Complete genome of the cellulolytic thermophile *Acidothermus cellulolyticus* 11B provides insights into its ecophysiological and evolutionary adaptations

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We present here the complete 2.4-Mb genome of the cellulolytic actinobacterial thermophile *Acidothermus cellulolyticus* 11B. New secreted glycoside hydrolases and carbohydrate esterases were identified in the genome, revealing a diverse biomass-degrading enzyme repertoire far greater than previously characterized and elevating the industrial value of this organism. A sizable fraction of these hydrolytic enzymes break down plant cell walls, and the remaining either degrade components in fungal cell walls or metabolize storage carbohydrates such as glycogen and trehalose, implicating the relative importance of these different carbon sources. Several of the *A. cellulolyticus* secreted cellulolytic and xylanolytic enzymes are fused to multiple tandemly arranged carbohydrate binding modules (CBM), from families 2 and 3. For the most part, thermophilic patterns in the genome and proteome of *A. cellulolyticus* were weak, which may be reflective of the recent evolutionary history of *A. cellulolyticus* since its divergence from its closest phylogenetic neighbor *Frankia*, a mesophilic plant endosymbiont and soil dweller. However, ribosomal proteins and noncoding RNAs (rRNA and tRNAs) in *A. cellulolyticus* showed thermophilic traits suggesting the importance of adaptation of cellular translational machinery to environmental temperature. Elevated occurrence of IVYWREL amino acids in *A. cellulolyticus* orthologs compared to mesophiles and inverse preferences for G and A at the first and third codon positions also point to its ongoing thermoadaptation. Additional interesting features in the genome of this cellulolytic, hot-springs-dwelling prokaryote include a low occurrence of pseudogenes or mobile genetic elements, an unexpected complement of flagellar genes, and the presence of three laterally acquired genomic islands of likely ecophysiological value.

[Supplemental material is available online at www.genome.org. The complete genome of *Acidothermus cellulolyticus* 11B (ATCC 43068) has been deposited in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) under accession no. NC_008578.]

Efforts are under way worldwide to develop renewable energy sources as alternatives to fossil fuels. Microorganisms capable of breaking down lignocellulosic plant matter, a bioenergy source, are of enormous interest in the global quest to identify enzymes that can convert biomass into biofuels. *Acidothermus cellulolyticus* was first isolated in enrichment cultures from acidic hot springs in Yellowstone National Park, in a screen for microorganisms that carry out efficient cellulose degradation at high temperature (Mohagheghi et al. 1986). *A. cellulolyticus* 11B is acid-tolerant (pH

4–6, with optimal pH 5.5) and thermophilic (growth between 37°C and 70°C; the optimal growth temperature [OGT] is 55°C). It produces many thermostable cellulose-degrading enzymes (Tucker et al. 1989; Baker et al. 1994; Adney et al. 1995; Ding et al. 2003). One of the endoglucanases, E1, which has been crystallized, is highly thermostable to 81°C and has very high specific activity on carboxymethylcellulose (Thomas et al. 1995; Sakon et al. 1996). E1 has been expressed in several plants and shows promise for generating genetically improved feedstock for the production of affordable cellulosic ethanol (Sticklen 2008). Hydrolytic enzymes from *A. cellulolyticus* have great potential in the biofuels industry because of their thermostability and activity at low pH (Rubin 2008).

A. cellulolyticus is a member of the Frankineae, a high G+C, primarily Gram-positive Actinobacterial group (Rainey

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and Stackebrandt 1993). All of the characterized strains of *A. cellulolyticus* are thermophilic and do not grow below 37°C (Mohagheghi et al. 1986). This makes the evolutionary context of *A. cellulolyticus* interesting, because its closest known phylogenetic neighbor is the mesophilic actinobacterium *Frankia*, based on the analysis of the 16S rRNA, *recA*, and *shc* nucleotide sequences (Supplemental Fig. S1; Normand et al. 1996; Marechal et al. 2000; Alloisio et al. 2005). *Frankia* is a mesophilic (OGT 25°C–28°C), nitrogen-fixing soil organism that forms symbiotic root nodule associations with plants (Benson 1988). The genetic distance between *A. cellulolyticus* and three *Frankia* strains—ACN14a, Ccl3, and EAN1pec—is very small and comparable to that found between certain strains within the *Frankia* species. Thus, although *Acidothermus* and *Frankia* share a close phylogenetic relationship at the DNA sequence level, they have evolved to live in dramatically diverse environments over the last 200–250 million years (Myr) since their last common ancestor (Normand et al. 2007). Complete genome sequences of three *Frankia* strains—ACN14a, Ccl3, and EAN1pec—as well as those of other close relatives of *A. cellulolyticus* are now available, including the mesophilic *Streptomyces avermitilis*, *Streptomyces coelicolor*, and the terrestrial thermophilic *Thermobifida fusca* (Omura et al. 2001; Bentley et al. 2002; Ikeda et al. 2003; Lykidis et al. 2007; Normand et al. 2007). Genomic comparison of *A. cellulolyticus* with the mesophilic as well as thermophilic actinobacteria could provide insight into the nature of adaptation of this aquatic thermophile and add to our understanding of evolution within the actinobacteria.

We present analysis of the complete genome of *Acidothermus cellulolyticus* 11B (ATCC 43068; GenBank accession NC_008578). Insights into the biomass degradation capabilities of the organism as well as thermophilic features of its genome and proteome are discussed. In addition, we discuss three laterally acquired genomic islands with genes of likely ecophysiological value, as well as the unexpected presence of flagellar genes in the genome.

Results

General genome characteristics

The 2.44-Mb genome of *A. cellulolyticus* is encoded on a single circular chromosome (Fig. 1) and is ~66.9% G+C rich. The G+C content of the noncoding region (68.41%) is higher than the G+C content of the coding region (66.76%). The total GC-skew analysis revealed a potential origin of replication (OriC) upstream of the *dnaA* gene and a terminus at ~1.2 Mb from the origin. A single *rrn* operon containing the genes for the 16S, 23S, and 5S rRNAs is located toward the replication terminus, an unusual position. Forty-five tRNAs representing 43 different anticodons are encoded in the genome (Supplemental Table S1; Supplemental Material). The *A. cellulolyticus* genome contains only four annotated pseudogenes (Acel_0124, Acel_0186, Acel_0477, Acel_1066) that do not encode any protein products. The protein-coding sequence constitutes ~90% of the genome and encodes 2157 predicted proteins. No identifiable prophages or phage-related proteins were found in the genome, and only two genes encoding fragments of a single transposase (Acel_1666, Acel_1667) were found in the genome. One-fifth of all the predicted proteins have no decipherable function. Approximately 8% of the proteins (171 proteins) do not show sequence similarity to any sequences in the NCBI database and thus appear to be ORFans unique to *A. cellulolyticus* (Supplemental Fig. S2). Analysis of the phyletic distribution of BLAST hits of the remaining proteins revealed that

Figure 1. Schematic of the *A. cellulolyticus* 11B genome. The outermost circle gives the genome coordinates. The next two inner rings show the predicted genes on the leading (outer circle) and the lagging (inner circle) strands. Color scheme is as follows: dark gray, hypothetical proteins; light gray, conserved hypothetical and unknown function; brown, general function prediction; red, replication and repair; green, energy metabolism; blue, carbon and carbohydrate metabolism; cyan, lipid metabolism; magenta, transcription; yellow, translation; orange, amino acid metabolism; pink, metabolism of cofactors and vitamins; light red, purine and pyrimidine metabolism; lavender, signal transduction; sky blue, cellular processes; pale green, structural RNAs. Ring 4 displays the positions of the glycoside hydrolases (black bars), the three GIs (triangles), the flagellar biosynthetic genes (red star), and the rRNA operon (blue star). Ring 5 shows the G+C content along the genome. The innermost ring, ring 6, displays the GC skew.

~80% of the *A. cellulolyticus* proteins show highest sequence similarity to proteins from other actinobacteria (Supplemental Fig. S2). Within the actinobacterial hits, the highest number of best BLAST hits, surprisingly, were to the phylogenetically more remote *Streptomyces* spp. (~18%), more so than to its closest phylogenetic neighbor *Frankia* spp. (~17%), and followed by *T. fusca* (~13%). Interestingly, 18 *A. cellulolyticus* proteins bear highest sequence similarity to archaeal proteins, and seven proteins show highest sequence similarity to eukaryotic proteins (Supplemental Table S2).

Based on the distribution of the top BLAST hits to *Frankia*, *Streptomyces*, and *T. fusca*, sequenced genomes of these organisms were used for comparative genome analyses. An overview of the *A. cellulolyticus* genome features in comparison with the genomes of *Frankia*, *Streptomyces*, and *T. fusca* is provided in Table 1.

Carbohydrate active enzymes

The genome of *A. cellulolyticus* contains at least 43 genes encoding 35 glycoside hydrolase (GH) and eight carbohydrate esterase (CE) enzymes (Table 2). Of these, 28 predicted enzymes break down structural or storage carbohydrates found in plant and fungal cells, including cellulose, xylan, starch, and chitin. The GHs belong to 17 families, while the CEs span five families as per the CAZy database (Henrissat 1991; Coutinho and Henrissat 1999)

Table 1. Comparative features of *Acidothermus cellulolyticus* 11B and close actinobacterial relatives^a

Features	<i>Acidothermus cellulolyticus</i> 11B	<i>Frankia</i> sp. Ccl3	<i>Frankia alni</i> ACN14a	<i>Streptomyces avermitilis</i> MA-4680	<i>Streptomyces coelicolor</i> A3(2)	<i>Thermobifida fusca</i> YX
OGT	55°C	27°C	28°C	28°C	30°C	50°C
Genome size (Mb)	2.4	5.4	7.5	9.0	8.7	3.6
G+C of the genome	66.9%	70.1%	72.8%	70.7%	72.1%	67.5%
Coding DNA fraction	89%	84%	86%	86%	88%	85%
Predicted proteins	2157	4499	6711	7577	7769	3110
rRNA operons	1	2	2	6	5	4
tRNA genes	46	46	46	68	64	52
Pseudogenes	4	50	12	0	56	7
Transposase/IS elements	2 ^b	145	33	110	55	5
Phage/viral proteins	0	6	24	20	8	3

^aThe genomes chosen for comparison were based on two attributes: (1) The majority of the top BLAST hits of *A. cellulolyticus* proteins were from these species (see Supplemental Fig. S2); and (2) both mesophilic and thermotolerant species were represented.

^bThe two transposase genes are frame-shifted fragments of an intact gene found in *Frankia* and thus are unlikely to encode a functional transposase in *A. cellulolyticus*.

(<http://www.cazy.org/>). At least 15 GHs belonging to families 1, 3, 5, 6, 9, 10, 12, 16, 48, and 74; and three CEs from families 1 and 7 may be important for plant biomass deconstruction in *A. cellulolyticus*. Two or more representatives of several of these enzyme families occur in the genome, except for GH1, 16, 48, and 74 and CE7 (Table 2).

Five previously described carbohydrate active enzymes (Ding et al. 2003) could be correctly mapped in the genome (Table 2). While these known cellulolytic enzymes are encoded in a large gene cluster (Ding et al. 2003), genes encoding many newly identified enzymes occur scattered throughout the genome (Fig. 1). The genome revealed six new cellulose-degrading enzymes including four endoglucanases and two beta-glucosidases. In addition, six enzymes for hemicellulose decomposition were identified including two xylanases, three xylan esterases, and a xylosidase. Except for the GH1 beta-glucosidase and the GH3 xylosidase that are predicted to be cytoplasmic as well as the CE7 esterase, the rest of the plant cell-wall-degrading enzymes are either predicted to be secreted or contain a signal peptide (Table 2).

In addition to the 17 plant cell-wall-degrading enzymes, the genome encodes 10 proteins potentially associated with the breakdown of fungal cell wall components. Two beta-N-acetylhexosaminidases and a chitoooligosaccharide deacetylase were predicted to be cytoplasmic, while the other seven proteins are either predicted to be secreted or have a signal sequence indicating that they are likely to be secreted. These include four chitinases, an N-acetylglucosaminidase, a GH16 endo-1,3-beta-glucanase, and a CenC-domain-containing putative chitin-binding protein.

Sixteen enzymes are involved in either glycogen and trehalose biosynthesis and degradation (eight enzymes) or related cellular metabolic functions (Table 2). The GH13 alpha amylase (Acel_0679) may additionally participate in starch metabolism. None of these enzymes contains a signal sequence and is predicted to be cytoplasmic except the two GH23 lytic transglycosylases that may be cell-wall associated.

Carbohydrate-binding modules (CBMs)

Catalytic domains of two-thirds of the 21 secreted biomass-degrading enzymes in *A. cellulolyticus* were found fused to one or more CBM types (Table 2). Furthermore, members of the same GH families carry varying numbers and combinations of fused CBMs. Only one of the esterases (CE1) was fused to CBMs. The

cellulose- and xylan-degrading *A. cellulolyticus* enzymes contain C-terminally fused CBM2 domains, a feature that was found to be similar to other actinobacterial homologs. However, many *A. cellulolyticus* enzymes additionally contain CBM3 domains. Curiously, CBM3 was always found to occur N-terminal relative to CBM2, but never C-terminal to it. In general, the two CBM types were found to occur in tandem (as X-CBM3-CBM2, where X is GH, CE, or CBM3 domain), except in the case of the Gux1 exoglucanase and the GuxA cellulase where the two CBMs are separated by a GH domain (CBM3-X-CBM2). Although two endoglucanases, the previously characterized endoglucanase E1 (GH5) and a newly identified GH12 endoglucanase, contain just the CBM2, no enzymes with only the CBM3 module occur in the genome.

Overall, the *A. cellulolyticus* genome encodes about equal numbers of the two CBM types—10 CBM2 and nine CBM3 modules. Comparative genome analysis revealed that *Frankia alni* ACN14a and Ccl3 lack either CBMs, while a single CBM2 fused to a chitinase was found in *Frankia* sp. EAN1pec. However, the three *Frankia* genomes also lack cellulolytic enzymes. The genomes of two close actinobacterial relatives with multiple cellulolytic enzymes, *Streptomyces* and *Thermobifida*, contain 11–14 CBM2 modules but just one to two CBM3 modules. In contrast, the genome of the anaerobic cellulosome-forming bacterium *Clostridium thermocellum* encodes about 24 CBM3 domains but no CBM2 homologs. Analysis of each of the two CBM types revealed that the sequences are highly conserved in *A. cellulolyticus*. In contrast, the different CBM2 domains in *Streptomyces* or *Thermobifida*, or the several CBM3 domains in *C. thermocellum*, exhibit sequence diversity.

In addition to the two CBM families, a single copy of CBM6 was found attached to a GH16 endo-1,3-beta-glucanase. Three of the secreted chitinases also contained CBM5 and/or CBM16 domains. A few of the cytoplasmic enzymes involved in glycogen/trehalose metabolism contain one to two CBM48 modules.

Genomic islands

Three major genomic islands (GIs) with significantly lower G+C and deviant dinucleotide signature were identified (Fig. 2). Several proteins encoded in these islands have no recognizable orthologs in close relatives of *A. cellulolyticus*. GI1 consists of 15 genes with an average G+C of 58% (Table 3). The first five genes likely

Table 2. Carbohydrate active enzymes encoded in the *A. cellulolyticus* 11B genome

Locus tag	MW ^a	Domains ^b	Known or predicted function	Role ^c	Sig ^d	Loc ^e	Reference ^f
Acel_0072	60	GH20	Beta-N-acetylhexosaminidase (EC 3.2.1.52)	Fun	Y	Cyt	
Acel_0128	50	GH3	Beta-N-acetylhexosaminidase	Fun	N	Cyt	
Acel_0129	49	GH16-CBM6	Endo-1,3-beta-glucanase	Fun	Y	U	
Acel_0133	53	GH1	Beta-glucosidase (EC3.2.1.21)	Cel	N	Cyt	
Acel_0135	51	GH6	Beta-1,4-endoglucanase (CelB; EC 3.2.1.4)	Cel	Y	U	
Acel_0179	68	CE1-CBM3-CBM2	Acetyl-xylan esterase	Hem	Y	Sec	
Acel_0180	71	GH10-CBM3-CBM2	Beta-1,4-xylanase	Hem	Y	Sec	
Acel_0372	43	GH10	Endo-1,4-beta-xylanase (EC 3.2.1.8)	Hem	Y	Sec	
Acel_0374	27	CE14	Putative deacetylase	M	N	Cyt	
Acel_0424	83	GH18	N-Acetylglucosaminidase	Fun	Y	Sec	
Acel_0557	40	CE9	N-Acetylglucosamine 6-phosphate deacetylase	M	N	Cyt	
Acel_0603	51	GH18	Chitinase	Fun	Y	U	
Acel_0614	61	GH5-CBM2	Endo-1,4-glucanase E1 (Cel5A; EC 3.2.1.4)	Cel	Y	Sec	1, 2
Acel_0615	125	GH6-CBM3-GH12-CBM2	Cellulase (GuxA; EC3.2.1.4)	Cel	Y	Sec	2
Acel_0616	80	GH5-CBM3-CBM2	Mannanase (ManA)	Hem	Y	Sec	2
Acel_0617	119	CBM3-GH48-CBM2	Exoglucanase (Gux1)	Cel	Y	Sec	2
Acel_0618	134	GH74-CBM3-CBM2	Avicelase (Cel74A)	Cel	Y	Sec	2
Acel_0619	41	GH12-CBM2	Endoglucanase	Cel	Y	Sec	
Acel_0676	82	CBM48-CBM48-GH13	1,4-Alpha-glucan branching enzyme	G/T	N	Cyt	
Acel_0678	65	GH13	Trehalose synthase	G/T	N	Cyt	
Acel_0679	73	GH13	Alpha amylase	G/T	N	Cyt	
Acel_0681	78	CBM48-GH13	Glycogen debranching enzyme GlgX	G/T	N	Cyt	
Acel_0767	41	CE1	Putative esterase	Hem	Y	Sec	
Acel_0846	33	NLPC_P60-GH23	Lytic transglycosylase	M	N	Sec	
Acel_0970	95	GH9-CBM3-CBM2	Beta-1,4-endoglucanase	Cel	N	Sec	
Acel_1143	71	GH15	Trehalase/glucoamylase/glucoamylase	G/T	N	Cyt	
Acel_1157	41	GH23	Lytic transglycosylase	M	N	U	
Acel_1363	38	GH32	Putative beta-fructosidase	M	N	Cyt	
Acel_1372	80	CBM48-GH13	Glycogen debranching enzyme GlgX	G/T	N	Cyt	
Acel_1373	85	GH13	Malto-oligosyltrehalose synthase	G/T	N	Cyt	
Acel_1374	64	CBM48-GH13	Malto-oligosyltrehalose trehalohydrolase	G/T	N	Cyt	
Acel_1458	47	GH18-CBM16	Chitinase (EC 3.2.1.14)	Fun	N	SW	
Acel_1459	26	CBM16	Carbohydrate-binding CenC domain protein	Fun	Y	U	
Acel_1460	80	GH18-CBM5-CBM16	Chitinase (EC 3.2.1.14)	Fun	Y	SW	
Acel_1601	83	GH77	4-Alpha-glucanotransferase (EC2.4.1.25)	M	N	Cyt	
Acel_1659	93	GH3-GH3C-PA14-GH3C	Beta-glucosidase (EC3.2.1.21)	Cel	Y	Sec	
Acel_1701	120	GH9-CBM3-CBM3-CBM2	Endoglucanase	Cel	Y	Sec	
Acel_1868	31	CE14	Putative deacetylase	M	N	Cyt	
Acel_1886	36	CE14	Putative deacetylase	M	N	Cyt	
Acel_1917	27	CE4	Putative chitoooligosaccharide deacetylase	Fun	N	Cyt	
Acel_2033	61	GH18-CBM5-CBM16	Chitinase (EC 3.2.1.14)	Fun	Y	SW	
Acel_2045	35	CE7	Acetyl xylan esterase	Hem	N	U	
Acel_2050	88	GH3-GH3C	Beta-D-xylosidase (EC 3.2.1.37)	Hem	N	Cyt	

^a(MW) Calculated molecular weight of the protein in kilodaltons, rounded to a whole number.

^bDomain architecture was deciphered using the CAZy database (Henrissat 1991; Coutinho and Henrissat 1999) (<http://www.cazy.org/>) and the Conserved Domains Search tool (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Marchler-Bauer et al. 2007). The GH (glycoside hydrolase), CBM (carbohydrate binding module), and CE (carbohydrate esterase) family numbers are based on the CAZy classification.

^cA predicted role for the enzyme in (Cel) cellulose, (Hem) hemicellulose, and (Fun) fungal cell wall degradation. (G/T) Glycogen/trehalose metabolism; (M) cellular metabolism.

^d(Sig) Signal peptide; the (Y) presence or (N) absence of a predicted signal peptide in the protein sequence. The SignalP 3.0 software was used to predict the occurrence of signal peptides (Bendtsen et al. 2004).

^e(Loc) Localization. The pSORTb prediction software (Gardy et al. 2005) (<http://www.psort.org/psortb/>) was used to predict the subcellular localization of the protein. (Cyt) Cytoplasmic; (Sec) secreted/extracellular; (U) unknown localization. (SW) Proteins are predicted to be secreted as well as cell-wall-associated; therefore, they could occupy multiple locations.

^fReferences: (1) Baker et al. (1994); (2) Ding et al. (2003).

constitute an operon that encodes fumarate reductase/succinate dehydrogenase, arylalkylphosphatase, a short-chain dehydrogenase, deoxyribose-phosphate aldolase, and a ROK-family protein, respectively. The second half of GI1 contains genes involved in sugar uptake and metabolism.

GI2 contains 18 genes (average G+C of 62.5%) flanked by tRNA genes (Table 3). Half of the genes do not have a recognizable function, while many of the remaining genes encode putative homologs of the *vrl* locus of *Dichelobacter nodosus*. The *VrII* and *J* homologs in *A. cellulolyticus* have DNA-binding and ATPase

domains, respectively, and the *VrIK*, *P*, and *Q* homologs do not have any identifiable domains. With respect to the four intervening proteins, one is a transcriptional regulator containing a helix–turn–helix motif, another shows weak homology with DNA methylases, a third is a hypothetical protein, and the fourth has a helicase domain and could be a *VrIO* homolog although the homology is undetectable at sequence level. Most proteins encoded in this island show highest similarity to proteins from low G+C Gram-positives, namely, *Bacteroides*, *Nitrosococcus*, and *Thermoanaerobacter*.

Figure 2. Genomic signature plot. A sliding window plot of the percent G+C content (top line, y-axis on the left) as well as the deviation in genomic signature (ΔGS ; bottom line, secondary y-axis on right) along the chromosome. Regions 1, 2, and 3 on the plot indicate the location of the three GIs: GI1, GI2, and GI3, respectively. Arrow indicates the location of the flagellar and motility genes.

GI3 carries 31 genes (average G+C of 61.7%) and is flanked by the *tRNA^{Arg}* gene upstream and by the *tRNA^{His}* gene downstream (Table 3). One-third of the proteins encoded on this island have no recognizable function. Of the remaining genes, three encode proteins involved in ABC transport, two of which may be involved in the uptake of amino acids. *Acel_1633*–*Acel_1639* form an operon of seven genes: The first two genes encode proteins with unknown function; the third and the last encode enzymes involved in amino acid metabolism; while the rest encode subunits of the carbon monoxide (CO) dehydrogenase family proteins. Another likely operon of four genes encodes an aldehyde oxidase, a coenzyme A transferase, glutamate coA-transferase, and a luciferase family protein. Six genes in this GI (namely, *Acel_1626*, *Acel_1628*, *Acel_1634*, *Acel_1639*, *Acel_1643*, and *Acel_1644*) encode proteins that bear highest sequence similarity to proteins from thermophilic bacteria and archaea. With the exception of *Acel_1626*, homologs of these six proteins do not occur in *Frankia*.

In addition to the three major islands, 21 smaller genomic regions (GRs) were identified. Characteristics of the predicted regions are detailed in Supplemental Table S3.

Flagella and motility

Mohagheghi et al. (1986) reported that *A. cellulolyticus* cells were nonmotile based on microscopic observations. Surprisingly, immediately downstream from GI2, we identified a stretch of 37 genes (*Acel_0828*–*Acel_0864*) that did not have any homologs in *Frankia*, *Streptomyces*, or *T. fusca*. This region encoded a complete set of genes coding for flagellar biosynthesis and motility. The genes are organized into two divergent gene clusters (Fig. 3). Most of the flagellar structural genes are organized in the larger cluster containing 31 genes on the leading strand. The regulatory gene *csrA*, recently shown to encode a regulator of flagellar biosynthesis (Yakhnin et al. 2007), is encoded by the last gene in the smaller cluster containing five genes. Thus far, only three other actinomycetes—*Nocardioide* sp. J5514, *Kineococcus radiotolerans*, and *Leifsonia xyli*—encode sequence homologs of the flagellar genes (Fig. 3). The gene content and order of the flagellar operon is highly conserved between *A. cellulolyticus* and *Nocardioide*, while minor differences in gene order are observed in *Kineococcus*. Several flagellar genes in *L. xyli* are pseudogenes, in agreement with the observation that the organism is nonmotile and does not produce a flagellum (Monteiro-Vitorello et al. 2004); the

presence of motility or flagella has not been well studied in the other two organisms. Although in the original study no motility was observed in *A. cellulolyticus* (Mohagheghi et al. 1986), the possibility of motility, perhaps under specific growth conditions, is being carefully reexamined.

Thermoadaptation

Principal component analysis (PCA) of global as well as synonymous codon usage revealed that *A. cellulolyticus*, surprisingly, did not contain patterns typically observed in thermophilic prokaryotes (Supplemental Fig. S3A,B). It was clearly positioned amidst mesophiles along the PC2 axis that correlated with OGT. Codon usage differences between *Acidothermus* and *Frankia* were very subtle (Supplemental Table S4). Differences in the codon usage of the six actinobacteria compared in our study did not always follow differences in G+C content in the coding region of their genomes (Supplemental Table S4), suggesting a physiological pressure influencing these differences. A detailed comparison of the relative abundances of the four nucleotides at each of the three codon positions showed that the relative proportion of G was higher and that of A was lower at the first codon position in the two thermophiles as compared to the four mesophiles (Table 4). In addition, an opposite but slightly weaker trend was observed at the third codon position, that is, the relative proportion of A was higher and that of G was lower in the two thermophiles as compared to the mesophiles (Table 4). Interesting differences were observed for the GNA and ANG codons (see Supplemental Table S4). Of the four GNA codons, the GAA codon (for glutamate) showed the most prominent increase in the two thermophiles. Of the four ANG codons, the AGG codon (for arginine) was clearly less preferred in *A. cellulolyticus* and *T. fusca*.

Noncoding RNAs, ribosomal RNAs (rRNAs), and transfer RNA (tRNAs), in *A. cellulolyticus* had a higher G+C content than mesophilic species with similar genomic G+C (Fig. 4). Confidence intervals of the prediction of a linear model (RNA G+C content as a function of genomic G+C content) for mesophilic species showed that *A. cellulolyticus* was clearly an outlier when compared to the mesophilic species in the study. The G+C content of functional RNAs has been shown to correlate positively with OGT (Galtier and Lobry 1997).

Similar to the codon-usage PCA results (Supplemental Fig. S3A,B), PCA of the amino acids usage did not reveal thermophilic trends in the *A. cellulolyticus* proteome (Supplemental Fig. S4). Contrary to our expectation that it should segregate with other thermophiles, *A. cellulolyticus* was positioned near mesophiles along the PC2 axis that correlated with OGT. However, in a more detailed analysis of the amino acid composition of ribosomal proteins, *A. cellulolyticus* was placed nearer to the thermophiles than *Frankia* or *Streptomyces* and was at the same level as *T. fusca* (Fig. 5).

The total fractions of IVYWREL amino acids in the *A. cellulolyticus* proteome and cytosolic subproteome were higher than those in *Frankia* sp. and *Streptomyces* sp. (Supplemental Table S5). Furthermore, analysis of the amino acid composition of 478 conserved orthologous proteins in these six actinobacteria clearly revealed that both *A. cellulolyticus* and *T. fusca* orthologs contain

Table 3. Genes encoded on the three genomic islands found in the *A. cellulolyticus* 11B genome

Locus tag	S	Percent GC	Size	Product description	Function
Genomic Island 1					
Acel_0569	+	58.7	446	Fumarate reductase/succinate dehydrogenase flavoprotein	Respiration
Acel_0570	+	53.5	333	Aryldialkylphosphatase	Organophosphate detoxification
Acel_0571	+	57.6	288	Short-chain dehydrogenase/reductase SDR	Metabolism
Acel_0572	+	59.7	236	Deoxyribose-phosphate aldolase	Nucleotide metabolism
Acel_0573	+	62.8	342	ROK family protein	Repressor/kinase/ORF
Acel_0574	—	59.3	254	Transcriptional regulator, GntR family	Regulation
Acel_0575	—	61.9	421	ROK family protein	Repressor/kinase/ORF
Acel_0576	+	58.2	283	SIS (Sugar ISomerase) phosphosugar binding domain protein	Carbohydrate metabolism
Acel_0577	+	58.9	359	Periplasmic binding protein/LacI transcriptional regulator	ABC transport
Acel_0578	+	59.0	489	ABC transporter related	ABC transport
Acel_0579	+	58.6	335	Inner-membrane translocator	ABC transport
Acel_0580	+	56.6	330	Inner-membrane translocator	ABC transport
Acel_0581	+	58.9	391	Oxidoreductase domain protein	ABC transport
Acel_0582	+	53.8	306	Xylose isomerase domain protein TIM barrel	Sugar interconversion
Acel_0583	—	59.1	397	Oxidoreductase domain protein	Metabolism
Genomic Island 2					
Acel_R0021	+	58.7		Xaa tRNA	
Acel_0810	+	59.0	61	DNA binding domain, excisionase family	VrII homolog
Acel_0811	+	59.3	159	Conserved hypothetical protein	VrII homolog
Acel_0812	+	62.6	1244	Conserved hypothetical protein	VrIK homolog
Acel_0813	+	61.0	468	Putative transcriptional regulator	Transcriptional regulation
Acel_0814	+	60.5	993	Conserved hypothetical protein	
Acel_0815	+	51.1	268	Hypothetical protein	
Acel_0816	+	64.7	934	Helicase domain protein	VrIO homolog?
Acel_0817	+	57.5	678	Conserved hypothetical protein	VrIP homolog
Acel_0818	+	57.0	261	Conserved hypothetical protein	VrIQ homolog
Acel_0819	+	67.2	64	Hypothetical protein	
Acel_0820	+	68.8	446	Metallophosphoesterase	DNA repair
Acel_0821	+	67.6	918	SMC domain protein	DNA repair
Acel_0822	+	66.7	502	Acyltransferase 3	Metabolic enzyme
Acel_0823	—	66.5	548	Diguanylate cyclase/phosphodiesterase	Metabolic enzyme
Acel_0824	—	65.0	122	Hypothetical protein	
Acel_0825	—	66.2	206	Protein of unknown function DUF421	
Acel_R0022	+	66.2		Met tRNA	
Genomic Island 3					
Acel_R0044	+	68.5		Arg tRNA	
Acel_1621	+	51.1	92	Hypothetical protein	
Acel_1622	+	62.8	162	Hypothetical protein	
Acel_1623	+	64.0	89	Transcriptional regulator, XRE family	Transcriptional regulation
Acel_1624	+	55.7	176	Hypothetical protein	
Acel_1625	+	66.5	180	Hypothetical protein	
Acel_1626	+	63.3	230	ABC transporter related	Transport
Acel_1627	+	65.8	426	Protein of unknown function DUF214	
Acel_1628	+	63.7	168	Methylglyoxal synthase	Enzyme
Acel_1629	—	64.9	483	Methyl-accepting chemotaxis sensory transducer	Chemotaxis
Acel_1630	—	65.9	213	Conserved hypothetical protein	
Acel_1631	—	65.5	358	Protein of unknown function DUF182	
Acel_1632	—	54.8	208	Conserved hypothetical protein	
Acel_1633	—	58.9	602	Purine catabolism PucR domain protein	Purine degradation regulator
Acel_1634	—	59.6	327	Conserved hypothetical protein	
Acel_1635	—	61.7	403	Pyridoxal-5'-phosphate-dependent enzyme, beta subunit	Metabolic enzyme
Acel_1636	—	62.2	238	Carbon monoxide dehydrogenase subunit G, CoxG	CO fixation?
Acel_1637	—	59.7	162	Carbon monoxide dehydrogenase small subunit, CoxS	CO fixation?
Acel_1638	—	61.4	296	Carbon monoxide dehydrogenase (acceptor), CoxM	CO fixation?
Acel_1639	—	59.2	231	Asp/Glu racemase	Amino acid metabolism
Acel_1640	—	58.9	560	Polar amino acid ABC transporter, inner membrane subunit	Amino acid transport
Acel_1641	—	57.2	303	Extracellular solute-binding protein, family 3	Solute uptake
Acel_1642	—	61.0	783	Aldehyde oxidase and xanthine dehydrogenase	Metabolic enzyme
Acel_1643	—	60.4	262	Coenzyme A transferase	Metabolic enzyme
Acel_1644	—	59.6	318	Glutaconate CoA-transferase	Metabolic enzyme
Acel_1645	—	55.6	316	Luciferase family protein	Metabolic enzyme
Acel_1646	+	62.8	230	NADPH-dependent F420 reductase	Metabolic enzyme
Acel_1647	+	67.7	505	Malate dehydrogenase (oxaloacetate-decarboxylating)	Metabolic enzyme
Acel_1648	+	66.1	363	Molybdenum cofactor biosynthesis protein A	Metabolic enzyme
Acel_1649	+	69.5	270	Exonuclease, RNase T, and DNA polymerase III	Metabolic enzyme
Acel_R0045	+	59.2		His tRNA	

+/- Indicates the DNA strand (S) that encodes the gene. The boxes indicate blocks of genes on the same strand with intergenic distance <50 bp. Size indicates the length of the predicted protein in amino acids. Product descriptions are based on automatic annotation of the gene. The last column provides a broad function of the protein.

Figure 3. Synteny and gene organization of the flagellar biosynthetic genes in actinobacteria. The *A. cellulolyticus* locus Acel_0827-Acel_0864 is displayed; the syntenic region ranges from Acel_0829 to Acel_0861. Ace, Kra, Lxy, and Noc denote *A. cellulolyticus*, *K. radiotolerans*, *L. xyli*, and *Nocardioides* sp. JS614, respectively. Chromosomal gene organization from each of the completely assembled genomes is shown, except in the case of *K. radiotolerans*, for which genes from two different contigs are shown. Therefore, the true order of the whole region in *K. radiotolerans* remains unclear. Synteny between the different chromosomal regions is indicated by green lines (for genes on the same strand) and red lines (for genes on opposite strands). The gene sizes in the different organisms are not drawn to scale. Also, the *K. radiotolerans* genes are colored differently than the genes in the other three organisms.

a higher proportion of IVYWREL amino acids compared to the four mesophilic organisms (Supplemental Table S5). The values of IVYWREL fractions in the orthologs showed even greater linear correlation with OGT than those from the cytosolic subproteomes or whole proteomes. In addition, an extended analysis of 46 conserved orthologous proteins from several mesophilic and thermophilic actinobacteria with varying G+C content showed a similar trend, namely, that orthologs from the thermophilic actinobacteria contain increased representation of IVYWREL amino acids compared to the mesophiles (Supplemental Table S6). It is to be noted that there are exceptions to a strict increase in IVYWREL with OGT. Thus, the content of IVYWREL is a reasonable but not a perfect predictor of the OGT, as noted also by Zeldovich et al. (2007).

Discussion

A. cellulolyticus has a small genome with very few pseudogenes or mobile genetic elements. The two transposase-encoding gene sequences in *A. cellulolyticus* encode frame-shifted fragments of an intact gene that is found in *Frankia* and other actinobacteria. As a result, *A. cellulolyticus* may not encode an active transposase. In

contrast, many of the terrestrial as well as aquatic actinobacterial relatives of *A. cellulolyticus*, such as *Frankia* sp., *S. avermitilis*, *S. coelicolor*, and *T. fusca* (see Table 1) as well as *K. radiotolerans*, and *Nocardioides* sp. (data not shown) possess multiple pseudogenes, as well as several transposase-encoding genes and interior sequence (IS) elements in their genomes. With the exception of *T. fusca*, the other actinobacteria also possess large genomes, ranging from 5 to 9 Mb. It is conceivable that the presence and abundance of transposase-related genes in the larger genomes reflect the role of these mobile elements in their genome expansion, as described for *Frankia* (Normand et al. 2007), but also that genome reduction events accompanied by the loss of mobile elements may have resulted in a small genome size of *A. cellulolyticus*.

With the renewed interest and growing quest for microbes that efficiently deconstruct plant cell wall carbohydrates for conversion to biofuels, the sequenced genome of *A. cellulolyticus* offers substantial potential for the discovery of valuable thermostable enzymes. In addition to five previously described cellulolytic enzymes, the *A. cellulolyticus* genome revealed many additional possibilities for biomass degradation. The *A. cellulolyticus* genome encodes genes for several enzymes that break down cellulose and xylans, while the absence of pectin degradation genes corroborates the reported lack of growth on pectin (Mohagheghi et al. 1986). The organism devotes about equal numbers of enzymes to the breakdown of cellulose (10 genes) and xylan (seven genes) in the plant cell wall, as well as chitin and other components in fungal cell walls (10 genes), and the metabolism of storage carbohydrates such as glycogen and trehalose (eight genes). This suggests that all these carbon sources are of comparable importance to the organism.

Complete enzymatic digestion of cellulose requires three types of glycosyl hydrolases, including cellulases (endoglucanases), cellobiohydrolases (exoglucanases), and cellobiosidases (beta-glucosidases). All three are present in multiple copies in the *A. cellulolyticus* genome. Specifically, there are six endoglucanases, two exoglucanases, and two beta-glucosidases. Efficient hydrolysis of crystalline cellulose requires the presence of at least

Table 4. Relative proportions of each nucleotide at each of the three codon positions in six actinobacteria

Organism	OGT (°C)	Nucleotide and codon base position											
		Position 1 (5'-end)				Position 2 (middle)				Position 3 (3'-end)			
		A	C	G	T	A	C	G	T	A	C	G	T
<i>A. cellulolyticus</i> 11B	55	0.362	0.280	0.425	0.235	0.457	0.291	0.213	0.533	0.181	0.429	0.362	0.232
<i>Frankia alni</i> ACN14	28	0.388	0.267	0.413	0.255	0.514	0.278	0.213	0.621	0.098	0.455	0.374	0.124
<i>Frankia</i> sp. Ccl3	27	0.382	0.277	0.408	0.247	0.487	0.282	0.216	0.580	0.131	0.441	0.376	0.173
<i>S. avermitilis</i> MA-4680	28	0.384	0.261	0.412	0.274	0.518	0.269	0.206	0.617	0.098	0.469	0.382	0.109
<i>S. coelicolor</i> A3(2)	30	0.381	0.258	0.417	0.275	0.534	0.264	0.208	0.644	0.086	0.478	0.375	0.081
<i>T. fusca</i> YX	50	0.357	0.272	0.424	0.256	0.481	0.265	0.212	0.591	0.163	0.463	0.364	0.153
R-squared value		0.900	0.331	0.885	0.342	0.631	0.085	0.024	0.521	0.795	0.196	0.854	0.484
P-value less than		0.004	0.232	0.005	0.223	0.059	0.575	0.772	0.105	0.017	0.380	0.008	0.125

Regression (R-squared) and P-values were calculated using the R software. A P-value <0.05 is considered significant.

Figure 4. Plot of the G+C content of noncoding RNAs (rRNA + tRNAs) versus the G+C of genome in prokaryotes. The following shapes and shades are used for distinguishing the organisms: black circles, hyperthermophiles; dark gray circles, thermophiles; open circles, mesophiles; filled squares, psychrophiles; inverted gray triangle, *A. cellulolyticus*; gray triangle, *T. fusca*; inverted open triangles, two *Frankia* sp. (ACN14a, Ccl3), and open triangles, two *Streptomyces* sp. (*S. avermitilis*, *S. coelicolor*). Black lines represent the regression line and 95% confidence intervals, computed for the mesophiles.

one endoglucanase and two types of exoglucanases. The *Acidothermus* genome contains both a reducing-end-specific GH48 exoglucanase and a nonreducing-end-specific GH6 exocellulase (Ding et al. 2003).

Based on sequence similarity of the *A. cellulolyticus* Acel_0129 protein to a characterized endo-1,3-beta-glucanase from *Streptomyces siayaensis*, we predict that this protein binds to and hydrolyzes 1,3-beta-D-glucan, a major constituent of fungal cell walls and laminarins of certain algal groups and diatoms (Hong et al. 2002). This enzyme likely helps the organism assimilate fungal cell walls as a food source. The functions of four putative chitinases remain to be confirmed experimentally. The capability to degrade chitin could permit degradation of fungal and insect biomass. After cellulose, chitin is the second most abundant structural cell wall polymer in nature. Unlike other eukaryotic cell-wall biopolymers, chitin contains nitrogen and hence could be used as a carbon and nitrogen source. Decaying plant matter as well as dead insects that fall into the thermal pools may provide sources of chitin and 1,3-beta-D-glucan. The ability to utilize a range of carbon sources could offer a survival edge under limiting nutritional conditions in the thermal pool. Chitinases have received increased attention recently owing to their wide applications in the agricultural, medical, and food industries. The potential for a source of thermostable chitinases elevates the industrial importance of *A. cellulolyticus* beyond its anticipated applications in cellulosic biofuel technologies.

The fact that secreted plant biomass-degrading enzymes in *A. cellulolyticus* contain two different types of CBM domains, from families 2 and 3, is interesting functionally as well as evolutionarily. Only 10 complete bacterial genomes, including *A. cellulolyticus*, encode both CBM types, of which six are Actinobacteria and one a Firmicute (<http://www.cazy.org>). This relatively low frequency suggests that the coexistence of both types of CBM domains is rare. Among these 10 genomes, there is a clear preference for either CBM2 (in Actinobacteria) or CBM3 (in the Firmicutes) but not for both. The *A. cellulolyticus* genome with equal

proportions of the two CBM types is clearly an exception to the pattern to date. The coexistence of CBM2 and CBM3 domains in a majority of the *A. cellulolyticus* modular enzymes as well as their restricted organization may suggest functional and/or thermostability constraints. It is possible that the presence of CBM3 alone or its location C-terminal to CBM2 may either destabilize the protein or affect the optimal activity of *A. cellulolyticus* enzymes. The high degree of sequence conservation within the two CBM families in *A. cellulolyticus* suggests duplication of each of these domains after speciation. Fusion of these duplicated domains to the GHs could indicate a selective pressure for localizing the secreted GHs on specific substrates. Both CBM2 and CBM3 bind predominantly to cellulose, with experimental evidence for binding to chitin in a few cases (Boraston et al. 2004). A few CBM2 members have also been observed to bind xylan (Boraston et al. 2004). Whether the two families of CBM domains in *A. cellulolyticus* bind cellulose, xylan or chitin, or multiple substrates remains to be determined functionally.

The *A. cellulolyticus* genome revealed three laterally acquired GIs characterized by a lower G+C content and a deviation from the genomic signature. Regions that deviate significantly from the genomic signature are thought to have been laterally transferred (Karlin 2001). In addition, the fact that the three islands are either flanked by tRNA genes and/or lack homologs in other actinobacteria strongly suggests that these DNA regions have been horizontally acquired in *A. cellulolyticus*. Several genes in these islands show highest sequence similarity to proteins from thermophilic organisms. Analysis of the genes encoded within the three GIs suggests a functional role for the acquired genes in the context of the organism's ecology. Aryldialkyl phosphatase (encoded on GI1) catalyzes the hydrolysis of an aryl-dialkyl phosphate to form dialkyl phosphate and an aryl alcohol. In cellulosytic fungi, aryl-alcohol dehydrogenase activity has been implicated in lignolysis (Reiser et al. 1994). GI2 carries homologs of the *vrl* genes found preferentially associated with more virulent isolates of *D. nodosus* and that are proposed to have been acquired horizontally possibly

Figure 5. Reduced dimensionality plot of PCA of amino acid usage in ribosomal proteins in 409 prokaryotes. The following shapes and shades are used for distinguishing the organisms: black circles, hyperthermophiles; dark gray circles, thermophiles; open circles, mesophiles; black squares, psychrophiles; inverted gray triangle, *A. cellulolyticus*; gray triangle, *T. fusca*; inverted open triangles, two *Frankia* sp. (ACN14a, Ccl3), and open triangles, two *Streptomyces* sp. (*S. avermitilis*, *S. coelicolor*).

from a bacteriophage or a plasmid (Billington et al. 1999). Although the precise function of the *vrl* locus is unclear, many of these genes could be involved in DNA restriction and modification, offering immunity to *A. cellulolyticus* against phage infection, similar to the *S. coelicolor* phage-resistance Pgl system (Sumbly and Smith 2002), which bears sequence similarity to the Vrl proteins. GI3 contains genes that may be involved in amino acid transport and metabolism as well as genes for three subunits of the CO dehydrogenase family. Homologs also occur in other actinobacteria such as *Arthrobacter* and *Mycobacteria* that have been shown to grow chemolithotrophically on CO as the sole carbon and energy source under aerobic conditions (Meyer and Schlegel 1983; Park et al. 2003), suggesting that a similar potential may be present in *A. cellulolyticus*. Since CO dehydrogenases share high sequence similarity with xanthine dehydrogenases, it is difficult to predict whether the *A. cellulolyticus* homologs function in carbon fixation or in purine salvage. However, either of these possibilities would add eco-physiological value for *A. cellulolyticus*.

Thermophilic adaptations have not been systematically examined within the actinobacteria, an ecologically diverse yet relatively understudied bacterial group. *A. cellulolyticus* grows optimally at 55°C, while most of its closest phylogenetic relatives are mesophilic. The use of PCA, or the similar technique, correspondence analysis (CA), to study the genomes of hyperthermophilic, thermophilic, and mesophilic prokaryotes has facilitated identification of their thermoadaptation characteristics (Kreil and Ouzounis 2001; Lynn et al. 2002; Singer and Hickey 2003; Suhre and Claverie 2003). Contrary to our expectations based on these previous studies, in our PCA results, neither the genome nor the proteome of *A. cellulolyticus* segregates with other thermophiles. The degree of separation along PC2 axis that correlates with OGT may suggest how recently a thermophile has evolved. In that case, the lack of unambiguous separation of *A. cellulolyticus* from mesophiles along PC2 could reflect the relatively short history of *A. cellulolyticus* in thermal pools, as its genome and proteome still show mesothermophilic features. This pattern suggests a recent and ongoing adaptation to the thermophilic environment. Alternatively, *A. cellulolyticus* may have evolved unique mechanisms of thermotolerance.

The subtle increase in the G and A nucleotides at the first and third codon positions, respectively, in the *A. cellulolyticus* genes could enhance thermostability of its mRNAs by probabilistically increasing the frequency of AG dinucleotides in its mRNAs, by a plausible increase in the frequency of NNA-GNN di-codons. The ApG dinucleotides are thought to stabilize DNA because of their low stacking energy and have been observed to occur at higher frequency in (hyper)thermophilic organisms compared to mesophiles (Zeldovich et al. 2007). The relatively lower frequency of AGG codons in *A. cellulolyticus* may, in turn, be due to the inverse purine preferences at the first and third codon positions and may explain the lack of separation of *A. cellulolyticus* from the mesophiles, along PC2 in our PCA (see Supplemental Fig. S3A,B). The AGG codon is known to strongly influence the separation between thermophiles and mesophiles (Lynn et al. 2002; Singer and Hickey 2003). *A. cellulolyticus* is clearly an exception in the use of AGG codons compared to other thermophiles.

The *A. cellulolyticus* proteome contained an elevated fraction of IVYWREL amino acids compared to both *Frankia* sp. and *Streptomyces* sp. A recently identified positive correlation between the total fraction of seven amino acids (Ile, Val, Tyr, Trp, Arg, Glu, Leu) in prokaryotic proteomes and the OGT of the organisms is another measure for thermoadaptation (Zeldovich et al. 2007).

Usage patterns of either the 20 individual amino acids (as studied using PCA) or the total fraction of IVYWREL amino acids likely represent alternative yet overlapping thermophilic signatures. This is because most hyperthermophiles and thermophiles separate well along the OGT axis in PCA and also contain a relatively elevated content of IVYWREL residues in their proteomes. Interestingly, *A. cellulolyticus* appears to show the latter but not the former thermophilic signature. It is possible that the elevated IVYWREL content in the proteome represents an overarching adaptation to thermophily and that usages of individual amino acids get fine-tuned with evolutionary time. The higher IVYWREL content in conserved *A. cellulolyticus* proteins compared to their orthologs in mesophilic actinobacteria rules out the possibility that the differences in IVYWREL residues in the proteome and cytosolic subproteome are due to a few proteins with skewed amino acid composition. This suggests that this biased amino acid usage in the *A. cellulolyticus* proteome may be reflective of its adaptation to the thermal environment. It is worth noting that there have been no findings of proteins unique to thermophiles that explain organismal adaptations to high temperature, and that proteins in thermophiles show biased amino acid compositions compared to orthologs in mesophiles (Takami et al. 2004).

Adaptation to thermophily is likely to be a slow and continuous process. Although the overall *A. cellulolyticus* proteome revealed no clear thermophilic tendency, a more detailed analysis revealed a preference for thermophilic amino acid usage in its ribosomal proteins. These results taken together with the fact that ribosomal proteins are essential for cellular viability, and that ribosomal RNAs and transfer RNAs in *A. cellulolyticus* contain distinct thermophilic features, suggest that evolution of a thermotolerant protein translation machinery may be an important early step in thermoadaptation. It has been reported that three characterized strains of *A. cellulolyticus* have different OGT (Mohagheghi et al. 1986). Conceivably, other strains of *A. cellulolyticus* that span a range of either lower or higher OGT exist in nature. Perhaps, the isolation of such strains in the future and the availability of genome sequence from multiple *A. cellulolyticus* strains may shed further light on genomic evolutionary processes for thermophilic adaptation.

Methods

Strains, culture, and DNA extraction

A. cellulolyticus 11B was grown at University of California, Davis, from DMSO stocks maintained and provided by the National Renewable Energy Laboratory (NREL, Golden, CO), derived from the original isolate of Mohagheghi et al. (1986). Cells were grown in shaking or rolling liquid cultures at 55°C, in LPBM medium (Mohagheghi et al. 1986) (also called ATCC medium 1473), pH 5.5, modified such that the carbon source was 0.25 g/L cellobiose + 0.25 g/L glucose, without cellulose. For isolation of high-molecular-weight genomic DNA from *A. cellulolyticus*, a protocol was devised to reduce the extensive nuclease activity: Cell pellets were suspended in 200 µL of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, preheated to 37°C) with 10 µL of lysozyme (100 mg/mL; MP Biomedicals), and incubated for 2 h at 37°C; 1200 µL of ATL solution (QIAGEN) plus 200 µL of protease K (10 mg/mL; QIAGEN) were added, followed by incubation for 2.5 h at 55°C. The supernatant was extracted with phenol-chloroform and chloroform, and DNA was precipitated, air-dried, and resuspended as in Sambrook et al. (1989). Genomic DNA was stored at -20°C

in the presence of 0.1 mg/mL RNase I (Promega), and its integrity was verified on 0.5% agarose gel.

Sequencing, gene prediction, and annotation

The *A. cellulolyticus* 11B genome (NCBI Record: NC_008578) was sequenced and annotated by the Joint Genome Institute, U.S. Department of Energy. Large (40 kb), medium (8 kb), and small (3 kb) insert DNA libraries were sequenced using the random shotgun method with an average success rate of 96% and average high-quality read lengths of 685 nucleotides (nt). After the shotgun stage, reads were assembled with parallel phrap (High Performance Software, LLC). Possible mis-assemblies were corrected with Dupfinisher (C. Han, unpubl.) or a transposon bomb of bridging clones (EZ-Tn5 <P6Kyor/KAN-2> Tnp Transposome kit; Epicentre Biotechnologies). Gaps between the contigs were closed by editing, custom primer walks, or PCR amplification. The completed genome sequence of *A. cellulolyticus* contains 59,147 reads, achieving an average of 18-fold sequence coverage per base with an error rate of <1 in 100,000. Automated annotation steps were performed as described previously (Chain et al. 2003).

Data acquisition

Genome sequence files, executable BLAST (Altschul et al. 1997) programs, and the “nr” database were obtained from the NCBI ftp site. In order to build a comprehensive data set spanning the entire known range of OGTs for our PCA analyses, we extracted all complete prokaryotic genome sequences available in the NCBI genome database, without making any a priori choice of the species to be included in our analyses. OGT information was extracted from the American Tissue Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ). Organisms with unknown OGT were removed, and our final data set contained 409 prokaryotes (Supplemental Table S7), including 17 hyperthermophilic species (OGT $\geq 80^\circ\text{C}$), 19 thermophilic species (OGT between 55°C and 80°C), 369 mesophiles (OGT between 20°C and 55°C), and four psychrophiles (OGT < 20°C).

To extract ribosomal proteins, we scanned the annotations of the complete genomes listed in the NCBI ftp sites for the following terms: “ribosomal,” “50S,” “30S,” “SSU,” or “LSU.” We then manually checked the annotations retrieved with this method, and we removed hits that did not correspond to ribosomal proteins per se (e.g., “ribosomal large subunit pseudouridine synthase D”).

Sequence analyses

The percent G+C of the genome and the noncoding RNAs were calculated from nucleotide sequences in the respective NCBI files. Short Perl codes were written and used for specific computational tasks, such as for calculating G+C in DNA and RNA sequences, amino acid composition of proteins, codon usage, and the like. The total fraction of IVYWREL residues was calculated by combining the fractions of the seven individual amino acids. The relative proportions of each nucleotide at each codon position were calculated from the codon usage tables. The genomic signature was calculated as described by Karlin (2001). The organization of flagellar genes in the different actinobacteria was obtained using the tools available on the Integrated Microbial Genomics (IMG) server (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>) (Markowitz et al. 2006).

All *A. cellulolyticus* proteins were searched against the nr database using the standalone BLASTP program, and the distri-

bution of organisms with the best hit was calculated from the BLAST results. Bidirectional top BLAST hits were used to identify the 478 conserved proteins (Supplemental Table S8) in six organisms listed in Table 1. Similarly, 46 orthologous proteins (Supplemental Table S9) were identified common to 45 completely sequenced actinobacteria.

Principal component analysis (PCA)

The amino acid compositions of ribosomal proteins from 409 prokaryotes with known OGTs were subjected to PCA using the R statistical software (<http://www.r-project.org/>). Global and synonymous codon usage in the genomes and amino acid usage in the whole proteomes of the 409 prokaryotes were also analyzed using PCA (see Supplemental Material). All statistical analyses were performed using the inbuilt functions in the R package (<http://www.r-project.org/>).

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