00293	thioesterase superfamily protein; Acyl-CoA hydrolase (EC 3.1.2.20)	29.148 up
	RAAC00294	NAD-dependent oxidoreductase	4.684 up

	RAAC00296	unassigned	5.654 up
	RAAC00300	unassigned	2.999 up
Aaci_2624	RAAC00301	Copper resistance protein, CopC	8.383 up
Aaci_2625	RAAC00302	Hypothetical membrane associated protein; nuclear export factor GLE1	5.977 up
	RAAC00303	Low-affinity inorganic phosphate transporter	5.658 up
	RAAC00304	Putative pit accessory protein	3.910 up
	RAAC00335	Purine-cytosine permease	2.025 up
	RAAC00336	Putative integral membrane protein	2.106 up
	RAAC00337	DNA adenine methylase (EC 2.1.1.72)	2.593 up
	RAAC00338	Copper chaperone copZ	2.519 up
	RAAC00339	Hypothetical cytosolic protein	2.386 up
	RAAC00366	unassigned	2.120 up
Aaci_2708	RAAC00397	LSU ribosomal protein L2P	2.106 up
Aaci_2712	RAAC00401	SSU ribosomal protein S10P	2.064 up
	RAAC00466	unassigned	2.089 up
Aaci_2814	RAAC00507	O-acetyl-L-homoserine sulfhydrolase (EC 2.5.1.49) / O-acetyl-L-serine sulfhydrolase (EC 2.5.1.47)	2.650 up
Aaci_2815	RAAC00508	Uncharacterized protein family UPF0324	7.577 up
	RAAC00509	Carotene isomerase/Phytoene desaturase homolog	2.813 up
	RAAC00510	GTP cyclohydrolase I (EC 3.5.4.16)	2.138 up
	RAAC00525	Kdg operon repressor	2.948 up
Aaci_2841	RAAC00537	Sulfate transport ATP-binding protein cysA	3.850 up
Aaci_2842	RAAC00538	Sulfate transport system permease protein cysW	5.053 up
Aaci_2843	RAAC00539	Sulfate transport system permease protein cysT	4.580 up
Aaci_2844	RAAC00540	Sulfate-binding protein	4.651 up
Aaci_2849	RAAC00547	FMN reductase (EC 1.5.1.29)	2.734 up

Aaci_2850	RAAC00548	Hypothetical protein; Protein of unknown function DUF2292	2.666 up
	RAAC00563	unassigned	2.747 up
	RAAC00577	Phosphohydrolase (MutT/nudix family protein)	5.434 up
	RAAC00578	unassigned	7.295 up
	RAAC00579	Transcriptional regulator, ArsR family	19.223 up
	RAAC00580	General substrate transporter	12.796 up
	RAAC00594	Alpha-xylosidase (EC 3.2.1.-)	3.817 up
	RAAC00618	unassigned	2.009 up
	RAAC00620	Peroxiredoxin (EC 1.11.1.15)	2.683 up
	RAAC00621	unassigned	2.760 up
Aaci_2204	RAAC00662	SSS sodium solute transporter superfamily	2.714 up
	RAAC00669	unassigned	2.607 up
	RAAC00679	Hypothetical membrane spanning protein	2.686 up
	RAAC00680	unassigned	2.758 up
	RAAC00683	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	3.112 up
	RAAC00695	Threonyl-tRNA synthetase (EC 6.1.1.3)	2.268 up
Aaci_2173	RAAC00697	protein of unknown function DUF395 YeeE/YedE	3.136 up
	RAAC00701	23S rRNA methyltransferase (EC 2.1.1.-)	2.924 up
Aaci_2144	RAAC00727	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	2.610 up
	RAAC00728	Dihydrolipoamide succinyltransferase component (E2) of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	2.221 up
	RAAC00777	unassigned	9.429 up
Aaci_2092	RAAC00778	Hypothetical protein	4.978 up
Aaci_2091	RAAC00779	Protoporphyrinogen oxidase (EC 1.3.3.4)	4.961 up
Aaci_2090	RAAC00780	Ferrochelatase (EC 4.99.1.1)	6.483 up
Aaci_2089	RAAC00781	Uroporphyrinogen decarboxylase (EC 4.1.1.37)	7.197 up

	RAAC00810	Queuosine biosynthesis protein QueF	2.165 up
	RAAC00811	Hypothetical protein	2.884 up
Aaci_2027	RAAC00847	protein of unknown function UPF0118; Hypothetical membrane spanning protein	4.711 up
	RAAC00900	Arginase family protein	2.274 up
	RAAC00901	unassigned	2.209 up
Aaci_1958	RAAC00916	Phosphate transporter	5.327 up
Aaci_1957	RAAC00917	Hypothetical protein	4.430 up
Aaci_1955	RAAC00920	Hypothetical protein	2.181 up
	RAAC00923	Small acid-soluble spore protein	2.696 up
	RAAC00924	2-haloalkanoic acid dehalogenase (EC 3.8.1.2)	2.653 up
	RAAC00925	Queuosine biosynthesis protein QueE	2.469 up
	RAAC00926	6-pyruvoyl tetrahydropterin synthase (EC 4.2.3.12)	2.409 up
Aaci_1938	RAAC00938	Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	69.773 up
Aaci_1937	RAAC00939	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	51.051 up
	RAAC00940	Transport ATP-binding protein cydD / Transport ATP-binding protein cydC	15.713 up
Aaci_1935	RAAC00941	ABC transporter, CydDC cysteine exporter (CydDC- E) family, permease/ATP-binding protein CydC	17.312 up
	RAAC00942	Hydroxyethylthiazole kinase (EC 2.7.1.50)	5.642 up
	RAAC00943	Phosphomethylpyrimidine kinase (EC 2.7.4.7) / Hydroxymethylpyrimidine kinase (EC 2.7.1.49)	2.507 up
	RAAC00944	Transcriptional activator tenA	2.711 up
	RAAC00945	unassigned	2.351 up
	RAAC00955	Isochorismate synthase (EC 5.4.4.2)	2.070 up
	RAAC00956	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase (EC 2.2.1.9) / 2-oxoglutarate decarboxylase (EC 4.1.1.71)	2.077 up
	RAAC00958	Phosphate-binding protein	14.022 up
	RAAC00959	Phosphate transport system permease protein pstC	15.696 up

	RAAC00960	Phosphate transport system permease protein pstA	9.060 up
Aaci_1906	RAAC00968	phosphate uptake regulator, PhoU	2.062 up
Aaci_1905	RAAC00969	phosphate ABC transporter, ATPase subunit; Phosphate transport ATP-binding protein pstB	3.609 up
Aaci_0515	RAAC00980	PAS/PAC sensor signal transduction histidine kinase	5.131 up
Aaci_0516	RAAC00981	two component transcriptional regulator, LuxR family	6.544 up
	RAAC01045	unassigned	2.027 up
	RAAC01046	Hypothetical exported protein	2.098 up
	RAAC01047	Integral membrane protein	2.919 up
	RAAC01088	Stage V sporulation protein B	2.911 up
	RAAC01089	UDP-glucose 4-epimerase (EC 5.1.3.2) / UDP-N-acetylglucosamine 4-epimerase (EC 5.1.3.7)	3.208 up
Aaci_1557	RAAC01095	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	7.206 up
Aaci_0072	RAAC01117	FAD dependent oxidoreductase	3.841 up
	RAAC01120	Glycerol-3-phosphate-binding protein	2.276 up
Aaci_2422	RAAC01238	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)	4.982 up
Aaci_2423	RAAC01239	Sulfite reductase [NADPH] hemoprotein beta-component (EC 1.8.1.2)	6.333 up
Aaci_2424	RAAC01240	adenylylsulfate reductase, thioredoxin dependent; Phosphoadenosine phosphosulfate reductase (EC 1.8.4.8)	9.062 up
	RAAC01295	Quinolinate synthase (EC 2.5.1.72)	2.105 up
	RAAC01296	L-aspartate oxidase (EC 1.4.3.16)	2.455 up
	RAAC01297	Nicotinate-nucleotide pyrophosphorylase [carboxylating] (EC 2.4.2.19)	2.743 up
	RAAC01299	Molybdopterin biosynthesis MoeB protein	2.548 up
	RAAC01300	Thiazole synthase (EC 2.8.1.-)	2.457 up
	RAAC01301	ThiS protein	2.969 up
	RAAC01302	Glycine oxidase (EC 1.4.3.19)	2.843 up
	RAAC01303	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)	3.053 up

	RAAC01366	Peroxide operon regulator	5.197 up
	RAAC01367	Peroxiredoxin (EC 1.11.1.15)	5.109 up
	RAAC01368	Peroxiredoxin reductase (NAD(P)H) (EC 1.8.1.-) / NADH oxidase H2O2-forming (EC 1.6.3.-)	8.152 up
	RAAC01374	Hydroxyacylglutathione hydrolase (EC 3.1.2.6)	2.012 up
Aaci_2421	RAAC01389	cobalamin (vitamin B12) biosynthesis CbiX protein	6.312 up
	RAAC01397	3-oxoacyl-[acyl-carrier-protein] synthase (EC 2.3.1.41)	2.092 up
Aaci_2406	RAAC01403	Na(+)/H(+) antiporter/exchanger	2.843 up
	RAAC01407	3-deoxy-7-phosphoheptulonate synthase (EC 2.5.1.54)	13.997 up
	RAAC01408	Acyl-CoA dehydrogenase, short-chain specific (EC 1.3.99.2)	18.286 up
	RAAC01409	Cyclohexane-1-carboxylate--CoA ligase (EC 6.2.1.-)	6.893 up
	RAAC01426	VEG protein	2.231 up
	RAAC01435	Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	2.147 up
	RAAC01456	Cell division protein ftsH (EC 3.4.24.-)	2.326 up
	RAAC01471	Lysine-specific permease	2.385 up
	RAAC01477	Multidrug/protein/lipid ABC transporter family, ATP-binding and permease protein	2.165 up
	RAAC01478	Multidrug/protein/lipid ABC transporter family, ATP-binding and permease protein	2.380 up
Aaci_2270	RAAC01494	Adenylosuccinate synthetase (EC 6.3.4.4)	5.294 up
Aaci_2271	RAAC01495	Exodeoxyribonuclease III (EC 3.1.11.2)	3.477 up
Aaci_2272	RAAC01496	Peptidase S41	4.470 up
Aaci_2273	RAAC01497	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	7.146 up
Aaci_2274	RAAC01498	transcriptional regulator, DeoR family; Central glycolytic genes regulator	7.502 up
	RAAC01566	Phosphoglycerate kinase (EC 2.7.2.3)	2.466 up
Aaci_0742	RAAC01567	Triosephosphate isomerase (EC 5.3.1.1)	3.578 up
Aaci_0743	RAAC01568	Phosphoglycerate mutase (EC 5.4.2.1)	3.221 up
Aaci_0744	RAAC01569	Enolase (EC 4.2.1.11)	2.816 up

	RAAC01570	Protein translocase subunit secG	2.228 up
Aaci_0746	RAAC01571	major facilitator superfamily MFS_1	3.142 up
Aaci_0753	RAAC01582	hypothetical protein	3.063 up
Aaci_0754	RAAC01583	Glycosyltransferase (EC 2.4.1.-)	4.332 up
Aaci_0755	RAAC01584	hypothetical protein	4.292 up
Aaci_0756	RAAC01585	hypothetical protein	3.939 up
Aaci_0757	RAAC01586	Hypothetical protein	3.948 up
	RAAC01587	Methyltransferase (EC 2.1.1.-)	2.226 up
	RAAC01633	unassigned	3.370 up
	RAAC01663	unassigned	2.175 up
	RAAC01688	L-aspartate beta-decarboxylase (EC 4.1.1.12)	2.002 up
	RAAC01696	Thioredoxin	2.291 up
	RAAC01697	Peptide methionine sulfoxide reductase msrA (EC 1.8.4.11)	2.770 up
Aaci_0864	RAAC01703	Sulfate adenylyltransferase (EC 2.7.7.4)	3.425 up
Aaci_0865	RAAC01704	transcriptional regulator, MarR family	2.507 up
Aaci_0866	RAAC01705	Protein of unknown function DUF2071	3.200 up
	RAAC01706	tRNA synthetases class I, catalytic domain	2.304 up
	RAAC01710	Pyrazinamidase (EC 3.5.1.-) / Nicotinamidase (EC 3.5.1.19)	2.726 up
	RAAC01711	unassigned	2.012 up
	RAAC01728	unassigned	2.662 up
	RAAC01753	NADH-dependent butanol dehydrogenase A (EC 1.1.1.-)	2.205 up
	RAAC01754	Transcriptional regulator, LacI family	3.632 up
	RAAC01758	Sugar transport system permease protein	3.714 up
	RAAC01759	Aconitate hydratase (EC 4.2.1.3)	9.221 up
	RAAC01760	Undecaprenyl-phosphate alpha-N-acetylglucosaminephosphotransferase (EC 2.7.8.33)	2.732 up

Aaci_0918	RAAC01761	Protoheme IX farnesyltransferase (EC 2.5.1.-)	10.026 up
	RAAC01765	cell wall degradation enzyme	2.565 up
	RAAC01766	ATP phosphoribosyltransferase regulatory subunit	6.807 up
Aaci_0924	RAAC01767	ATP phosphoribosyltransferase (EC 2.4.2.17)	7.382 up
	RAAC01768	Histidinol dehydrogenase (EC 1.1.1.23)	6.905 up
Aaci_0926	RAAC01769	Imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19)	6.812 up
Aaci_0927	RAAC01770	Imidazole glycerol phosphate synthase, glutamine amidotransferase subunit (EC 2.4.2.-)	6.071 up
Aaci_0928	RAAC01771	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	5.660 up
Aaci_0929	RAAC01772	imidazoleglycerol phosphate synthase, cyclase subunit	4.566 up
Aaci_0930	RAAC01773	phosphoribosyl-ATP diphosphatase	4.672 up
	RAAC01775	unassigned	2.562 up
	RAAC01778	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26) / 5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193)	2.772 up
	RAAC01779	Riboflavin synthase alpha chain (EC 2.5.1.9)	4.581 up
	RAAC01780	GTP cyclohydrolase II (EC 3.5.4.25) / 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.99.12)	3.687 up
	RAAC01781	6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.9)	3.023 up
	RAAC01789	Transketolase (EC 2.2.1.1)	2.287 up
Aaci_0947	RAAC01790	Transcriptional regulators, LysR family	2.068 up
	RAAC01821	Isoaspartyl dipeptidase (EC 3.4.19.15)	2.037 up
	RAAC01822	Isoaspartyl dipeptidase (EC 3.4.19.15)	2.581 up
	RAAC01826	RNA polymerase ECF-type sigma factor	2.973 up
	RAAC01827	unassigned	3.128 up
	RAAC01828	Alanine dehydrogenase (EC 1.4.1.1)	3.081 up
Aaci_1034	RAAC01876	cell cycle protein; Rod shape-determining protein rodA	2.799 up
Aaci_1035	RAAC01877	Aminotransferase	2.962 up

Aaci_1036	RAAC01878	hypothetical protein	2.567 up
	RAAC01879	unassigned	2.090 up
Aaci_1038	RAAC01880	Hypothetical protein	2.967 up
	RAAC01912	Transcriptional regulator, DeoR family	6.126 up
	RAAC01913	Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10)	6.703 up
	RAAC01914	Galactokinase (EC 2.7.1.6)	4.187 up
	RAAC01925	Hypothetical membrane spanning protein	2.751 up
	RAAC01926	N-acetylglucosaminyltransferase (EC 2.4.1.-)	3.150 up
	RAAC01927	unassigned	2.583 up
Aaci_2341	RAAC01933	3H domain protein; Transcriptional repressor for NAD biosynthesis in gram-positives	2.310 up
	RAAC01955	Cysteine synthase (EC 2.5.1.47)	2.642 up
	RAAC01964	Globin family protein	2.043 up
Aaci_2381	RAAC01975	Multicopper oxidase type 3	8.646 up
Aaci_2382	RAAC01976	Hemerythrin HHE cation binding domain protein	5.562 up
	RAAC01977	unassigned	2.306 up
	RAAC01986	Ferredoxin	2.046 up
	RAAC01987	unassigned	2.508 up
	RAAC02012	Transcriptional regulator, LytR family	2.163 up
	RAAC02014	Hypothetical cytosolic protein	2.166 up
	RAAC02015	unassigned	2.357 up
	RAAC02016	unassigned	2.050 up
	RAAC02017	Calcium/proton antiporter	2.684 up
	RAAC02018	Hypothetical membrane spanning protein	2.256 up
Aaci_1857	RAAC02031	Transcriptional regulator, GntR family	3.747 up
Aaci_1856	RAAC02032	Radical SAM superfamily protein	4.506 up

	RAAC02050	Glutamyl-tRNA reductase (EC 1.2.1.70)	4.191 up
	RAAC02051	unassigned	3.812 up
	RAAC02052	Porphobilinogen deaminase (EC 2.5.1.61)	5.755 up
	RAAC02053	unassigned	3.610 up
	RAAC02054	Delta-aminolevulinic acid dehydratase (EC 4.2.1.24)	3.789 up
	RAAC02055	Glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8)	3.312 up
	RAAC02093	Aminomethyltransferase (EC 2.1.2.10)	2.150 up
	RAAC02094	Glycine dehydrogenase [decarboxylating] (EC 1.4.4.2)	2.152 up
	RAAC02095	Glycine dehydrogenase [decarboxylating] (EC 1.4.4.2)	2.187 up
	RAAC02096	Phosphoglycerate mutase family protein	2.146 up
	RAAC02219	Hypothetical membrane spanning protein	2.983 up
	RAAC02226	O-methyltransferase (EC 2.1.1.-)	2.333 up
	RAAC02227	D-alanyl-D-alanine serine-type carboxypeptidase (EC 3.4.16.4)	2.088 up
Aaci_0662	RAAC02228	cytochrome oxidase assembly	4.964 up
Aaci_0661	RAAC02229	Cytochrome c oxidase polypeptide IV (EC 1.9.3.1)	5.628 up
Aaci_0661	RAAC02230	Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	4.871 up
	RAAC02231	Cytochrome c oxidase polypeptide I (EC 1.9.3.1)	4.500 up
	RAAC02232	Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	4.318 up
Aaci_0628	RAAC02261	hypothetical protein	8.499 up
	RAAC02263	ThiJ/PfpI family protein	2.057 up
	RAAC02293	unassigned	2.183 up
	RAAC02335	unassigned	2.753 up
	RAAC02396	S-adenosylmethionine synthetase (EC 2.5.1.6)	2.367 up
	RAAC02397	N-acetylglucosaminyldiphosphoundecaprenol N-acetyl-beta-D-mannosaminyltransferase (EC 2.4.1.187)	2.354 up
	RAAC02398	unassigned	2.197 up

	RAAC02399	Multidrug resistance efflux pump	2.547 up
	RAAC02425	Esterase (EC 3.1.1.-)	3.764 up
	RAAC02426	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	5.252 up
	RAAC02427	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	4.282 up
	RAAC02428	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	3.384 up
	RAAC02429	Dihydrolipoamide dehydrogenase (EC 1.8.1.4)	2.539 up
	RAAC02432	Transcriptional regulator, IclR family	2.819 up
	RAAC02433	3-isopropylmalate dehydrogenase (EC 1.1.1.85)	2.549 up
	RAAC02517	Hypothetical cytosolic protein	2.545 up
	RAAC02625	Ornithine carbamoyltransferase (EC 2.1.3.3)	3.990 up
	RAAC02626	Argininosuccinate synthase (EC 6.3.4.5)	3.522 up
Aaci_1229	RAAC02627	Argininosuccinate lyase (EC 4.3.2.1)	3.359 up
Aaci_1230	RAAC02629	Aspartokinase (EC 2.7.2.4)	2.384 up
	RAAC02834	LSU ribosomal protein L19P	2.257 up
	RAAC02835	GTP-binding protein	3.599 up
	RAAC02836	Ribonuclease HII (EC 3.1.26.4)	2.320 up
	RAAC02837	unassigned	2.008 up
	RAAC02839	Peptidoglycan N-acetylglucosamine deacetylase (EC 3.5.1.104)	2.012 up
	RAAC02942	unassigned	3.744 up
Aaci_0343	RAAC03013	MMPL domain protein	5.947 up
Aaci_0344	RAAC03014	protein of unknown function DUF1453; Cytochrome c biosynthesis protein, CcdC	5.391 up
Aaci_0345	RAAC03015	peptidase S11 D-alanyl-D-alanine carboxypeptidase 1	4.430 up
Aaci_0346	RAAC03016	Peptidoglycan glycosyltransferase	5.507 up
Aaci_0393	RAAC03064	Adenylosuccinate lyase (EC 4.3.2.2)	5.577 up
Aaci_0394	RAAC03065	Phosphoribosylamidoimidazole-succinocarboxamide synthase (EC 6.3.2.6)	5.492 up

Aaci_0395	RAAC03066	Phosphoribosylformylglycinamidine synthase, purS component (EC 6.3.5.3)	4.287 up
Aaci_0396	RAAC03067	Phosphoribosylformylglycinamidine synthase I(EC 6.3.5.3)	4.358 up
Aaci_0397	RAAC03068	Phosphoribosylformylglycinamidine synthase II(EC 6.3.5.3)	3.923 up
Aaci_0398	RAAC03069	Amidophosphoribosyltransferase (EC 2.4.2.14)	3.574 up
Aaci_0399	RAAC03070	Phosphoribosylformylglycinamidine cyclo-ligase (EC 6.3.3.1)	3.760 up
Aaci_0400	RAAC03071	Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3) / IMP cyclohydrolase (EC 3.5.4.10)	3.550 up
Aaci_0401	RAAC03072	Phosphoribosylamine--glycine ligase (EC 6.3.4.13)	2.948 up
Aaci_0402	RAAC03073	protein of unknown function DUF6 transmembrane; Transporter, drug/metabolite exporter family	2.087 up
	RAAC03112	unassigned	2.102 up
	RAAC03194	unassigned	2.213 up
	RAAC03420	unassigned	2.832 up
	RAAC03516	unassigned	2.360 up
	RAAC03610	unassigned	2.363 up
	RAAC03969	unassigned	5.911 up
	RAAC04057	unassigned	4.306 up
	RAAC04074	unassigned	2.317 up
	RAAC04113	unassigned	2.140 up
	RAAC04259	unassigned	2.200 up
	RAAC04314	unassigned	2.149 up
	RAAC04336	von Willebrand factor type A domain protein	2.104 up
	RAAC04378	unassigned	2.929 up
	RAAC04491	unassigned	2.594 up
	RAAC04554	Phosphotransferase system, phosphocarrier protein HPr	2.277 up
	RAAC04555	Mannitol-1-phosphate 5-dehydrogenase (EC 1.1.1.17)	2.525 up

	RAAC04556	Transcriptional regulator, DeoR family	2.853 up
	RAAC04650	unassigned	2.007 up
	RAAC04684	Cold shock protein	2.322 up
	RAAC04685	unassigned	3.656 up
	RAAC04767	unassigned	4.254 up
	RAAC04823	unassigned	2.115 up
	RAAC04872	unassigned	2.638 up
	RAAC04940	unassigned	3.123 up
	RAAC04967	unassigned	2.802 up
	RAAC04968	unassigned	2.296 up