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¹³C metabolic flux analysis of three divergent extremely thermophilic bacteria: *Geobacillus* sp. LC300, *Thermus thermophilus* HB8, and *Rhodothermus marinus* DSM 4252

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Abstract

Thermophilic organisms are being increasingly investigated and applied in metabolic engineering and biotechnology. The distinct metabolic and physiological characteristics of thermophiles, including broad substrate range and high uptake rates, coupled with recent advances in genetic tool development, present unique opportunities for strain engineering. However, poor understanding of the cellular physiology and metabolism of thermophiles has limited the application of systems biology and metabolic engineering tools to these organisms. To address this concern, we applied high resolution ¹³C metabolic flux analysis to quantify fluxes for three divergent extremely thermophilic bacteria from separate phyla: *Geobacillus* sp. LC300, *Thermus thermophilus* HB8, and *Rhodothermus marinus* DSM 4252. We performed 18 parallel labeling experiments, using all singly labeled glucose tracers for each strain, reconstructed and validated metabolic network models, measured biomass composition, and quantified precise metabolic fluxes for each organism. In the process, we resolved many uncertainties regarding gaps in pathway reconstructions and elucidated how these organisms maintain redox balance and generate energy. Overall, we found that the metabolisms of the three thermophiles were highly distinct, suggesting that adaptation to growth at high temperatures did not favor any particular set of metabolic pathways. All three strains relied heavily on glycolysis and TCA cycle to generate key cellular precursors and cofactors. None of the investigated organisms utilized the Entner-Doudoroff pathway and only one strain had an active oxidative pentose phosphate pathway. Taken together, the results from this study provide a solid foundation for future model building and engineering efforts with these and related thermophiles.

Keywords

Parallel labeling experiments; metabolic model validation; thermophiles; metabolic flux analysis

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1. INTRODUCTION

Extreme thermophiles are microorganisms that grow optimally at temperatures at or above 75 °C. Research on extremely thermophilic organisms has intensified in the past two decades, driven by technological advances in whole-genome sequencing, genetic tool development, and other breakthroughs in omics sciences (Counts et al., 2017). Given the unique physiological and metabolic features of thermophiles, they are increasingly investigated for their biotechnological potential in areas such as bioremediation (Feng et al., 2007), as a source of thermostable enzymes (Elleuche et al., 2014; Canakci et al., 2012; Ezeji and Bahl, 2006; Verma et al., 2013), and as novel platforms for the production of biofuels and chemicals from renewable feedstocks (Taylor et al., 2009; Chang and Yao, 2011). Key advantages of thermophiles include their broad substrate range, i.e. the ability to use simple and complex carbohydrates including cellulose, hemicellulose and xylans (Lynd, 1989; Blumer-Schuetz et al., 2008; Cordova et al., 2016); high specific substrate uptake rates and growth rates (Cordova et al., 2015; Marchant et al., 2002), which can be several-fold higher compared to mesophiles such as *E. coli* (Long et al., 2017a); and the ability to survive and maintain metabolic activity under harsh process conditions. Additionally, from a bioprocessing perspective, high process temperatures provide important economic benefits, including reduced cooling costs, reduced chances of contamination, easier processing of feedstocks, and the potential to integrate fermentation and product recovery (Lin and Xu, 2013; Lynd, 1989).

Extreme thermophiles are found in two of the three domains of life: bacteria and archaea. In the past decade, many genomes of extreme thermophiles have been sequenced and metabolic network models have been reconstructed using automated genome annotation tools. Based on these data, it is becoming apparent that the metabolic capabilities of extreme thermophiles vary greatly. Thermophiles can be aerobes, anaerobes, autotrophs, or heterotrophs. However, for the most part, the metabolism of extremely thermophilic organisms is still poorly understood. One reason is that enzymes from thermophiles have diverged evolutionarily from their mesophilic counterparts, resulting in weak homologies and thus making genome annotations challenging and error-prone. As a result, network model reconstructions contain many gaps and it is often unclear which metabolic pathways are present or absent.

To address this concern and advance our understanding of thermophile physiology, in this study we investigated the metabolism of three divergent extremely thermophilic aerobic bacteria from three separate phyla: *Geobacillus* sp. LC300 (from the phylum Firmicutes), *Thermus thermophilus* HB8 (from the phylum Deinococcus-Thermus), and *Rhodothermus marinus* DSM 4252 (from the phylum Bacteroidetes). We were particularly interested in investigating if the extreme conditions under which these organisms have evolved led to evolutionarily convergent or divergent metabolic features. To address this question, we applied a ¹³C-metabolic flux analysis (¹³C-MFA) approach called COMPLETE-MFA (Leighty and Antoniewicz, 2013) (short for complementary parallel labeling experiments technique for metabolic flux analysis) to validate network models for the three organisms and quantify precise metabolic fluxes (Crown et al., 2016). Using this technique, we resolved many questions regarding metabolic network structure and quantified pathway

fluxes. Overall, we found that the metabolic features of the three extreme thermophiles were highly distinct, suggesting that evolution at high temperatures did not favor any particular pathway. Looking forward, the validated network models and flux data generated in this study will serve as a valuable resource for strain engineering and model building efforts of these and related thermophiles.

2. MATERIALS AND METHODS

2.1. Materials

Media and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Glucose tracers, [1-¹³C], [2-¹³C], [3-¹³C], [4-¹³C], [5-¹³C], and [6-¹³C]glucose (99% ¹³C) were purchased from Cambridge Isotope Laboratories (Andover, MA). Wolfe's minerals (Cat. No. MD-TMS) and Wolfe's vitamins (Cat. No. MD-VS) were purchased from ATCC (Manassas, VA). Tris solution (1 mol/L) was purchased from Cellgro (Cat. No. 46-031-CM). Yeast extract was purchased from Fisher (Cat. No. BP-1422-500, lot 068366). Glucose stock solutions (20 wt%) and yeast extract stock solution (1 wt%) were prepared in distilled water. The base growth medium for all strains contained (per liter of medium): 0.50 g K₂HPO₄, 0.30 g KH₂PO₄, 0.50 g NH₄Cl, 0.50 g NaCl, 0.20 g MgCl₂·6H₂O, 0.04 g CaSO₄·2H₂O, 40 mL of 1 M Tris, 5 mL of Wolfe's minerals, 5 mL of Wolfe's vitamins, and 0.05 g/L of yeast extract. For growth of *Rhodothermus marinus*, the base growth medium was supplemented with 1% NaCl. Glucose was added as indicated in the text. All solutions were sterilized by filtration.

2.2. Strains and growth conditions

Thermus thermophilus HB8 (ATCC 27634) and *Rhodothermus marinus* DSM 4252 (ATCC 43812) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The *Geobacillus* strain LC300 was described in Cordova *et al.* (2015). For parallel labeling experiments, cells from -80 °C frozen stock were first pre-grown in medium containing 2 g/L of unlabeled glucose. Next, 50 µL of this pre-culture was used to inoculate six culture tubes containing 10 mL of growth medium with one of six ¹³C-glucose tracers, [1-¹³C], [2-¹³C], [3-¹³C], [4-¹³C], [5-¹³C], or [6-¹³C]glucose. The optical density (OD₆₀₀) of the inoculated cultures was about 0.01. Cells were then grown aerobically in custom mini-bioreactors as described previously (Swarup *et al.*, 2014). *Thermus thermophilus* HB8 was grown at 72 °C with 1.6 g/L glucose; *Rhodothermus marinus* DSM 4252 was grown at 77 °C with 2.5 g/L glucose; and *Geobacillus* sp. LC300 was grown at 72 °C with 3.2 g/L glucose. A high-precision multichannel peristaltic pump (Watson Marlow, Wilmington, MA) was used to control the air flow to the mini-bioreactors, which was set at 11 mL/min. Gas flow rates were monitored by a digital flow-meter (Supelco, Veri-Flow 500). Mixing in the mini-bioreactors was achieved through the rising gas bubbles, and a constant culture temperature was maintained by placing the tubes in a heating block (Fisher Isotemp Digital Dry-Bath 125D) (Cordova and Antoniewicz, 2016). Samples for isotopic labeling analysis were collected during mid-exponential growth phase when OD₆₀₀ was between 0.5 and 1.0.

2.3. Analytical methods

Samples were collected at multiple times during the exponential growth phase to monitor cell growth, substrate uptake and acetate production. Biomass concentration was determined by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Eppendorf BioPhotometer). The OD_{600} values were converted to cell dry weight concentrations using experimentally determined OD_{600} -dry cell weight relationships (using the techniques described in Long et al., 2016b): *Thermus thermophilus* HB8 ($1.0 OD_{600} = 0.34 \text{ g}_{\text{DW}}/\text{L}$), *Rhodothermus marinus* DSM 4252 ($1.0 OD_{600} = 0.32 \text{ g}_{\text{DW}}/\text{L}$), and *Geobacillus* sp. LC300 ($1.0 OD_{600} = 0.27 \text{ g}_{\text{DW}}/\text{L}$). Glucose concentration was determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH), and acetate concentration was determined using an Agilent 1200 Series HPLC (Gonzalez et al., 2017).

2.4. Gas chromatography-mass spectrometry

GC-MS analysis was performed on an Agilent 7890B GC system equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm -phase thickness; Agilent J&W Scientific), connected to an Agilent 5977A Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230°C, the MS quad temperature at 150°C, the interface temperature at 280°C, and the inlet temperature at 250°C. GC-MS analysis of *tert*-butyldimethylsilyl (TBDMS) derivatized proteinogenic amino acids was performed as described in (Antoniewicz et al., 2007a). Labeling of glucose in the medium was determined after aldonitrile propionate derivatization as described in (Antoniewicz et al., 2011; Sandberg et al., 2016). Labeling of fatty acids was determined after derivatization to fatty acid methyl esters (FAME) (Crown et al., 2015). Labeling of glucose (derived from glycogen) and ribose (derived from RNA) were determined as described in (Long et al., 2016a; McConnell and Antoniewicz, 2016). In all cases, mass isotopomer distributions were obtained by integration (Antoniewicz et al., 2007a) and corrected for natural isotope abundances (Fernandez et al., 1996).

2.5. Biomass composition analysis

Biomass composition was determined by GC-MS using the methods described in (Long and Antoniewicz, 2014b). Briefly, samples were prepared by three respective methods: hydrolysis of protein and subsequent TBDMS derivatization of amino acids; hydrolysis of RNA and glycogen and subsequent aldonitrile propionate derivatization of sugars (ribose and glucose, respectively); and hydrolysis of lipids and methyl ester derivatization for analysis of fatty acids. Quantification of all components was achieved by isotope ratio analysis using an isotopically labeled standard and unlabeled biomass sample. The isotopically labeled standard in this study was fully ^{13}C -labeled *E. coli* that was generated by growing *E. coli* on fully labeled $[\text{U}-^{13}\text{C}]$ glucose as described in (Long and Antoniewicz, 2014b).

2.6. Metabolic network models and ^{13}C -metabolic flux analysis

The metabolic network models used for ^{13}C -metabolic flux analysis (^{13}C -MFA) are provided in Supplemental Materials. For each organism, a core metabolic network model was constructed based on the pathways and reactions annotated in the KEGG and BioCyc

databases (Caspi et al., 2012; Kanehisa et al., 2012; Kanehisa and Goto, 2000). Because of uncertainties in genome annotations, several additional pathways were also included in the ^{13}C -MFA models, including the Entner-Doudoroff pathway, oxidative pentose phosphate pathway (not annotated in *Geobacillus* and *Thermus*), malic enzyme (not annotated in *Rhodothermus*), as well as alternative pathways for isoleucine, lysine and glycine biosynthesis (see results section for further details). The lumped biomass reaction for each organism was based on the measured biomass composition. To describe fractional labeling of metabolites, G-value parameters were included in the ^{13}C -MFA models. As described previously (Antoniewicz et al., 2007c), the G-value represents the fraction of a metabolite pool that is produced during the labeling experiment from glucose, while $1-G$ represents the fraction that is naturally labeled, i.e. from the incorporation of unlabeled amino acids from the yeast extract and from the inoculum. By default, one G-value parameter was included for each measured metabolite in each data set. The models also accounted for the dilution of intracellular labeling by incorporation of atmospheric unlabeled CO_2 (Leighty and Antoniewicz, 2012). Reversible reactions were modeled as separate forward and backward fluxes. Net and exchange fluxes were determined as follows: $v_{\text{net}} = v_f - v_b$; $v_{\text{exch}} = \min(v_f, v_b)$.

All flux calculations were performed using the Metran software (Yoo et al., 2008) which is based on the elementary metabolite units (EMU) framework (Antoniewicz et al., 2007b). Fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the experimentally measured and model predicted mass isotopomer distributions of biomass amino acids (from proteins), glucose (from glycogen), and ribose (from RNA) using non-linear least-squares regression (Antoniewicz et al., 2006). All mass isotopomers that were used for ^{13}C -MFA calculations are provided in Supplemental Materials. For integrated analysis of parallel labeling experiments, all six data sets for each organism were fitted simultaneously to a single flux model as described in (Crown et al., 2016) and (Antoniewicz, 2015). Flux estimation was repeated 10 times starting with random initial values for all fluxes to find a global solution. At convergence, accurate 95% confidence intervals were computed for the estimated fluxes by evaluating the sensitivity of the minimized SSR to flux variations. Precision of estimated fluxes was determined as follows (Antoniewicz et al., 2006):

$$\text{Flux precision (stdev)} = [(\text{flux}_{\text{upper bound 95\%}}) - (\text{flux}_{\text{lower bound 95\%}})] / 4$$

2.7. Goodness-of-fit analysis

To determine the goodness-of-fit, the ^{13}C -MFA fitting results were subjected to a χ^2 -statistical test. In short, assuming that the model is correct and data are without gross measurement errors, the minimized SSR is a stochastic variable with a χ^2 -distribution (Antoniewicz et al., 2006). The number of degrees of freedom is equal to the number of fitted measurements n minus the number of estimated independent parameters p . The acceptable range of SSR values is between $\chi^2_{\alpha/2}(n-p)$ and $\chi^2_{1-\alpha/2}(n-p)$, where α is a certain chosen threshold value, for example 0.05 for the 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1. Growth physiology and optimal growth conditions

Growth characteristics and optimal growth conditions were determined for *Geobacillus* sp. LC300, *Thermus thermophilus* HB8, and *Rhodothermus marinus* DSM 4252 in aerobic batch culture. First, various C6 and C5 sugars were evaluated as carbon sources, including glucose, galactose, mannose, xylose, and arabinose (Table 1). All three strains grew well on glucose (Supplemental Figure S1); *Geobacillus* and *R. marinus* also grew on galactose and xylose; and *Geobacillus* and *T. thermophilus* grew on mannose. None of the strains displayed growth on arabinose. In all experiments, the same growth medium was used for growing *Geobacillus* and *T. thermophilus*, and the medium was supplemented with 1% NaCl for growing the marine bacterium *R. marinus*, which could not grow without NaCl supplementation (Table 2). A small amount of yeast extract (0.05 g/L) was also added to all media to eliminate a lag phase that was sometimes observed when subculturing cells into fresh medium. The presence of the yeast extract did not impact the strains' substrate uptake rates or growth rates. The optimal growth temperature for *Geobacillus* sp. LC300 and *T. thermophilus* was around 75°C, and around 80°C for *R. marinus* (Table 2).

A more detailed characterization of the growth physiology was performed for growth on glucose (Table 3). During the exponential growth phase, the specific growth rate of *Geobacillus* sp. LC300 was $2.15 \pm 0.07 \text{ h}^{-1}$ (doubling time 19 min). To our knowledge, this is the fastest growth rate reported for any organism on glucose medium. The specific growth rates of *T. thermophilus* ($0.22 \pm 0.02 \text{ h}^{-1}$, doubling time 190 min) and *R. marinus* ($0.51 \pm 0.03 \text{ h}^{-1}$, doubling time 80 min) were significantly lower. The biomass yields were similar for *Geobacillus* sp. LC300 and *R. marinus* ($0.39 \pm 0.02 \text{ g}_{\text{DW}}/\text{g}$ and $0.38 \pm 0.03 \text{ g}_{\text{DW}}/\text{g}$, respectively) and slightly lower for *T. thermophilus* ($0.33 \pm 0.02 \text{ g}_{\text{DW}}/\text{g}$). During the exponential growth phase, *Geobacillus* sp. LC300 produced acetate as a by-product ($0.38 \pm 0.03 \text{ mol}$ of acetate produced per mol of glucose consumed), while no by-products were detected in the cultures of *T. thermophilus* and *R. marinus*.

3.2. Metabolic model construction and biomass composition analysis

To facilitate quantitative studies of metabolism, models of core metabolism were constructed for the three organisms based on reactions annotated in the KEGG and BioCyc databases. Metabolic network models play a central role in metabolic engineering and systems biology, as they are the basis for wide range of computational design and analysis approaches, including metabolic flux analysis (MFA) (Long and Antoniewicz, 2014a), constraint-based reconstruction and analysis (COBRA) approaches (Becker et al., 2007), and strain design algorithms such as OptKnock and others (Burgard et al., 2003; King et al., 2015). In this study, we aimed to experimentally validate the network models of the three thermophiles using isotope tracing and ^{13}C -MFA (Gonzalez and Antoniewicz, 2017).

The models used in this study for ^{13}C -MFA are provided in Supplemental Materials. The models included all major pathways of central carbon metabolism, lumped amino acids biosynthesis pathways, and a lumped biomass formation reaction. Fig. 1 shows the reconstructed central carbon metabolism for the three organisms. Based on current

annotations, the three organisms contain many well-known metabolic pathways such as glycolysis, pentose phosphate pathway, TCA cycle, glyoxylate shunt, and various anaplerotic and cataplerotic reactions. *R. marinus* has a complete oxidative pentose phosphate pathway (oxPPP), while *Geobacillus* sp. LC300 is missing the second enzyme in oxPPP (EC 3.1.1.31), and *T. thermophilus* is missing the first two oxPPP enzymes (EC 1.1.1.49 and EC 3.1.1.31). The Entner-Doudoroff pathway was not complete in the three organisms. Additionally, based on current annotations, *R. marinus* is believed to be missing a malic enzyme (EC 1.1.1.38). In order to experimentally validate the presence or absence of these reactions, all were included in the ^{13}C -MFA models so that fluxes through them could be estimated.

An important reaction in ^{13}C -MFA models is the lumped biomass reaction that captures the drain of precursor metabolites and cofactors needed for cell growth. To determine the coefficients in this reaction for each strain, biomass compositions were determined experimentally using the methods described in Long and Antoniewicz (2014b). The results of these analyses are provided in Supplemental Materials and are shown in Fig. 2, where the biomass composition of the three thermophiles is also compared to the composition of the model mesophilic microbe *E. coli*, as previously reported (Long et al., 2016b). Proteins were the most abundant component in all strains (ranging from 46% of dry weight in *R. marinus*, to 60% in *T. thermophilus*), followed by RNA, fatty acids and glycogen. The RNA content of the fast-growing *Geobacillus* LC300 strain was especially high (28%), compared to the other two thermophiles (12–13%). It has been observed previously that RNA content is generally higher for faster growing strains (Long et al., 2016b), which is thought to reflect the need for more ribosomes to support higher growth rates. *R. marinus* was unique among the three thermophilic strains in that it had relatively high amounts of fatty acids (16%, compared to 4–10% for the other strains) and glycogen (14%, compared to 1–4% for the other strains). The relative distribution of fatty acids was similar for the three thermophiles, with the most abundant fatty acids being fully saturated even-chain (C16:0 and C18:0) and odd-chain fatty acids (C17:0 and C15:0). No unsaturated fatty acids were detected in the three thermophiles, in contrast with *E. coli* where the mono-unsaturated fatty acids C16:1 and C18:1 are abundant. The relative distributions of amino acids in biomass were also relatively similar for all organisms, with a few notable exceptions, e.g. relatively low abundances of isoleucine and aspartate/asparagine and relatively high abundances of leucine and glycine in *T. thermophilus*; and relatively high abundances of proline in *T. thermophilus* and *R. marinus*.

3.3. ^{13}C Metabolic flux analysis

Precise intracellular fluxes for the three thermophilic organisms were determined during growth on glucose using the ^{13}C -MFA approach called COMPLETE-MFA (Leighty and Antoniewicz, 2013). The analysis consisted of performing six parallel labeling experiments for each organisms, using all singly labeled ^{13}C -glucose tracers (i.e. [1- ^{13}C], [2- ^{13}C], [3- ^{13}C], [4- ^{13}C], [5- ^{13}C], and [6- ^{13}C]glucose), and simultaneously fitting the isotopic labeling data from proteinogenic amino acids, the ribose moiety of RNA, and the glucose moiety of glycogen to the network models described in the previous section. Statistically acceptable fits were obtained for all three strains. The minimized sum of squared residuals

for *Geobacillus* LC300 (SSR = 224), *T. thermophilus* (SSR = 575), and *R. marinus* (SSR = 564) were lower than the maximum acceptable SSR value of 804 at 95% confidence level, assuming a constant measurement error of 0.4 mol% for all GC-MS measurements. The estimated metabolic fluxes and 95% flux confidence intervals for all fluxes are provided in Supplemental Materials.

Fig. 3 shows the estimated fluxes in central metabolism for *Geobacillus* sp. LC300, *T. thermophilus*, and *R. marinus* during aerobic growth on glucose (fluxes were normalized to glucose uptake rate of 100). The flux map of *Geobacillus* sp. LC300 was characterized by high glycolytic flux (62 ± 0), and moderate activities of oxPPP (37 ± 0), TCA cycle (31 ± 1), anaplerosis from pyruvate to oxaloacetate (32 ± 2), and acetate secretion (38 ± 2). Inactive (or nearly inactive) pathways included the ED pathway (0 ± 0), glyoxylate shunt (1 ± 1), malic enzyme (3 ± 2), and phosphoenolpyruvate carboxykinase (4 ± 1). The flux map of *T. thermophilus* was characterized by two main active metabolic pathways, glycolysis (98 ± 0) and TCA cycle (85 ± 3), while many other pathways were inactive (or nearly inactive), including: oxPPP (0 ± 0), ED pathway (0 ± 0), glyoxylate shunt (0 ± 1), and malic enzyme (1 ± 1). ^{13}C -MFA revealed a futile cycle in *T. thermophilus* that was the result of simultaneous activity of phosphoenolpyruvate carboxylase (51 ± 1 , $\text{PEP} \rightarrow \text{oxaloacetate} + \text{CO}_2$) and phosphoenolpyruvate carboxykinase (28 ± 1 , $\text{oxaloacetate} + \text{CO}_2 + \text{ATP} \rightarrow \text{PEP}$). At each turn of this cycle 1 ATP is consumed. The flux map of *R. marinus* was similar to that of *T. thermophilus*, with two main active pathways, i.e. glycolysis (90 ± 0) and TCA cycle (58 ± 1), and several inactive (or nearly inactive) pathways, including: oxPPP (2 ± 0), ED pathway (0 ± 0), glyoxylate shunt (5 ± 1), malic enzyme (0 ± 0), and phosphoenolpyruvate carboxykinase (0 ± 0). Overall, the ^{13}C -MFA results provided supporting evidence for the genome annotations described in the previous section, e.g. absence of the ED pathway in all three organisms, absence of malic enzyme in *R. marinus*, and absence of oxPPP in *T. thermophilus*. Although no gene encoding for 6-phosphogluconolactonase (EC 3.1.1.31) was identified in the genome of *Geobacillus* sp. LC300, ^{13}C -MFA predicted a significant oxPPP flux for this organism, which is also evident from inspection of RNA labeling (Supplemental Figure S2). Tang et al (2009a) also described an active oxPPP in a related *Geobacillus thermoglucosidasius* strain. These results may be explained by the fact that the 6-phosphogluconolactonase reaction is well known to proceed spontaneously (Kupor and Fraenkel, 1969; Thomason et al., 2004; Zimenkov et al., 2005), especially at the elevated temperatures under which *Geobacillus* was grown.

3.4. Elucidating amino acid biosynthesis pathways

In addition to elucidating gaps in central carbon metabolism, we also focused on elucidating gaps in amino acid biosynthesis pathways. Figures 4A–C show the annotated metabolic pathways for biosynthesis of lysine, threonine, serine, glycine, and isoleucine, for the three organisms. Based on current knowledge, *Geobacillus* sp. LC300 was expected to utilize the classical bacterial amino acid pathways (i.e. similar to *E. coli*). The ^{13}C -MFA results (Fig. 4D) were consistent with this hypothesis. Lys was produced exclusively from Asp and Pyr via the classical diaminopimelate (DAP) pathway, Ser was derived from 3PG, and Ile was produced from Thr. There was no flux from Thr to Gly (0.2 ± 0.1), consistent with the absence of threonine aldolase (EC 4.1.2.48), and no flux from Pyr and AcCoA to Ile via the

citramalate pathway (0.2 ± 0.1), consistent with the absence of citramalate synthase (EC 2.3.1.182). For *T. thermophilus*, the ^{13}C -MFA results were also largely consistent with the current pathway annotations (Fig 4E). For example, Lys was produced exclusively from AKG and AcCoA via the yeast-like lysine biosynthesis pathway that was previously described for this organism (Kobashi et al., 1999; Nishida et al., 1999; Miyazaki et al., 2001), Ser was produced from 3PG, and Gly was produced both from Ser and Thr, consistent with the presence of both glycine hydroxymethyltransferase (EC 2.1.2.1) and threonine aldolase (EC 4.1.2.48). Interestingly, the ^{13}C -MFA results suggested that the majority of Ile was produced via the citramalate pathway in *T. thermophilus*, although no citramalate synthase (the first enzyme in this pathway) has been identified so far in *T. thermophilus*. The same was true for *R. marinus*, where the majority of Ile was derived via the citramalate pathway with no known citramalate synthase. The other amino acid pathway fluxes for *R. marinus* were consistent with the current genome annotation, i.e. Lys was exclusively produced via the classical bacterial DAP lysine biosynthesis pathway, Ser was derived from 3PG, and Gly was produced both from Ser and Thr (Fig 4F).

The suggested presence of citramalate synthase in *T. thermophilus* and *R. marinus* was investigated in more detail. In past studies, citramalate synthase has been described mainly in anaerobic organisms (Tang et al., 2009b; Tang et al., 2010; Feng et al., 2009; Feng et al., 2010; Au et al., 2014). It is known that citramalate synthase is sometimes incorrectly annotated as 2-isopropylmalate synthase (EC 2.3.3.13) (Au et al., 2014), a closely related enzyme that is part of the leucine biosynthesis pathway. To identify which genes in *T. thermophilus* and *R. marinus* could be coding for the citramalate synthase, BLASTp analysis was performed. The results of this analysis are summarized in Fig 5. First, BLASTp analysis was performed against the known 2-isopropylmalate synthase from *E. coli* (b0074) (Fig 5D). *T. thermophilus* had one gene with a strong homology to b0074 (TTHA1210) and one gene with a moderate homology (TTHA1208); *R. marinus* also had one gene with a strong homology (Rmar_0740) and one gene with a moderate homology (Rmar_0743); in contrast, *Geobacillus* sp. LC300 only had one gene with a strong homology (IB49_05170). Next, BLASTp analysis was performed using the protein sequences of TTHA1208 (from *T. thermophilus*) and Rmar_0743 (from *R. marinus*) against the entire NCBI protein database. This time, many of the top hits were well-known citramalate synthases. Additional inspection of the gene location of TTHA1208 and Rmar_0743 within their respective genomes revealed that in both organisms these genes resided in an operon where the other genes coded for enzymes of the isoleucine biosynthesis pathway (Fig 5A). In summary, the ^{13}C -MFA and BLASTp analysis results, as well as location of the genes within the genome, all strongly suggested that TTHA1208 in *T. thermophilus* and Rmar_0743 in *R. marinus* code for citramalate synthase (EC 2.3.1.182), the first enzyme of the citramalate pathway for isoleucine biosynthesis (Fig 5B).

3.5. Redox and energy metabolism

To provide additional insights into the physiology and metabolism of the three thermophiles, we analyzed the production and consumption rates of key co-factors in metabolism based on the ^{13}C -MFA estimated fluxes, and calculated the overall carbon balance. In Fig. 6, the results are summarized (see Supplemental Materials for additional details). The overall

carbon balance was similar for the three thermophiles, with the majority of glucose being converted to biomass (47–60%), and the remaining part to CO₂ (28% for *Geobacillus* LC300, and 41–49% for *T. thermophilus* and *R. marinus*) and acetate (12% for *Geobacillus* LC300 only). Fig. 6B shows the normalized (per mol of glucose consumed) production and consumption rates of NADH/FADH₂, NADPH and ATP, with contributions by the various pathways (absolute contributions in units of mmol/g_{DW}/h are shown in Supplemental Materials). For *Geobacillus* sp. LC300, NADH was produced about equally via glycolysis and TCA cycle and the majority of NADH was oxidized to generate ATP via oxidative phosphorylation. For *T. thermophilus* and *R. marinus*, the TCA cycle produced >2-fold more NADH than glycolysis, resulting in greater amounts of NADH oxidation and ATP generation via oxidative phosphorylation. The NADPH metabolism was significantly different for the three organisms. For *Geobacillus* sp. LC300, the majority of NADPH was produced via oxPPP, while for *T. thermophilus* the majority of NADPH was produced in the TCA cycle, and for *R. marinus* the majority of NADPH was produced by transhydrogenase. For *Geobacillus* sp. LC300 the net transhydrogenase flux was estimated to be nearly zero. Based on current genome annotation, *Geobacillus* sp. LC300 is believed to have no transhydrogenase genes, while *T. thermophilus* and *R. marinus* both have a single transhydrogenase gene (EC 1.6.1.2). Analysis of the ATP fluxes revealed that energy management was relatively similar for the three organisms, with ATP being mainly produced via oxidative phosphorylation in all three organisms, with additional contribution from substrate-level phosphorylation in glycolysis. The ATP was mainly utilized for cell growth, maintenance, and substrate uptake. It should be noted that the calculated ATP production rates and maintenance costs here should be viewed with caution, since little information is available about the effective P/O ratios for the three thermophiles studies here. For simplicity, we assumed the same P/O ratio of 1.5 for all organisms. Once more reliable estimates of P/O ratios are available, the ATP analysis results reported here should be re-evaluated.

4. CONCLUSIONS

Thermophiles are promising organisms with a wide range of potential biotechnological applications. In this study, the physiology and metabolism of three extremely thermophilic bacteria, *Geobacillus* sp. LC300, *Thermus thermophilus* HB8, and *Rhodothermus marinus* DSM 4252, were elucidated in detail using state-of-the-art ¹³C-flux analysis. One of the investigated thermophiles, *Geobacillus* sp. LC300, is the fastest known organism on glucose (doubling time 19 min), with a growth rate and substrate uptake rate that is >3-fold higher than the workhorse organism *E. coli* (doubling time ~60 min), and 30–40% higher than the fast-growing marine bacterium *Vibrio natriegens* (Long et al., 2017b). The physiological and fluxomic data presented in this study will serve as important foundational components for future model building and strain engineering efforts. For example, the verified intracellular metabolic pathways and flux maps will enable the use of COBRA model-based analysis and design tools to engineer these thermophilic organisms and related strains for biotechnological applications.

This study also clearly demonstrates the power and importance of ¹³C-MFA for model validation. For example, based on genome annotations alone, our initial hypothesis was that

of the three investigated organisms *R. marinus* would be the only one to utilize the oxidative pentose phosphate pathway (oxPPP). Instead, ¹³C-MFA results revealed that only *Geobacillus* sp. LC300 had an active oxPPP, and that this pathway played an important role in the overall redox (NADPH) balance for this organism. The ¹³C-MFA results also allowed us to resolve many model inconsistencies and fill in gaps in e.g. pathways for amino acid biosynthesis. For example, citramalate synthase was identified as playing an important role in the biosynthesis of isoleucine in both *T. thermophilus* and *R. marinus*. We hope that the validated network models described in this study will enable more accurate genome annotations in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Central metabolic pathways of *Geobacillus* sp. LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252, based on current genome annotations. EC numbers of identified enzymes are shown.

Figure 2.

Biomass composition of *Geobacillus* sp. LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252. The fractional amounts of major biomass components were determined, as well as the distributions of fatty acids and amino acids.

Figure 3.

Metabolic flux maps for *Geobacillus* sp. LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252 during aerobic growth on glucose. Fluxes were determined using ^{13}C -MFA by simultaneously fitting labeling data from six parallel labeling experiments for each organism. Fluxes were normalized to glucose uptake rate for each organism. Complete flux results are provided in Supplemental Materials.

Figure 4. (A–C) Metabolic pathways for biosynthesis of selected amino acids in *Geobacillus* sp. LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252, based on current genome annotations. EC numbers of identified genes (blue arrows) and apparently missing enzymes (red arrows) are shown. (D–F) Estimated metabolic fluxes, determined using ^{13}C -MFA by simultaneously fitting labeling data from six parallel labeling experiments for each organism. Fluxes shown here were normalized to glucose uptake rate. Complete flux results are provided in Supplemental Materials.

Figure 5.

(A) Identification of putative citramalate synthases (EC 2.3.1.182) in *Thermus thermophilus* HB8 (TTHA1208) and *Rhodothermus marinus* DSM 4252 (Rmar_0743). (B) Citramalate pathway for biosynthesis of isoleucine from pyruvate and acetyl-CoA. (C) Fractional contributions of the citramalate pathway and threonine pathway towards biosynthesis of isoleucine. Fluxes were determined using ^{13}C -MFA by simultaneously fitting labeling data from six parallel labeling experiments for each organism. (D) BLASTp homology analysis of 2-isopropylmalate synthase genes (i.e. b0074 from *E. coli*; IB49_05170 from *Geobacillus* sp. LC300; TTHA1210 from *Thermus thermophilus* HB8; and Rmar_0740 from *Rhodothermus marinus* DSM 4252) and putative citramalate synthase genes (i.e. TTHA1208 from *Thermus thermophilus* HB8; and Rmar_0743 from *Rhodothermus marinus* DSM 4252).

Figure 6.

Overview of carbon and cofactor balances for *Geobacillus* sp. LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252. (A) The overall carbon balance reflects the fate of glucose on a C-mol basis. (B) Metabolic pathways responsible for the production and consumption of the cofactors NADH/FADH₂, NADPH, and ATP are summarized, on a normalized (per unit glucose) basis. “Other” in the NADPH panel represents the contribution of malic enzyme to NADPH production. “Other” in the ATP panel represents the estimated ATP maintenance cost. The absolute production rates from oxidative phosphorylation and the calculated maintenance cost are based on an assumed effective P/O ratio of 1.5 for all three organisms. Since the P/O ratios have not been reliably measured for these organisms, the results shown here should be interpreted with caution.

Table 1

Growth rates of *Geobacillus* LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252 determined in aerobic batch cultures on different C6 and C5 sugars.

Sugar	<i>Geobacillus</i> LC300 ^a	<i>Thermus thermophilus</i> HB8 ^b	<i>Rhodothermus marinus</i> DSM 4252 ^c
Glucose	2.15 h ⁻¹	0.22 h ⁻¹	0.51 h ⁻¹
Galactose	0.83 h ⁻¹	No growth	0.54 h ⁻¹
Mannose	1.15 h ⁻¹	0.16 h ⁻¹	No growth
Xylose	1.52 h ⁻¹	No growth	0.58 h ⁻¹
Arabinose	No growth	No growth	No growth

^aThe growth medium contained 30 g/L of the respective sugar and 0.05 g/L of yeast extract. The growth temperature was 72°C (data taken from Cordova et al, 2015).

^bThe growth medium contained 2 g/L of the respective sugar and 0.05 g/L of yeast extract. The growth temperature was 72°C.

^cThe growth medium contained 2 g/L of the respective sugar, 0.05 g/L of yeast extract and 1% NaCl. The growth temperature was 77°C.

Table 2

Effects of various growth conditions on the growth phenotypes of *Geobacillus* LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252 in aerobic batch cultures with glucose as the carbon source.

Condition	<i>Geobacillus</i> LC300	<i>Thermus thermophilus</i> HB8	<i>Rhodothermus marinus</i> DSM 4252
0.05% NaCl	++	++	–
1% NaCl	+	W	++
2% NaCl	+	–	+
4% NaCl	–	–	+
6% NaCl	–	–	w
75°C	++	++	+
80°C	–	+	++
85°C	–	+	–

“++” indicates optimal growth condition; “+” indicates good growth; “w” indicates weak growth, defined as growth rate less than half of maximum growth rate; and “–” indicates no growth.

Table 3

Growth characteristics of *Geobacillus* LC300, *Thermus thermophilus* HB8 and *Rhodothermus marinus* DSM 4252 in aerobic batch culture with glucose as the carbon source.

Sugar	<i>Geobacillus</i> LC300	<i>Thermus thermophilus</i> HB8	<i>Rhodothermus marinus</i> DSM 4252
Growth rate	$2.15 \pm 0.07 \text{ h}^{-1}$	$0.22 \pm 0.02 \text{ h}^{-1}$	$0.51 \pm 0.03 \text{ h}^{-1}$
Biomass yield	$0.39 \pm 0.02 \text{ g}_{\text{DW}}/\text{g}$	$0.33 \pm 0.02 \text{ g}_{\text{DW}}/\text{g}$	$0.38 \pm 0.03 \text{ g}_{\text{DW}}/\text{g}$
Acetate yield	$0.38 \pm 0.03 \text{ mol/mol}$	Not detected	Not detected
Glucose uptake rate	$30.6 \text{ mmol}/\text{g}_{\text{DW}}/\text{h}$	$3.7 \text{ mmol}/\text{g}_{\text{DW}}/\text{h}$	$7.5 \text{ mmol}/\text{g}_{\text{DW}}/\text{h}$