

## L-Alanine Production from Glucose Fermentation by Hyperthermophilic Members of the Domains *Bacteria* and *Archaea*: a Remnant of an Ancestral Metabolism?

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New members of the order *Thermotogales* were isolated from nonvolcanically heated geothermal environments, including oil fields and waters of the Great Artesian Basin of Australia, thereby extending their known habitats, previously recognized primarily as volcanic. The hyperthermophilic and thermophilic members of *Thermotogales* of volcanic origin, together with the recently described nonvolcanic species of this order and three new isolates described in this paper, were all found to produce L-alanine from glucose fermentation, in addition to acetate, lactate, CO<sub>2</sub> and H<sub>2</sub>. L-Alanine production from glucose is a trait in common with *Pyrococcus furiosus* and *Thermococcus profundus*. We propose that L-alanine production from sugar fermentation be regarded as an ancestral metabolic characteristic.

Members of the order *Thermotogales* were first found in natural ecosystems associated with active volcanism (14, 20). Recently, the range of their known habitat was extended to nonvolcanically heated geothermal environments such as oil fields and subsurface aquifers (2, 10, 15, 21). In order to improve our knowledge of the biodiversity of the *Thermotogales*, we conducted studies aimed at isolating new strains from such environments. Three new strains were isolated from oil fields and the Great Artesian Basin of Australia. They were phylogenetically placed as members of the *Thermotogales* (Fig. 1) and designated *Thermotoga* strain SEBR 7054, *Thermotoga* strain SEBR 2665, and *Fervidobacterium gondwanense* AB39<sup>T</sup>. Their isolation indicates that there is greater phylogenetic diversity and distribution of the *Thermotogales* than was previously recognized (11). Interestingly, our screening did not record the presence of *Fervidobacterium* species in oil fields and conversely did not record the presence of *Thermotoga* species in the artesian waters of Australia, though the genera were reported to coexist in volcanic hot springs (11). This distribution of *Thermotogales* genera is intriguing and may perhaps be due to the absence of elemental sulfur in nonvolcanic geothermal environments; elemental sulfur may serve as a common electron acceptor for growth in volcanic environments (3, 18). However, the growth of the *Thermotogales* in nonvolcanic environments could also result from an adaptive fermentative process.

During our studies of glucose-grown cultures of members of the *Thermotogales*, not only did we detect acetate, lactate, CO<sub>2</sub>, and H<sub>2</sub> as fermentation end products, as already reported by other researchers (11), but we also observed an unidentified compound. This, together with our inability to calculate accu-

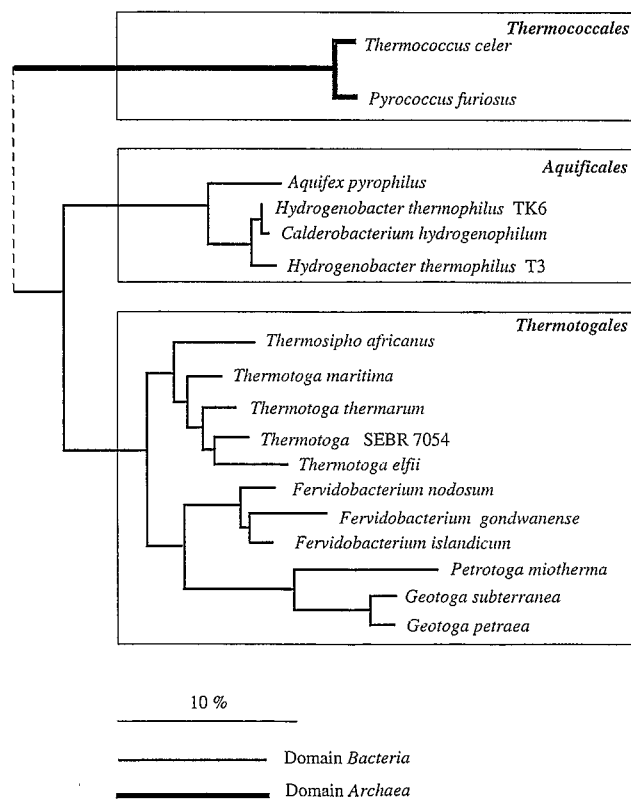


FIG. 1. Dendrogram, based on 16S rRNA gene sequence analysis, showing the phylogenetic positions of two new members (*F. gondwanense* and *Thermotoga* strain SEBR 7054) among the *Thermotogales* and related bacteria. The sequences used in the analysis were obtained from the Ribosomal Database Project, version 4.1, and from the GenBank database (for *Hydrogenobacter thermophilus* [Z30214], *Calderobacterium hydrogenophilum* [Z30242], and *Hydrogenobacter* strain TS3 [Z30189]). The sequences of *Thermotoga* strain SEBR 7054 and *T. profundus* are unpublished. Bars indicate evolutionary distance.

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TABLE 1. Acetate and L-alanine production from glucose metabolism by members of the order *Thermotogales* in the absence or presence of thiosulfate<sup>a</sup>

Species or strain <sup>b</sup>	Growth condition <sup>c</sup>	OD <sub>580</sub> <sup>d</sup>	Amt of glucose consumed (mmol)	Amt of product (mmol) <sup>e</sup>				Ratio	
				Acetate	L-Alanine	H <sub>2</sub>	H <sub>2</sub> S	Acetate/Glc	Alanine/Glc
Group I									
<i>F. islandicum</i> <sup>f</sup>	Glc	0.84	14.20	6.25	3.98	21.58	ND	0.44	0.28
	Glc + thio	0.96	16.20	20.25	1.22	ND	34.02	1.25	0.08
<i>T. africanus</i>	Glc	0.29	7.20	7.92	3.74	16.77	ND	1.10	0.52
	Glc + thio	0.36	7.70	12.40	0.00	ND	14.55	1.61	0.00
<i>T. elfii</i>	Glc	0.10	2.75	3.49	1.05	7.70	ND	1.27	0.38
	Glc + thio	0.30	8.15	12.63	0.41	ND	14.55	1.55	0.05
<i>Thermotoga</i> strain SEBR 7054	Glc	0.30	8.60	4.49	1.71	29.03	ND	0.52	0.20
	Glc + thio	0.91	14.39	19.70	1.06	2.29	15.08	1.37	0.07
Group II									
<i>T. maritima</i>	Glc	0.28	13.70	15.62	0.69	36.09	ND	1.14	0.05
	Glc + thio	0.73	13.55	15.99	0.81	4.02	14.45	1.18	0.06
<i>T. neapolitana</i>	Glc	0.57	14.00	18.27	0.87	31.67	ND	1.30	0.06
	Glc + thio	0.94	13.90	16.12	0.60	16.07	7.39	1.16	0.04
<i>Thermotoga</i> strain SEBR 2665	Glc	0.61	12.20	15.74	0.61	33.63	ND	1.29	0.05
	Glc + thio	1.11	15.50	19.22	0.51	1.23	18.77	1.24	0.03

<sup>a</sup> Type cultures including *T. maritima*, *T. neapolitana*, *T. africanus*, and *F. islandicum* were obtained from the Deutsche Sammlung von Mikroorganismen, *Ferrobacterium nodosum* was purchased from the American Type Culture Collection, and *Thermotoga elfii* was from our culture collection. They all were grown on a glucose-based medium (16) but the concentration of both yeast extract and bio-Trypcase was decreased to 1 g · liter<sup>-1</sup>. Fermentation end products were analyzed as described previously (15), and L-alanine was measured by high-pressure liquid chromatography (12).

<sup>b</sup> Group I, strains which produced more than 0.2 mol of L-alanine per mol of glucose consumed; group II, strains which produced less than 0.2 mol of L-alanine per mol of glucose consumed.

<sup>c</sup> Glc, glucose; thio, thiosulfate (20 mM).

<sup>d</sup> OD<sub>580</sub>, optical density at 580 nm. Optical densities of control cultures were subtracted.

<sup>e</sup> ND, not detected.

<sup>f</sup> *F. nodosum* and *F. gondwanense* also produced L-alanine from glucose fermentation.

rate carbon recoveries and oxidation-reduction balances from the analyzed data, indicated that an end product was still unaccounted for. This compound was later identified as L-alanine. The amount of L-alanine produced per mole of glucose consumed permitted us to divide the tested *Thermotogales* into two broad groups (Table 1). Thermophiles within group I—viz., *Ferrobacterium islandicum*, *Thermosiphon africanus*, *Thermotoga elfii*, and *Thermotoga* strain SEBR 7054—produced more than 0.2 mol of L-alanine per mol of glucose, whereas hyperthermophiles of group II—viz., *Thermotoga neapolitana*, *Thermotoga maritima*, and the thermophile *Thermotoga* strain SEBR 2665—produced less than 0.2 mol of L-alanine per mol of glucose consumed.

Our studies further indicated that several *Thermotoga* isolates from oil fields, including *T. elfii* (15) and *Thermotoga* strain SEBR 7054, utilized thiosulfate but not sulfur as an electron acceptor. In contrast, all isolates from volcanic hot springs utilized thiosulfate or sulfur except *Thermotoga thermarum*, which is currently not reported to use sulfur compounds as electron acceptors (11). The addition of thiosulfate to the carbohydrate-containing growth medium is known to improve the growth rate and cell yields of the tested *Thermotogales* (16). Data in Table 1 provide further evidence that the use of thiosulfate as an electron acceptor changed the end-product profile of glucose metabolism by the thermophiles *F. islandicum*, *T. africanus*, *T. elfii*, and *Thermotoga* strain SEBR 7054. For both groups I and II, the addition of thiosulfate reduced the H<sub>2</sub> concentration with a concomitant increase in H<sub>2</sub>S concentration (Table 1). However, the L-alanine concen-

tration decreased drastically and the acetate concentration increased for members of group I when thiosulfate was added. A similar behavior was described for *Pyrococcus furiosus* grown in the presence of elemental sulfur (8). On the other hand, in group II, there was no significant change in L-alanine and acetate production when thiosulfate was added, thus indicating a possible involvement of the oxidative phosphorylation process in glucose metabolism. The reason for the much stronger effect of thiosulfate on glucose metabolism in members of group I than group II could be related to the different H<sub>2</sub> sensitivities that members of each group have (1, 6).

Phylogenetic studies, determination of physiological properties, and sequence comparisons of ferredoxins suggested that *T. maritima* (domain *Bacteria*, order *Thermotogales*) was closely related to members of the domain *Archaea* and in particular to *P. furiosus* (4). Both *P. furiosus* and *Thermococcus profundus*, which belong to the order *Thermococcales*, one of the deepest branches within the domain *Archaea*, have been reported to produce L-alanine from carbohydrate fermentation (8, 9). Our findings provide further evidence that the *Thermotogales*, also one of the deepest branches within the domain *Bacteria*, are closely related to the *Thermococcales* (Fig. 1). Therefore, L-alanine production can be regarded as an ancestral metabolism. Both *T. maritima* and *P. furiosus* are known to ferment glucose via the Embden-Meyerhof pathway (7, 19). In *P. furiosus*, L-alanine production results from alanine aminotransferase activity. Coupling of alanine aminotransferase with glutamate dehydrogenase was ascertained by Kengen and Stams (8). Whether this mechanism is operational in members of the

*Thermotogales* has yet to be investigated. It is known that glutamate dehydrogenase is the key enzyme which links the carbon and nitrogen metabolic pathways in *P. furiosus* during carbohydrate fermentation (17). It is possible that a similar situation exists in all members of the *Thermotogales*; this is worthy of further investigation. L-Alanine production from sugar fermentation has been reported for the intestinal parasite *Giardia lamblia* (5), the genetically modified bacterium *Zymomonas mobilis* (22), and more recently a moderately thermophilic *Clostridium* species (13). On the basis of our report, it can be proposed that L-alanine production from sugar fermentation may be more widespread among the three domains of bacteria than previously believed.

**Nucleotide sequence accession number.** The 16S rRNA sequence for *F. gondwanense* AB39<sup>T</sup> has been deposited in the EMBL database under accession number Z49117.

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