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ORIGINAL ARTICLE

Multiple syntrophic interactions in a terephthalate-degrading methanogenic consortium

This paper was corrected on 21st December 2010 to include contributing authors inadvertently ommitted from the original version of the paper

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Terephthalate (TA) is one of the top 50 chemicals produced worldwide. Its production results in a TA-containing wastewater that is treated by anaerobic processes through a poorly understood methanogenic syntrophy. Using metagenomics, we characterized the methanogenic consortium inside a hyper-mesophilic (that is, between mesophilic and thermophilic), TA-degrading bioreactor. We identified genes belonging to dominant *Pelotomaculum* species presumably involved in TA degradation through decarboxylation, dearomatization, and modified β-oxidation to H_2/CO_2 and acetate. These intermediates are converted to CH_4/CO_2 by three novel hyper-mesophilic methanogens. Additional secondary syntrophic interactions were predicted in *Thermotogae*, *Syntrophus* and candidate phyla OP5 and WWE1 populations. The OP5 encodes genes capable of anaerobic autotrophic butyrate production and *Thermotogae*, *Syntrophus* and WWE1 have the genetic potential to oxidize butyrate to CO_2/H_2 and acetate. These observations suggest that the TA-degrading consortium consists of additional syntrophic interactions beyond the standard H_2-producing syntroph–methanogen partnership that may serve to improve community stability.
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Introduction

Terephthalate (TA) is used as the raw material for the manufacture of numerous plastic products (for example, polyethylene TA bottles and textile fibers). During its production, TA-containing wastewater is discharged in large volumes (as high as 300 million m^3 per year) and high concentration (up to 20 kg COD (chemical oxygen demand) m^{-3}) (Razo-Flores *et al.*, 2006). This wastewater is generally treated by anaerobic biological processes under mesophilic conditions (~35 °C). However, anaerobic processes operated at hyper-mesophilic (46–50 °C) and thermophilic (~55 °C) temperatures may be preferable because of the ability to achieve higher loading rate (van Lier *et al.*, 1997; Chen *et al.*, 2004), which reduces the reactor volume. Moreover, TA wastewater is usually generated at 54–60 °C, and does not require additional energy input for maintaining reactor temperature (Chen *et al.*, 2004). The microbial biomass usually occurs in the form of granules or biofilms attaching on the surface of porous media. Under such environments, TA degradation has been hypothesized (Kleerebezem *et al.*, 1999) to be based on a syntrophic microbial relationship whereby fermentative H_2-producing bacteria (syntrophs) convert TA through benzoate to acetate and H_2/CO_2, and acetoclastic and hydrogenotrophic methanogens further convert the intermediates to methane by physically positioning themselves close to the syntrophs to overcome the

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thermodynamic barrier (Stams, 1994; Conrad, 1999; Dolfing, 2001).

In practice, the complexities of TA-degrading communities are not as well known. The communities require a long maturation phase (>200–300 days), are difficult to maintain, and do not always result in a successful syntrophic interaction. If the syntrophic interaction is disturbed and the treatment rendered ineffective, the resulting high-concentration effluent must be treated with a more energy-intensive down-stream aerobic biological process. These factors can significantly increase the operational cost of the process and limit its application on a wider scale.

Several studies have investigated the microbial populations present in methanogenic TA degradation, often using laboratory-scale reactors operated at various temperatures (Kleerebezem *et al.*, 1999; Wu *et al.*, 2001; Chen *et al.*, 2004). Using ribosomal RNA (rRNA)-based molecular methods, these studies have found that TA-degrading consortia in bioreactors are dominated by two to three bacterial populations and two types of methanogens (Wu *et al.*, 2001; Chen *et al.*, 2004). The methanogens are relatively straightforward to classify being mainly acetoclastic *Methanosaeta*-related species and a novel hydrogenotrophic methanogenic species in the family *Methanomicrobiales*. The syntrophic bacteria, however, are difficult to identify based on phylogenetic classification, and extremely difficult to obtain in pure culture. In the last decade, only three bacterial species that can degrade TA and its isomers have been successfully co-cultured with methanogens under mesophilic conditions (Qiu *et al.*, 2006, 2008), and these isolates are different from those found under thermophilic conditions (Chen *et al.*, 2004). This greatly limits the effort to understand the microbial interaction and function in the TA-degrading consortia.

A metagenome analysis was chosen for this study since it has been proven as an effective method for retrieving nearly complete microbial genomes of dominant populations in relatively simple microbial ecosystems (Tyson *et al.*, 2004; Martin *et al.*, 2006). In particular, this study aims to elucidate the microbiology underpinning anaerobic TA-degrading processes, including improved knowledge of the diversity and physiology of participating syntrophs and methanogens, and the mechanism behind the establishment and maintenance of the partnership. This knowledge may lead to the generation of principles, which could be applied to establish different consortia for treating other chemicals discharged from industrial production lines, or to treat contaminants in other environments.

Materials and methods

The anaerobic microbial consortium that degrades TA was selectively enriched using a 1-l laboratory-scale hybrid bioreactor (Figure 1a) as described previously (Angelidaki *et al.*, 1990; Chen *et al.*, 2004) (see Supplementary Text). Biomass was collected from only porous packing filters on days 221 and 280, and from both filters and sludge bed on days 346 and 430 for further analyses. These biomass samples were used for genomic DNA extraction, library construction and sequencing according to standard protocols (http://www.jgi.doe.gov/sequencing/protocols/prots_production.html) (see Supplementary Text). Detailed metagenome analysis methods are described in the Supplementary Text. Data can be accessed through the Integrated Microbial Genome/Microbiome (http://img.jgi.doe.gov/m/) system.

Results and discussion

Bioreactor operation and performance

An anaerobic hybrid reactor was successfully operated with TA as the only carbon and energy source for 480 days. This reactor was constructed with an upper section filled with ring-shape porous filters to support the growth of microbial biofilms, and a lower section for the development of anaerobic granular sludge (Figure 1a). This reactor is unusual and novel, in that it was the first methanogenic reactor operated in the hyper-mesophilic temperature zone (46–50 °C), whereas previously published studies of TA-degrading communities were at mesophilic (~35 °C) or thermophilic (~55 °C) temperatures (Kleerebezem *et al.*, 1999; Wu *et al.*, 2001; Chen *et al.*, 2004). After achieving a good TA removal efficiency (Figure 1b), sludge samples were taken from the surface of the filter media at days 221, 280, 346 and 430. Samples were also taken from the sludge bed at days 346 and 430. These samples taken were used for 16S rRNA and metagenome analysis.

16S rRNA-based community profiling

The rarefaction curve analysis indicates that the bacterial population diversity is much higher in terms of operational taxonomic unit number than the archaeal population diversity in any given sample taken from the TA reactor (Supplementary Figure 1). The phylogenetic distribution of bacterial 16S rRNA clones (Figure 2) indicates that *Peptococcaceaea* (mostly *Pelotomaculum*), *Thermotogae*, *Syntrophaceae*, and candidate phyla OP5 and WWE1 were the dominant bacterial lineages present. Between the biofilm samples (rings 1, 2, 3 and 5) and sludge bed samples (rings 4 and 6) taken, differences in the abundances of major phyla including *Firmicutes*, *Thermotogae*, *Proteobacteria* and OP5 were observed. These differences are likely attributed to the differences in growth temperature (46 °C vs 50 °C) and form (biofilms vs granules). Archaeal representatives were less diverse and consisted of two major types of methanogens belonging to the orders *Methanomicrobiales* and *Methanosarcinales* (Supplementary Figure 2). Differences in growth temperature may further

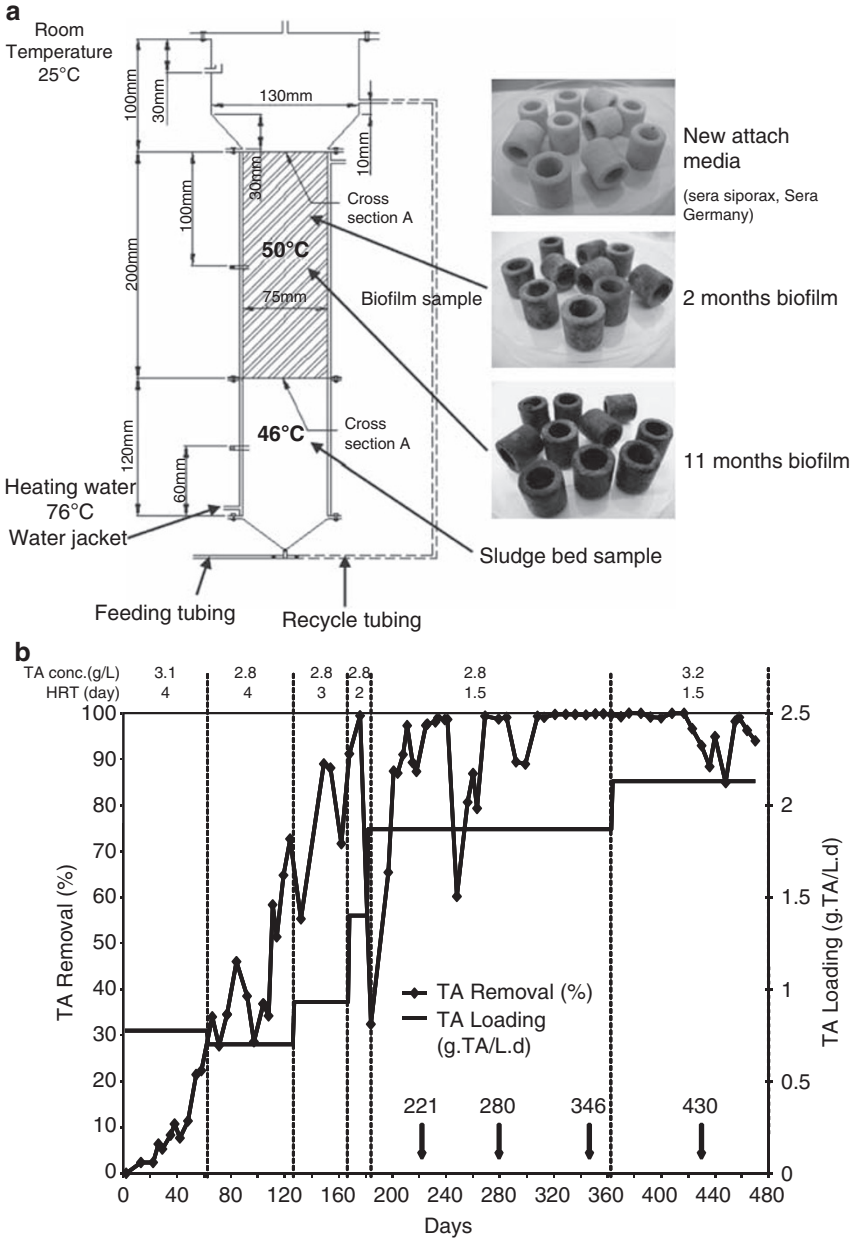

Figure 1 TA-degrading laboratory-scale anaerobic hybrid bioreactor. (**a**) Schematic of the laboratory-scale anaerobic TA-degrading hybrid reactor operated with a temperature gradient from ~46 °C at the bottom to 50 °C in the upper zone. Inserts illustrate freshly grown biofilm biomass on the surface of the media after 2 and 11months of enrichment; and (**b**) performance of the reactor over 480-day operation. Under the initial operational conditions (that is, TA-loading rate of 0.70–0.78 gTA/d.l, and hydraulic retention time (HRT) of 4 days), TA removal efficiency was gradually improved to 72.7% by day 124. By shortening the HRT (3 d on day 127, 2 d on day 168 and then to 1.5 d on day 182) and increasing the TA loading concentration (to 3.2 g on day 364), the TA loading rate was increased to 2.13 gTA/d·l by day 364. Concurrently, the TA removal efficiency increased over the operation period reaching a 99% removal efficiency by day 308. During the entire operation no sulfate reduction activity was detected. Samples were removed at the indicated time points (arrows) and the genomic DNA was extracted for 16S rRNA clone library construction and metagenomics analysis.

explain the variations observed in the microbial populations enriched in previous studies (Supplementary Figure 3). Using the 16S rRNA gene and *McrA* gene as biomarkers, temperature-dependent variations were also observed with acetoclastic methanogen populations found in the order *Methanosarcinales* (Supplementary Figure 4). The hydrogenotrophic methanogens identified here are closely related to methanogens found in mesophilic and thermophilic TA-degrading reactors (Wu *et al.*, 2001; Chen *et al.*, 2004), and together with *Methanolinea tarda* NOBI-1 recently isolated from anaerobic digestion processes (Imachi *et al.*, 2008), form a novel cluster separate from other known hydrogenotrophic methanogens. The comparison of *McrA* genes further suggests that the methanogens found in the new cluster are likely different from *M. tarda* NOBI-1 (Supplementary Figure 4B).

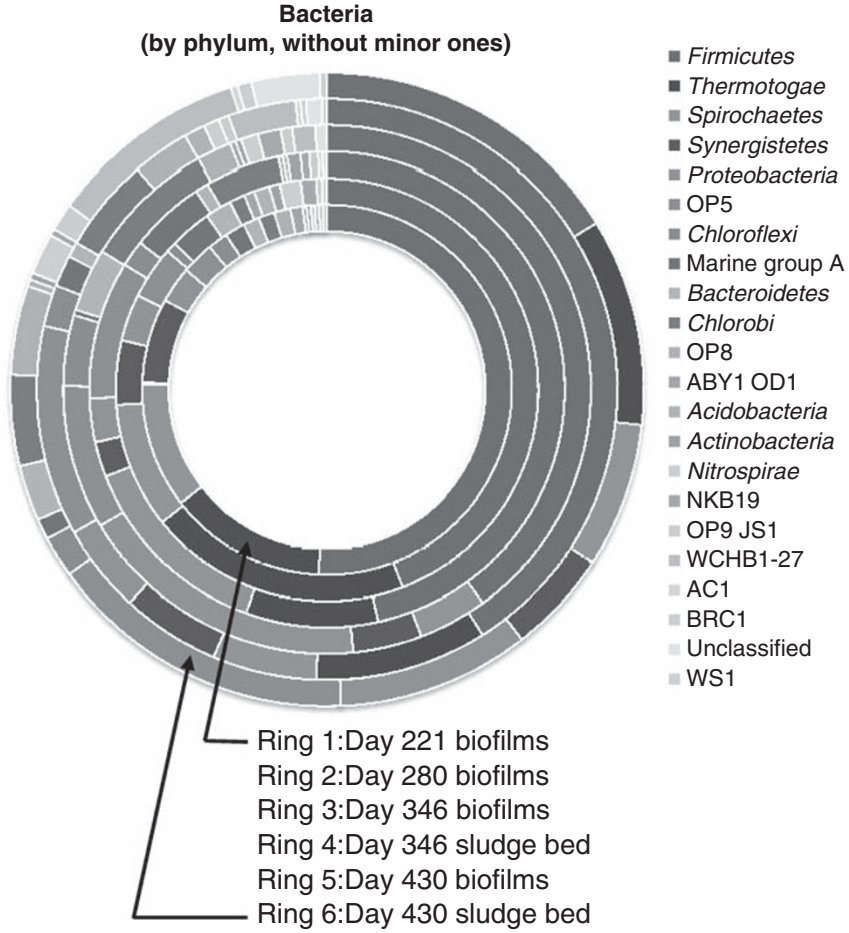

Figure 2 Bacterial population dynamics of the TA-degrading bioreactor as revealed by 16S rRNA clone library. Samples (number of 16S rRNA sequences) from inner to outer of the ring chart were day 221 biofilms (287), day 280 biofilms (254), day 346 biofilms (337), day 346 sludge bed (289), day 430 biofilms (352) and day 430 sludge bed (287).

Shotgun sequencing

The assembled sequence data contained 37 818 singlets and 14 526 contiguous fragments of intermediate length (the largest fragment was approximately 240 kb (Supplementary Figure 5) and 45 fragments between 24 and 167 kb). Gene prediction on the entire data set using Genemark resulted in the prediction of 93 104 protein-coding genes. A composition-based classifier, PhyloPythia (McHardy *et al*., 2007), was used to assign those contigs and singlets into major phylogenetic groups (Table 1), including *Pelotomaculum* species, candidate phylum OP5 species, *Methanolinea* species and *Methanosaeta* species. The highest coverage of an isolate reference genome was observed for *M. thermophila* (~80%) followed by *Pelotomaculum thermopropionicum* (~60%) (Supplementary Figure 6). However, the *Methanolinea* population appears to be the best covered one as the average read depth is 5.3× with many contigs having 10× read depth (Supplementary Figure 5). In the case of OP5, in which a closely related microbial genome was not present in the database, the occurrence was calculated with the phylogenetic marker clusters of orthologous genes in the OP5 bin (1.41 Mb; G+C content, 28%). Approximately 50% of the OP5 genome is estimated to be covered by the metagenomic data (Table 1).

Pelotomaculum

As a known catabolic-degrading organism abundant in the reactor, *Pelotomaculum* is assumed to be largely responsible for catabolic degradation of TA to CO_2, H_2 and acetate. With an average read depth of 3.2×, 1083 contigs were assigned to the *Pelotomaculum* population, comprising 4.3 Mb in total (Table 1). We first searched for genes with known decarboxylase functions that are responsible for the first decarboxylation step of TA degradation (Supplementary Figure 7). Two gene sets (tadcc27178-79-80 and tadcc16349) from the *Pelotomaculum* bin were identified to have high sequence similarity and a subunit complement with a known 4-hydroxybenzoate decarboxylase, EC 4.1.1.61, from *Sedimentibacter hydroxybenzoicum* that consists of three subunits (AAD50377, AAY67850 and AAY67851) and belongs to the UbiD family of proteins (Lupa *et al*., 2005). Two mechanisms have been described for the subsequent fermentation of

Table 1 Phylogenetic affiliations of major bins in the TA data set identified with the composition-based classifier, PhyloPythia (McHardy *et al*., 2007)[a]

Phylogenetic affiliation	*No of DNA contigs*	*Total sequence (Mb)*	*Average read depth*	*Expected genome size (Mb)*[b]
Bacteroidetes (class)	70	0.155	2.3 ± 1.1	—
Bacteroidales	120	0.235	2.2 ± 0.9	—
Betaproteobacteria	73	0.096	1.7 ± 0.6	—
Deltaproteobacteria	160	0.343	2.1 ± 0.8	—
Uncultured *Syntrophus*	196	0.724	2.9 ± 1.3	1.9
Geobacter	264	0.578	2.1 ± 0.7	—
Firmicutes	430	0.628	2.1 ± 0.7	—
Clostridia	66	0.372	3.3 ± 1.6	—
Uncultured *Pelotomaculum* spp[c]	1083	4.256	3.2 ± 1.5	3.6
OP5	228	1.411	3.8 ± 1.9	2.8
Spirochaetes (class)	81	0.177	2.3 ± 0.9	—
Euryarchaeota	1560	2.648	2.1 ± 0.9	—
Thermoplasmata	71	0.148	1.9 ± 0.8	—
Methanomicrobiales	36	0.098	2.4 ± 1.8	—
Uncultured *Methanolinea* spp[c]	78	2.162	5.3 ± 3.9	3.7
Methanosarcinales	15	0.095	3.7 ± 1.9	—
Uncultured *Methanosaeta*	1180	2.613	2.6 ± 1.1	3.1
Uncultured *Methanosaeta*	351	2.361	4.2 ± 1.3	2.8
Unclassified	46 280			

Abbreviations: rRNA, ribosomal RNA; SNP, single-nucleotide polymorphism; TA, terephthalate.
[a]No DNA contigs were binned to WWE1 related to *C. acidaminovorans* because of insufficient training data for PhyloPythia. 16S rRNA clone library indicted that132 sequences were affiliated with WWE1 and grouped into two different clusters. One of the clusters (37/132) was closely related to *C. acidaminovorans* (similarity = 96–98.8%).
[b]Expected genome size was calculated based on the percent coverage of the corresponding isolate genomes. For example, there are 1735 genes in *Pelotomaculum thermopropionicum* that are best-BLAST matches to genes from the metagenome dataset. Given that *P. thermopropionicum* contains 2920 genes, we estimate the genome size of the uncultured *Pelotomaculum* sp. was 7.16 Mb (4.256 *2920/1735). However, there are at least two strains of *Pelotomaculum* present in the sample. Therefore, the individual genome size for each strain is estimated to be around 3.6 Mb. For estimating the *Methanolinea* genome size we used as a reference genome *Methanoculleus marisnigri*. For *Methanosaeta* genome size, 2.6 Mb of sequences give hits to 1438 proteins in *M. thermophila* genome has 1730 coding sequences predicted so the expected genome size would be $(2.6 \times 1730)/1438 = 3.1$ Mb. In the case of OP5, expected genome size was calculated based on the occurrence of phylogenetic marker clusters of orthologous genes (COGs) that are defined as COGs having one or mostly one member in the genomes that are present and are available in Integrated Microbial Genome/Microbiome (IMG/M). The OP5 bin contained 91 out of 180 phylogenetic marker COGs.
[c]At least two species/strains were observed in each bin. With SNP frequencies of at least 0.03–0.07% (data not shown), we concluded that these species/strains are not clonal.

benzoate to acetate and CO_2: the well-known benzoyl-CoA reductase (BCR, EC 1.3.99.15) route used by *Thauera aromatica* (Boll and Fuchs, 1995) and the less understood BCR-independent mechanism for reductive dearomatization (Wischgoll *et al*., 2005). Examining the TA decarboxylase data set did not show any clear homologs of the *Thauera* BCRs. Instead, homologs are found in the alternative BCR-independent mechanism within a set of 44 genes that have been postulated to operate in *Geobacter metallireducens* and 'Syntrophus aciditrophicus' (Butler *et al*., 2007; McInerney *et al*., 2007; Peters *et al*., 2007). The key BCR enzyme in *G. metallireducens* was successfully characterized *in vitro* (Kung *et al*., 2009). The metagenome analysis further predicted the pathways that are used for conversion of benzoate to hydroxypimelyl-CoA and subsequent conversion of 3-hydroxypimelyl-CoA via β-oxidation to acetyl-CoA, which in turn gives rise to acetate through substrate-level phosphorylation (Supplementary Figure 7; Figure 3). The *Pelotomaculum* bin also contains genes and pathways for the production of butyrate (Supplementary Figure 7). These observations indicate that TA fermentation by *Pelotomaculum* may lead to the formation of butyrate in addition to acetate. Two genes assigned to *Pelotomaculum* (tadcc25255 and tadcc12813) belong to the Fe-only hydrogenase protein family and are potentially involved in hydrogen generation.

Methanogens

Three major groups or bins of methanogens belonging to the genera *Methanosaeta* and *Methanolinea* were identified and are known to be syntrophic partners of *Pelotomaculum* (Table 1). Complete pathways for both acetoclastic and hydrogenotrophic methanogenesis were identified (Supplementary Table 1). The first step in acetoclastic methanogenesis is the formation of acetyl-CoA from acetate. It has been proposed (Smith and Ingram-Smith, 2007) that acetoclastic methanogenesis in *Methanosaeta* proceeds with a modified version of the pathway compared with *Methanosarcina*, which utilize the acetate kinase/phosphotransacetylase pathway to convert acetate to acetyl-CoA. In contrast, the *M. thermophila* genome does not include a readily identifiable acetate kinase and it has been

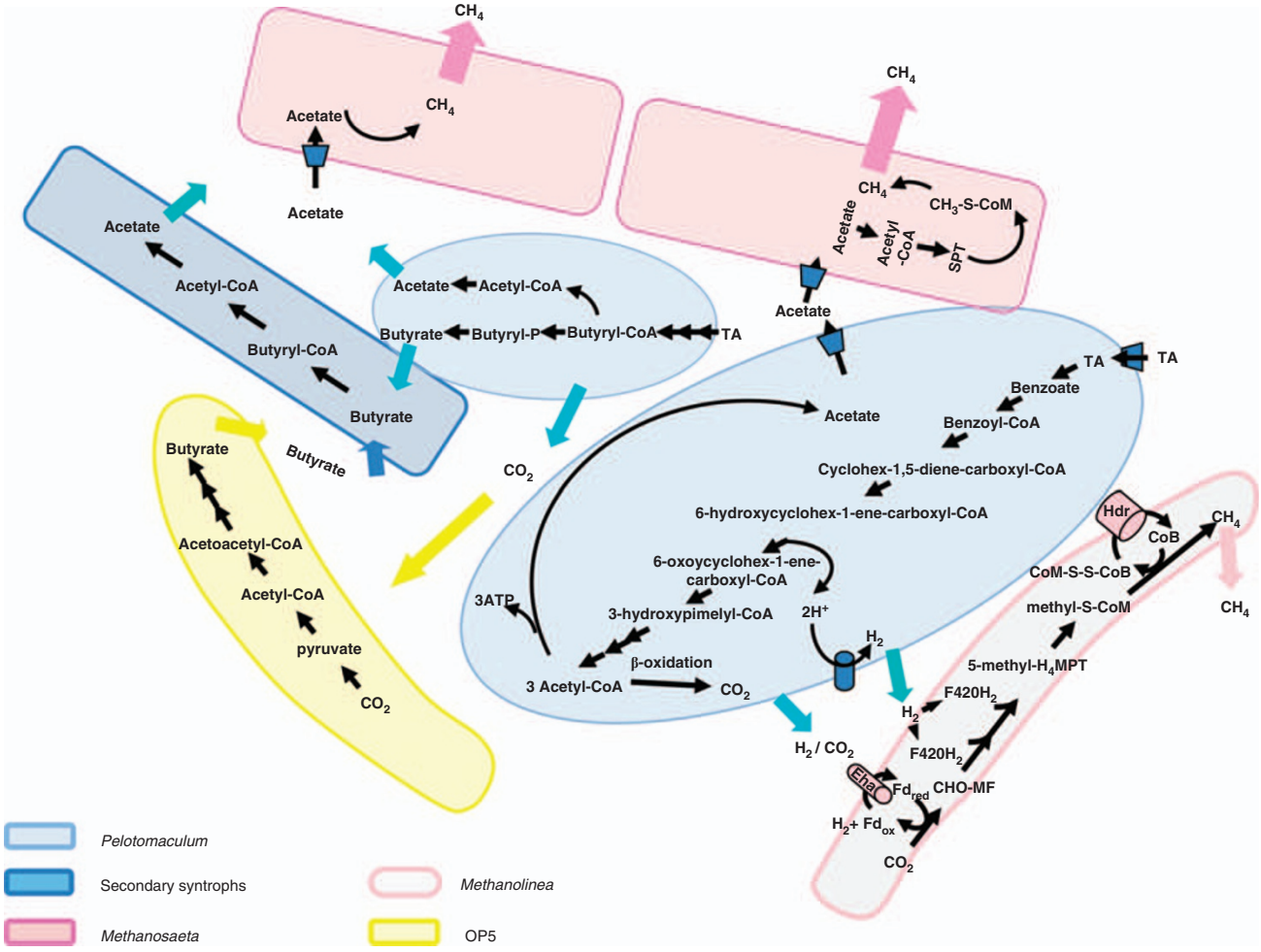

Figure 3 Metabolic reconstruction of the TA-degrading syntrophic community. The metagenomic data revealed pathways for the degradation of the aromatic (TA) compound, the recycling of the intermediates (H_2/CO_2, acetate and butyrate) and the subsequent syntrophic methanogenesis.

proposed that this species utilizes an acetate transporter coupled with acetyl-CoA synthetases to convert acetate to acetyl-CoA (Smith and Ingram-Smith, 2007). Analysis of the TA data set indicates the presence of acetate transporters (tadcc8417) and acetyl-CoA synthetases, EC 6.2.1.1, (tadcc27524, tadcc27520, tadcc27522, tadcc21744, tadcc21743) in the *Methanosaeta* bin. A complete set of the five acetyl-CoA decarbonylase subunits (EC 1.2.99.2) was identified as well as genes for the remaining steps of methanogenesis (Supplementary Table 1).

Hydrogenotrophic methanogenesis in the TA community is performed by the *Methanolinea* group. A complete set of Eha hydrogenase enzyme subunits is found in the *Methanolinea* bin. This set is adjacent to formyl-methanofuran dehydrogenase, which reduces CO_2 to formyl-methanofuran (tadcc39592–39604), suggesting that it may be the enzyme reducing the ferredoxin used by the dehydrogenase (Anderson *et al.*, 2008). In addition, complete sets of *ech* (tadcc3040–tadcc3045) and *mbh* (tadcc17854–tadcc17865) hydrogenases can be found in the *Methanolinea* bin. These hydrogenases are proposed to provide H_2 for the reduction of heterodisulfide (CoM-S-S-CoB) by the heterodisulfide reductase in the absence of MvhADG hydrogenase (Anderson *et al.*, 2008; Thauer *et al.*, 2008). In this way they link the regeneration of CoM to the reduction of ferredoxin. No homologs to the MvhADG hydrogenase were identified in the *Methanolinea* bin, suggesting that this organism couples ferredoxin and CoB-S-S-CoM reduction to hydrogen.

OP5

Analysis of the gene content in the OP5 bin (Table 1) revealed the existence of a gene fragment (tadcc9232) related to the *Archaeoglobus* type III RuBisCO. This fragment contains 181 amino acids and exhibits 60% identity to the N-terminus of the large subunit of the *Archaeoglobus* ribulose 1, 5-bisphosphate carboxylase, EC 4.1.1.39, raising a link between OP5 and autotrophic CO_2 fixation through the Calvin–Benson–Bassham cycle. Previous work has established that type III RuBisCOs are functional enzymes *in vitro* and also complement RuBisCO deletion in photosynthetic organisms

indicating their functionality *in vivo* (Tabita *et al.*, 2007). However, other experiments have shown that type III RuBisCO enzymes are involved in adenosine monophosphate metabolism (Sato *et al.*, 2007). Thus, future experiments are required to validate whether OP5 species can use type III RuBisCO enzymes for autotrophic CO_2 fixation. Although organisms that contain type III RuBisCOs usually lack recognizable phosphoribulokinases (as is the case for *Archaea*), the OP5 bin contains a gene (tadcc30466) that belongs to the phosphoribulokinase protein family (Pfam domain 00485), which provides the second substrate for the RuBisCO reaction, ribulose 1,5-bisphosphate. These are the two unique enzymatic activities required for CO_2 assimilation. The OP5 bin also contains genes encoding phosphoglycerate kinase, EC 2.7.2.3, (tadcc33464), glyceraldehyde-3-phosphate dehydrogenase, EC 2.7.1.12, (tadcc33465), and phosphoglycerate mutase, EC 5.4.2.1, (tadcc16672) although no representatives of the remaining Calvin–Benson–Bassham cycle genes are readily recognizable in the OP5 bin.

OP5 also contains phosphate butyryltransferase, EC 2.3.1.19, (tadcc17546) and two copies of butyrate kinase, EC 2.7.2.7, (tadcc17547 and tadcc17544) indicating its ability to produce butyrate and gain energy by substrate-level phosphorylation. No acetate kinases or adenosine diphosphate-forming acyl-CoA synthetases were detected in the OP5 genes binned by PhyloPythia. However, inspecting unassigned contigs with GC content $<31\%$ identified an acetate kinase (EC 2.7.2.1) gene (tadcc15543 on contig taComm3_C5047) that may originate from OP5. On the basis of these observations, it is proposed that the OP5 populations within the TA community participate in the syntrophic interactions by removing CO_2 and H_2 and producing butyrate and potentially acetate. An operon on contig C11376 binned in OP5 was found to contain a system of Ni-hydrogenases (tadcc33916 and tadcc3391) potentially involved in hydrogen utilization.

Syntrophaceae, Thermotogae *and WWE1*

Syntrophaceae are members of syntrophic communities and are a minor component of the TA-degrading community (Figure 2). To our knowledge, no known *Syntrophaceae* isolates have been reported to degrade TA and most of the isolates utilize propionate, long-chain fatty acids and benzoate. The *Thermotogae* and WWE1 groups were estimated to constitute a significant proportion of the community based on the 16S rRNA analysis. Both for WWE1 and *Thermotogae*, the respective sample populations could not be modeled directly in composition-based binning, because of a lack of sample-specific training data, for WWE1 there was also not sufficient data to directly model the clade (Table 1). A protein-similarity comparison with sequenced members of the phylum *Thermotogae* (utilizing the distribution of BLAST (Basic Local Alignment Search Tool) matches for protein-coding genes in the data set) resulted in 1066 genes with a BLAST matches $>60\%$, with additional 1646 genes having BLAST matches $>30\%$. Among these, acetate kinase (tadcc6136) (Supplementary Figure 8) and a phosphotransacetylase (tadcc64919) were identified, suggesting that members of the *Thermotogae* in the TA community may participate in the syntrophic interactions by producing acetate from an intermediate molecule. This intermediate molecule may be the butyrate produced by the OP5 population. A fragment encoding butyryl-CoA dehydrogenase, EC 1.3.99.2, (tadcc28367) further suggests the existence of the butyrate utilizing pathway, and a contig encoding a Fe-only hydrogenase (tadcc1433) suggests the ability of this population to generate H_2. On the basis of these observations, we hypothesize that the *Thermotogae* species may oxidize butyrate to acetate and H_2.

The sequence similarity-based phylogenetic profiler tool of IMG identified a set of genes from the TA-degrading community with high similarity to *Candidatus* Cloacamonas acidaminovorans, which presents the only sequenced bacterial genome of the WWE1 candidate phylum through genome sequence reconstruction and is predicted as a syntrophic bacterium in anaerobic digesters (Pelletier *et al.*, 2008). Comparing the common genes between the TA community data set and the *C. acidaminovorans* genome identified 1607 and 3228 genes with sequence identity greater than 80% or 60%, respectively. These genes are likely to originate from the WWE1 population in the TA-degrading community. Among them, acetate kinases (tadcc38857) and acetyl phosphotransferases, EC 2.3.1.8, (tadcc25853, tadcc25854) were identified, suggesting an oxidative pathway generating acetate and energy through substrate-level phosphorylation and Fe-only hydrogenases (tadcc1522, tadcc13376 and tadcc38378), which presumably produce hydrogen. The substrate for this oxidative pathway may be butyrate because members of the butyrate-oxidizing pathway can be identified in the data set.

Methanogenic syntrophy

Methanogenic syntrophy has a critical role in the complete degradation of TA to methane (Figure 3). Thermodynamic considerations suggest a low and narrow-range hydrogen concentration as the essential regulator to establish the syntrophic association between the H_2-producing bacteria and the H_2-consuming methanogens (Schink, 1997; Conrad, 1999). This is because the first reaction from TA to acetate and CO_2/H_2 ($TA + 8H_2O \rightarrow 3\ Acetate + 3H^+ + 2HCO_3^- + 3H_2$, $\Delta G^{o'} = 43.2\ kJ\ mol^{-1}$) can occur only at a low pH_2 (<5 Pa, 1 a.t.m. $= 10\,1325$ Pa) by coupled with methanogenesis ($4TA + 35\ H_2O \rightarrow 17HCO_3^- + 9H^+ + 15CH_4$, $\Delta G^{o'} < -151.9$) (Schink, 1997). Also, a minimal threshold pH_2 is required for the H_2-dependent methanogenesis step to produce the minimum amount of energy required for cell maintenance

(Conrad, 1999; Dolfing, 2001). When H_2 concentration is higher than this threshold level, H_2-dependent methanogenesis ceases. Such a low, narrow-range pH_2 is thought to be maintained by 'interspecies hydrogen transfer' (Stams, 1994), in which H_2-producing syntrophs and H_2-consuming methanogens cooperate intimately by arranging themselves in close physical proximity in flocs or in a biofilm with short diffusion distances to facilitate hydrogen transfer. TA community metagenomic data revealed a set of hydrogenases, which generate hydrogen in *Pelotomaculum* and consume hydrogen in methanogens.

Using fluorescence *in situ* hybridization analysis (Supplementary Figure 9), we observed not only close physical proximity among methanogens and *Pelotomaculum* but also the presence of other microbes (that is, OP5, WWE1, *Thermotogae* and *Syntrophus*), as illustrated in Figure 3, associating with syntrophs and methanogens. Metagenomic analysis suggests that these populations can actively participate in the syntrophic interactions to tightly regulate pH_2. OP5 are likely to consume CO_2 and H_2 that are produced by *Pelotomaculum* through the degradation of TA, and produce butyrate. This concept is supported by the $\Delta G^{o'}$ value ($-198.05\,kJ\,mol^{-1}$) for the conversion of $CO_2 + H_2$ to butyrate ($10H_2 + 4CO_2 \rightarrow C_4H_8O_2 + 6H_20$), which is even more favorable under hyper-mesophilic conditions than mesophilic conditions. OP5 and methanogens can compete for H_2 but the competition is likely to be pH_2 dependent.

Populations of *Syntrophus*, *Thermotogae* and WWE1 may be involved in utilizing and recycling butyrate produced by OP5 probably through the secondary β-oxidation pathway. The presence of hydrogenases indicates that both *Thermotogae* and WWE1 gain energy through substrate-level phosphorylation. Although there is no clear evidence for the carbon source that these populations utilize, butyrate (produced by OP5) may serve as a key carbon source. This would suggest that, like *Syntrophus*, some members of *Themotogae* and WWE1 are possibly syntrophs. However, they are not persistently dominant populations and their abundance varies throughout the reactor operation (Figure 2). TA metagenomics data indicate the presence of butyrate kinases and phosphotransacetylases in the *Pelotomaculum* bin, suggesting that this population may ferment TA not only to acetate but also butyrate. Our previous study also observed a detectable level of butyrate by using 2-bromoethanesulfonate to inhibit the methanogenesis step in a mesophilic TA-degrading consortium (Wu *et al.*, 2001). It is possible that this type of fermentation results in the production of a second end product (butyrate in addition to acetate) and triggers a secondary syntrophic interaction involving butyrate-oxidizing organisms.

Several studies (Chan, 2000; Qiu *et al.*, 2006; Imachi *et al.*, 2008) have observed the existence of multiple bacterial populations in highly enriched methanogenic cultures degrading carbon substrates like formate, acetate, propionate and phthalate isomers. These observations were shown through a defined mixed culture (Dolfing *et al.*, 2008), suggesting that syntrophic interactions in methanogenic enrichments are more complex than simple pairwise syntroph–methanogen relationships. Rather, they include other members that maintain and regulate the interspecies hydrogen transfer, which is the cornerstone of syntrophy. In conclusion, our overall observations imply that degradation of organic carbon is not simply a syntrophic interaction between H_2-producing syntrophs and methanogenic archaea. They further support the hypothesis that additional secondary interactions take place to maintain the stability of the TA-degrading community. (Supplementary Table 2).

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