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Microbial analysis and enrichment of anaerobic phenol and *p*-cresol degrading consortia with addition of AQDS

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ABSTRACT

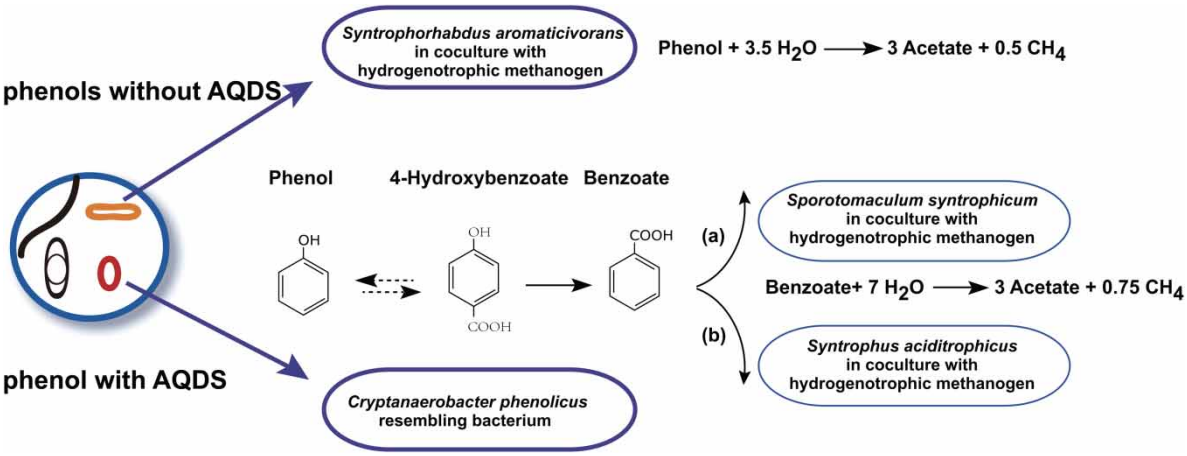
Quinones and humus are ubiquitous in the biosphere and play an important role in the anaerobic biodegradation and biotransformation of organic acids, poisonous compounds as well as inorganic compounds. The impact of humic model compound, anthraquinone-2, 6-disulfonate (AQDS) on anaerobic phenol and *p*-cresol degradation were studied. Four methanogenic AQDS-free phenol and *p*-cresol enrichments and two phenol-AQDS enrichments were obtained using two sludges with potential biodegradability of phenol and cresol isomers as inoculum. 16S rRNA gene-cloning analysis combined with fluorescence *in situ* hybridization revealed that syntrophic aromatic compound degrading bacterium *Syntrophorhabdus aromaticivorans* was dominant in four AQDS-free enrichments, whereas phenol degrading *Cryptanaerobacter phenolicus* was dominant in two phenol-AQDS enrichments. Neither co-culture of *S. aromaticivorans* with *Methanospirillum hungatei* nor two phenol-AQDS enrichments could metabolize phenol using AQDS as the terminal electron acceptor. Further degradation experiments suggested that *C. phenolicus* related microbes in two phenol-AQDS enrichments were responsible for the conversion of phenol to benzoate, and benzoate was further degraded by benzoate degraders of *Syntrophus aciditrophicus* or *Sporotomaculum syntrophicum* to acetate.

Key words: anaerobic syntrophism, AQDS, *Cryptanaerobacter phenolicus*, phenols degradation, *Syntrophorhabdus aromaticivorans*

HIGHLIGHTS

- Anaerobic degradation of phenol and *p*-cresol were faster than *o*- and *m*-cresols.
- 2 mM AQDS mitigated phenol and completely inhibited *p*-cresol degradation.
- The dominant bacteria in AQDS-free and phenol-AQDS enrichments were different.
- Neither *S. aromaticivorans* nor *C. phenolicus* affinities could use AQDS as TEA.
- The characteristic of key phenol degraders in phenol-AQDS enrichments was described.

GRAPHICAL ABSTRACT



INTRODUCTION

Phenol and cresol isomers (*ortho-*, *meta-*, and *para*-cresols) are important petrochemical products and commonly found in wastewaters from coal gasification, petrochemical manufacturing, oil-refining, synthetic chemicals, pesticides, and pharmaceuticals industries. Phenols are also found in municipal landfill leachates, lignin residues in anaerobic agroecosystems, and methanogenic bioreactors treating organic solid wastes, such as slaughter house waste, animal manure, food wastes and crop residues (Levén *et al.* 2012; Qiao *et al.* 2013). Therefore, studying anaerobic degradation of phenols is necessary to the industrial wastewater treatment processes and the biogeochemical process of natural phenols.

Thermodynamically, the oxidation of phenol to acetate, carbon dioxide, and hydrogen is unfavorable, and phenol is thought to be metabolized only at a very low hydrogen partial pressure. Under methanogenic conditions, phenol degradation requires the coupling of fermentative bacteria with methanogens to overcome thermodynamics limit. $C_6H_6O + 6.5H_2O \rightarrow 3.5CH_4 + 2.5HCO_3^- + 2.5H^+$ ($\Delta G^{0'} = -155.3$ kJ reaction^{-1}); $C_7H_8O + 7.5H_2O \rightarrow 4.25CH_4 + 2.75HCO_3^- + 2.75H^+$ ($\Delta G^{0'} = -187.5$ kJ reaction^{-1}) (Cervantes *et al.* 2000a).

The anaerobic degradation of phenols have been reported under methanogenic conditions. Previous studies showed that anaerobic degradation of phenols can be observed at either ambient (26 °C) (Fang *et al.* 2004), mesophilic (37 °C) (Chen *et al.* 2008; Franchi *et al.* 2018; Levén & Schnürer 2010) or thermophilic temperatures (55 °C) (Fang *et al.* 2006; Chen *et al.* 2008; Sierra *et al.* 2018, 2020). Investigations of the methanogenic phenolic compound-degrading microbial community revealed that the majority of functional bacteria belong to *Clostridia* and subcluster Ih of *Desulfotomaculum* cluster I within phylum *Firmicutes*, and *Syntrophorhabdaceae* and *Syntrophus* spp. in *Deltaproteobacteria* (Fang *et al.* 2006; Chen *et al.* 2008; Levén *et al.* 2012). Levén *et al.* (2012) reviewed that temperature had a strong impact on the degradation efficiency of phenols and phenol-degrading microbes. *Desulfotomaculum* subcluster Ih was the dominant phenol-degrading population in the thermophilic temperature, while *Syntrophorhabdus* was the dominant population in the mesophilic temperature (Chen *et al.* 2008; Levén & Schnürer 2010; Li *et al.* 2014; Franchi *et al.* 2018).

Humus is the stable organic matter accumulating in sediments and soils. Lovley *et al.* (1996) first proposed the concept of humus respiration. They found that Fe(III)-reducing bacteria *Geobacter metallireducens* and *Shewanella alga* were able to use humus model AQDS as the sole electron acceptor to oxidize organic compounds or hydrogen, supporting the growth of bacteria. In humus respiration, some quinones act as electron receptors, hence humus respiration is also called quinone respiration (Lovley *et al.* 1996; Cervantes *et al.* 2000a). It has been reported that quinones play an active role in the anaerobic transformation of organic acids, poisonous compounds, such as azo dyes (Hong *et al.* 2007), pentachlorophenol (Zhao *et al.* 2019), vinyl chloride and dichloroethene (Bradley *et al.* 1998), polycyclic aromatic hydrocarbons (Ma *et al.* 2011), benzene (Cervantes *et al.* 2011) and toluene (Cervantes *et al.* 2001). Recently, AQDS and AQS were found to serve as the terminal electron acceptor (TEA) for anaerobic oxidation of methane by ANME-2d (Bai *et al.* 2019). Anaerobic degradation of phenol and *p*-cresol can be coupled to the reduction of AQDS as TEA. When the humic model compound AQDS was included as an alternative electron acceptor in the cultures inoculated with anaerobic granular sludge and sediment, the

oxidation of the phenols was coupled to the reduction of the model humic compound to its corresponding hydroquinone, anthrahydroquinone-2,6-disulfonate. The equations of the theoretical reactions are shown as follows: $C_6H_6O + 17H_2O + 14AQDS \rightarrow 14AH_2QDS + 6HCO_3^- + 6H^+$ ($\Delta G^{0'} = -302.0$ kJ reaction^{-1}); $C_7H_8O + 20H_2O + 17AQDS \rightarrow 17AH_2QDS + 7HCO_3^- + 7H^+$ ($\Delta G^{0'} = -365.7$ kJ reaction^{-1}) (Cervantes *et al.* 2000a). However, the anaerobes responsible for the degradation reaction are unknown.

To date, two anaerobic phenol-degrading bacteria have been described: *Cryptanaerobacter phenolicus* belongs to subcluster Ih of *Desulfotomaculum* cluster I within phylum *Firmicutes* has been found transform phenol into benzoate via 4-hydroxybenzoate (Juteau *et al.* 2005); *Syntrophorhabdus aromaticivorans* belongs to the family *Syntrophorhabdaceae* in *Deltaproteobacteria,* an anaerobic microbe that degrades phenol to acetate in syntrophic associations with the hydrogenotrophic methanogen *Methanospirillum hungatei* (Qiu *et al.* 2008). The utilization of AQDS by *Cryptanaerobacter phenolicus* and *S. aromaticivorans* is unknown.

In this study, we reported the impact of AQDS on phenol and *p*-cresol degradation by enrichment cultures. The dominant microbial populations were identified by 16S rRNA gene cloning analysis and fluorescence *in situ* hybridization (FISH). In addition, partial physiological properties of dominant phenol degrading consortia belonging to subcluster Ih of *Desulfotomaculum* cluster I were characterized.

MATERIALS AND METHODS

Inoculum sources, microorganisms and cultivation

Methanogenic sludges were taken from two lab-scale, mesophilic (35 °C), upflow anaerobic sludge blanket (UASB) reactors: (i) sludge C treated cresols for one year; (ii) sludge N treated nitro-phenol for three months (Zhang *et al.* 2005). Two sludges were washed with phosphate buffer (10 mM, pH 7.2) and homogenized briefly for primary enrichment. A total volume of 100 mL sludges were washed with phosphate buffer and then centrifuged at 7,000 × g for 15 min. The pellets were collected and resuspended in 20 mL of fresh medium.

S. aromaticivorans (JCM 13376, DSM 1777) was the first cultured anaerobe capable of degrading phenol to acetate in syntrophic associations with a hydrogenotrophic methanogen (Qiu *et al.* 2008). Co-culture of *S. aromaticivorans* with *M. hungatei* was obtained from our lab and routinely cultured at 37 °C with 2 mM phenol and 0.01% yeast extract (Qiu *et al.* 2008).

The medium used for cultivation was prepared as described previously (Sekiguchi *et al.* 2000). All batch experiments were carried out at 37 °C under dark anaerobic conditions, 60 mL serum vials containing 20 mL of medium ($pH_{25\,°C}$ 7.0) under an atmosphere of N_2-CO_2 (80:20, v/v) without shaking.

Degradability experiments of methanogenic sludges

To test the degradability of phenol (2 mM) and cresol isomers (1 mM each) by methanogenic sludges C and N, batch experiments were carried out in 60-mL serum vials with sludges as inoculum at 37 °C without shaking. The experiment lasted 160 days. Concentration of phenol and cresol isomers was measured every 15–20 days.

Enrichment culture

For enrichment of phenol and *p*-cresol degrading anaerobes, either phenol (2 mM) or *p*-cresol (1 mM) was added to the basal medium as the sole energy source with sludges C and N, respectively. Four methanogenic AQDS-free enrichment cultures CP (enriched on phenol medium with sludge C), NP (enriched on phenol with sludge N), CPC (enriched on *p*-cresol with sludge C) and NPC (enriched on *p*-cresol with sludge N) were obtained. To evaluate the effect of quinone on phenol and *p*-cresol degradation, another set of enrichment cultures with addition of 2 mM AQDS were prepared in parallel. The cultures were transferred into fresh medium when approximately 50% of phenol and *p*-cresol were degraded. For enrichments, large volume of inoculum was always needed (30–50%).

FISH

Fixation of cells in the enrichment cultures and subsequent whole-cell *in situ* hybridization were performed as described previously (Sekiguchi *et al.* 1999). The following 16S rRNA-targeted oligonucleotide probes were used in this study (Table 1): (i) EUB338 mix, specific for *Bacteria* (Amann *et al.* 1990); (ii) Ih820, specific for subcluster Ih of *Desulfotomaculum* cluster I, which was used to detect dominant phenol-degrading bacteria in enrichment culture NPA (enriched on phenol-AQDS with

Table 1 | Fluorescently Cy3-labeled oligonucleotide probes used in this study

Probe name	Target group	Sequence (5′ to 3′)[a]	*E. coli* position	% FA[b]	References
EUB338	*Bacteria*	GCWGCCWCCCGTAGGWGT	338–355	10	Amann *et al.* (1990)
Ih820	*Desulfotomaculum* subcluster Ih	ACCTCCTACACCTAGCAC	820–837	30	Imachi *et al.* (2006)
UI178	*Syntrophorhabdus aromaticivorans*	GTGTCGTGTGGTCTTATC	178–196	15	Qiu *et al.* (2004)
DEM1164-ar.[c]	Clones CPA01 and CPA02 in an undefined subcluster of *Desulfotomaculum* cluster I	CCTTCCTCCGGTTTGTCA	1,164–1,181	0	Stubner & Meuser (2000)

[a]W = A:T, Y = C:T.
[b]Percentage of formamide (FA) in buffer.
[c]Probe DEM1164-ar. is specific for clones CPA01 and CPA02 (obtained from enrichment culture CPA grown on phenol-AQDS), which belong to an undefined subcluster of *Desulfotomaculum* cluster I. The probe was arranged from *Desulfotomaculum* cluster I specific probe DEM1164 with a slight modification.

sludge N) (Imachi *et al.* 2006); (iii) UI178, specific for *S. aromaticivorans*, which was used to detect dominant phenols degrading bacteria in enrichment cultures CP, NP, CPC and NPC (Qiu *et al.* 2004); (iv) DEM1164-ar. (5′-CCTTCCTCCGGTTTGTCA-3′), specific for clones CPA01 and CPA02 obtained from phenol-AQDS enrichment culture CPA (enriched on phenol-AQDS with sludge C), which belong to a new undefined subcluster of *Desulfotomaculum* cluster I. The probe DEM1164-ar. was arranged from *Desulfotomaculum* cluster I specific probe, DEM1164 (5′-CCTTCCTCCGTTTTGTCA-3′) with a slight modification (Stubner & Meuser 2000) (Figure 3). To test the specificity of probe DEM1164-ar., *Sporotomaculum syntrophicum* (DSM 14795) and *Pelotomaculum terephthalicum* (DSM 16121) were used as reference organisms. Hybridization stringency was adjusted by adding formamide to the hybridization buffer (30% [v/v] for Ih820; 15% [v/v] for UI178; 10% [v/v] for EUB338 mix; 0% [v/v] for DEM1164-ar.).

16S rRNA gene clone library and phylogenetic analysis

DNA extraction, PCR amplification, cloning, and sequencing procedures for constructing 16S rRNA gene clone libraries were performed as described previously (Sekiguchi *et al.* 1998) with slight modifications. The following primer sets for PCR amplification of bacterial 16S rRNA genes were used: *Bacteria*-specific primer EUB8F (5′-AGAGTTTGATCCTGGCTCAG-3′, 8 to 27 in *Escherichia coli* position) and prokaryote-specific primer UNIVI1492R (5′-TACGGYTACCTTGTTACGACTT-3′; positions 1,492 to 1,513 in *E. coli*) (Weisburg *et al.* 1991). The PCR products were purified with a TIAN quick MiDi purification kit (Tiangen Inc., Beijing, China), followed by cloning into plasmids using the TA cloning kit (Promega Inc., Beijing, China). For each enrichment culture, 10–40 clonal rRNA genes were randomly picked and screened by comparing restriction fragment length polymorphism (RFLP) patterns with *Hae*III and *Hha*I restriction endonuclease. Representative clones having different RFLP patterns were then subjected to sequencing.

The phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbor-joining method (Zhang & Sun 2008) implemented in the MEGA5 computer software program (Tamura *et al.* 2007). The confidence values of branches in the phylogenetic tree were determined using bootstrap analysis based on 1,000 resamplings (Felsenstein 1985).

Utilization of AQDS as the terminal electron acceptor

To test whether AQDS could be used as electron acceptor, batch experiments were conducted with enrichment cultures CPA, NPA and syntrophic co-culture of *S. aromaticivorans* with *M. hungatei*. In this test, 5 mM 2-bromoethane-sulfonate (2-BES) was added to inactivate the methanogenesis in enrichment cultures. The concentrations of AQDS were set at 5 and 30 mM respectively, according to the theoretic chemical equation $C_6H_6O + 17H_2O + 14AQDS \rightarrow 14AH_2QDS + 6HCO_3^- + 6H^+$ ($\Delta G^{0'} = -302.0$ kJ reaction^{-1}) and previous studies (Cervantes *et al.* 2000a, 2000b). In total, six sets of assays were run as follows: (i) phenol; (ii) phenol-BES; (iii) phenol-AQDS (5 mM); (iv) phenol-AQDS (5 mM)-BES; (v) phenol-AQDS (30 mM); (vi) phenol-AQDS (30 mM)-BES (Table 2). Sterilized controls were also included to evaluate the chemical transformation. For enrichment cultures CPA & NPA (more than 20 successive transfers over three years), a total volume of 200 mL exponential phase cultures in the exponential phase were collected by centrifugation at 7,000 × *g* for 15 min at

Table 2 | AQDS utilization by two enrichment cultures CPA and NPA originally grown on phenol/AQDS, and co-culture of *Syntrophorhabdus aromaticivorans* with *M. hungatei* after 4 months of incubation

Culture name	Substrate[a]	Final phenol conc. (mM)	Products formed (mM)[b]				Degradability (%)
			Benzoate	Methane[c]	Acetate	AH_2QDS	
Enrichment CPA (87% similarity with *C. phenolicus*)	Phenol	0	0	7.5	0	–	100
	Phenol-BES	0	1.2	0	2.5	–	100
	Phenol-AQDS (5 mM)	1.7	0.2	0.5	0	0	17
	Phenol-AQDS (5 mM)-BES	1.6	0	0	1.2	0	20
	Phenol-AQDS (30 mM)	2.0	0	0	0	0	0
	Phenol-AQDS (30 mM)-BES	2.0	0	0	0	0	0
Enrichment NPA (96% similarity with *C. phenolicus*)	Phenol	0	0	7.1	0	–	100
	Phenol-BES	0	0.7	0	3.7	–	100
	Phenol-AQDS (5/30 mM)	2.0	0	0	0	0	0
	Phenol-AQDS (5/30 mM)-BES	2.0	0	0	0	0	0
Syntrophorhabdus aromaticivorans with *M. hungatei*	Phenol	0	0	1.1	5.9	–	100
	Phenol-BES	2.0	0	0	0	–	0
	Phenol-AQDS (5/30 mM)	2.0	0	0	0	0	0
	Phenol-AQDS (5/30 mM)-BES	2.0	0	0	0	0	0

[a]Initial concentrations of phenol and BES are 2 and 5 mM, AQDS are 5 and 30 mM, respectively.

[b]The values were corrected by subtracting the amounts of products formed in the control vial without substrate. The methane values are expressed as millimoles of methane formed in 1 L of culture.

[c]Reaction involved in the degradation of phenol under anaerobic conditions: $C_6H_6O + 6.5\ H_2O \rightarrow 3.5\ CH_4 + 2.5\ HCO_3^- + 2.5\ H^+$ ($\Delta G^0 = -155.3$ kJ·reaction^{-1}).

35 °C, and then used for inoculation (inoculum size, 30%) (Qiu *et al.* 2004). All the experiments were performed in duplicate at 37 °C and pH 7.0 for four months.

Substrate utilization by two phenol-AQDS enrichment cultures

It is widely recognized that 4-hydroxybenzoate and benzoate are intermediates during anaerobic phenol degradation (Juteau *et al.* 2005; Qiu *et al.* 2008; Levén *et al.* 2012). To test the degradation of phenol, *o*, *m*, *p*-cresols, 4-hydroxybenzoate and benzoate by enrichment cultures CPA and NPA, batch experiments were performed with highly enriched dense cell suspensions (more than three years successive transfers). The degradability was evaluated by percent substrate degradation in 1–4 months' incubation. For the other possible aromatic substrates such as catechol, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, hydroquione, 2-hydroxybenzoate, 3-hydroxybenzoate, 3-phenylpropionate, and phthalate isomers, the utilization was evaluated by turbidity of cultures and methane production over four months' incubation.

Analytical methods

An Olympus microscope equipped for epifluorescence was used for studies of cell morphology and epifluorescence (Olympus BX50F). Concentrations of phenol, *o*-, *m*-, *p*-cresols, benzoate and 4-hydroxybenzoate were analyzed by high-performance liquid chromatography (HPLC) with an UV detector as described previously (Qiao *et al.* 2013). Short-chain fatty acids, methane, and hydrogen were measured as described previously (Yuan *et al.* 2011). Concentrations of AH_2QDS were determined spectrophotometrically by monitoring the absorbance at 450 nm in an anaerobic chamber as described previously (Cervantes *et al.* 2000a; Li *et al.* 2019).

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences of phenol-degrading clones are AB853905 to AB853925.

RESULTS AND DISCUSSION

Degradability of phenol and *o*-, *m*-, *p*-cresols by two sludges

To assess the biodegradability of two sludges C and N, batch experiments were performed with either phenol or *o*-, *m*-, *p*-cresols as sole carbon source. Both sludges were able to degrade phenol and three cresol isomers within 150 days of incubation (Figure 1), suggesting functional phenol and cresol isomers degrading organisms existed in the sludges. Phenols are toxic to microorganisms, that cannot be degraded easily under anaerobic conditions. Therefore, in most cases, long lag period is often required. Phenol and *p*-cresol were completely degraded by both sludges within three months. *o*-Cresol was completely degraded by sludge C, while partially (25%) degraded by sludge N within five months. Only 20% *m*-cresol was degraded by both sludges within five months of incubation. Phenol and *p*-cresol were easy to degrade, whereas *o*- and *m*-cresols were more difficult to degrade. Our result was consistent with the previous study (Veeresh *et al.* 2005).

Enrichment of phenol and *p*-cresol degrading microbes

To enrich phenol and *p*-cresol degrading microbes, primary enrichment was made with each phenol (2 mM) and *p*-cresol (1 mM) as the sole carbon and energy source with two sludges C and N as inoculum. Four methanogenic AQDS-free enrichment cultures CP, NP, CPC and NPC were obtained. To evaluate the effect of quinone on phenol and *p*-cresol degradation, another set of enrichment cultures with addition of 2 mM AQDS was prepared in parallel. 2 mM AQDS completely inhibited *p*-cresol degradation, thus, two phenol-AQDS enrichment cultures CPA and NPA were obtained.

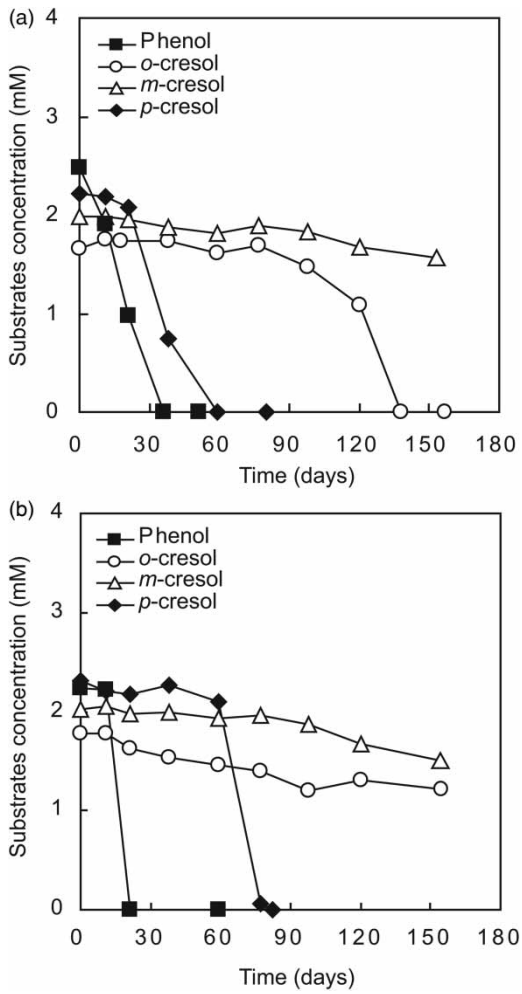


Figure 1 | Degradation of phenol and *o*-, *m*-, *p*-cresols by two types of anaerobic phenolic compounds degrading sludges C (a) and N (b).

In methanogenic AQDS-free enrichment cultures, the growth of microbes and phenol or *p*-cresol degradation accompanied by methane production were observed after 1–2 months of incubation. In total, four successful methanogenic enrichment cultures were obtained: (i) enrichment CP with phenol from sludge C; (ii) enrichment NP with phenol from sludge N; (iii) enrichment CPC with *p*-cresol from sludge C; (iv) enrichment NPC with *p*-cresol from sludge N. After two years of successive transfers, short rods (later identified as *S. aromaticivorans* by FISH) were the major morphotypes in all four enrichment cultures by microscopic observation. *Methanospirillum*-like F_{420}-autofluorescent rods and *Methanosaeta*-like thick rods in some cases were also detected.

The addition of 2 mM AQDS diminished phenol degradation and completely inhibited *p*-cresol degradation. The growth rate of phenol-AQDS cultures was approximately 1.5–2 times slower than that of AQDS-free phenol enrichment cultures, and phenol was degraded after 3–4 months. Methane production was detected along with phenol degradation no matter with or without AQDS addition. In total, two stable phenol-AQDS enrichment cultures were obtained: (i) enrichment CPA with phenol-AQDS from sludge C; (ii) enrichment NPA with phenol-AQDS from sludge N. Microscopic observation revealed that major morphotypes in two phenol-AQDS enrichment cultures CPA and NPA were slightly different. In enrichment culture CPA, short rods (later identified as *C. phenolicus* resembling bacterium with 87% sequence similarity, and *Syntrophus aciditrophicus* with 99% similarity) were observed as major morphotypes. In enrichment culture NPA, at least two major bacterial cell morphotypes were observed: one was short rods (later identified as *C. phenolicus* resembling bacterium with 96% similarity), the other was sporulating rods (later identified as *Sporotomaculum syntrophicum* with 100% similarity). *Methanospirillum*-like F_{420}-autofluorescent rods and *Methanosaeta*-like thick rods were presented in both CPA and NPA enrichment cultures.

FISH of dominant microbes in four methanogenic AQDS-free enrichments

After two years of successive transfers, short rods *S. aromaticivorans* like microbes were major morphotypes in all four AQDS-free enrichment cultures CP, NP, CPC and NPC by microscopic observation. To confirm whether *S. aromaticivorans* actually represented the predominant bacterial populations in four enrichments, FISH with *S. aromaticivorans*-specific16S rRNA-targeted oligonucleotide probe UI178 (Qiu *et al.* 2004) was done (Figure 2). *Bacteria*-specific probe EUB338 mix was also applied. At least 10 microscopic fields were randomly acquired. FISH analysis indicated that short rods in all of the four cultures reacted with the probe UI178, and represented approximately 80–90% of the total bacteria detected by probe EUB338 mix.

Phylogenetic analysis of bacterial community in phenol-AQDS enrichment cultures

To identify bacterial populations in two phenol-AQDS enrichments CPA and NPA, 16S rRNA gene clone libraries were constructed as described above. RFLP analysis indicated that three RFLP patterns were found in enrichment culture CPA. Phylogenetic analysis indicated that clones CPA01 (3/9 clones) and CPA02 (3/9 clones) were affiliated with phenol degrading bacterium *C. phenolicus* (87% similarity), clone CPA06 (3/9 clones) was affiliated with syntrophic benzoate degrading bacterium *Syntrophus aciditrophicus* (99% similarity) (Jackson *et al.* 1999) (Figure 3 and Table S1 in Supplementary Information). For enrichment culture NPA, eight RFLP patterns were found. Phylogenetic analysis indicated that clones NPA01 (4/41 clones) and NPA02 (2/41 clones) were affiliated with phenol degrading bacterium *C. phenolicus* (96% similarity) and benzoate-degrading bacterium *Sporotomaculum syntrophicum* (100% similarity) (Qiu *et al.* 2003), respectively.

Phylogenetic analysis indicated that dominant microbes in cultures NPA and CPA were affiliated with two different groups in *Desulfotomaculum* cluster I of *Firmicutes*: clone NPA01 belonged to subcluster Ih, whereas clones CPA01 and CPA02 represented an undefined subcluster in *Desulfotomaculum* cluster I (Figure 3). To detect dominant microbes in enrichment culture NPA, FISH with *Desulfotomaculum* subcluster Ih-specific probe Ih820 was used (Imachi *et al.* 2006) (Table 1). To detect clones CPA01 and CPA02, an arranged probe DEM1164-ar was applied to enrichment culture CPA (Stubner & Meuser 2000). *Bacteria*-specific probe EUB338 mix and *S. aromaticivorans*-specific probe UI178 were also applied to the enrichment cultures. In enrichment culture NPA, non-spore forming short rodshaped microbes reacted with probe Ih820, indicating that bacteria resembling *C. phenolicus* in *Desulfotomaculum* subcluster Ih were represented (Figure 2). In enrichment culture CPA, short rod-shaped bacteria reacted with probe DEM1164-ar, but did not react with probe Ih820 (data not shown), confirming that the dominant populations belonged to a new sucluster in *Desulfotomaculum* cluster I (Figure 2). Both enrichment cultures CPA and NPA did not react with *S. aromaticivorans*-specific probe UI178, indicating that *S. aromaticivorans* were not present in the two phenol-AQDS cultures (data not shown).

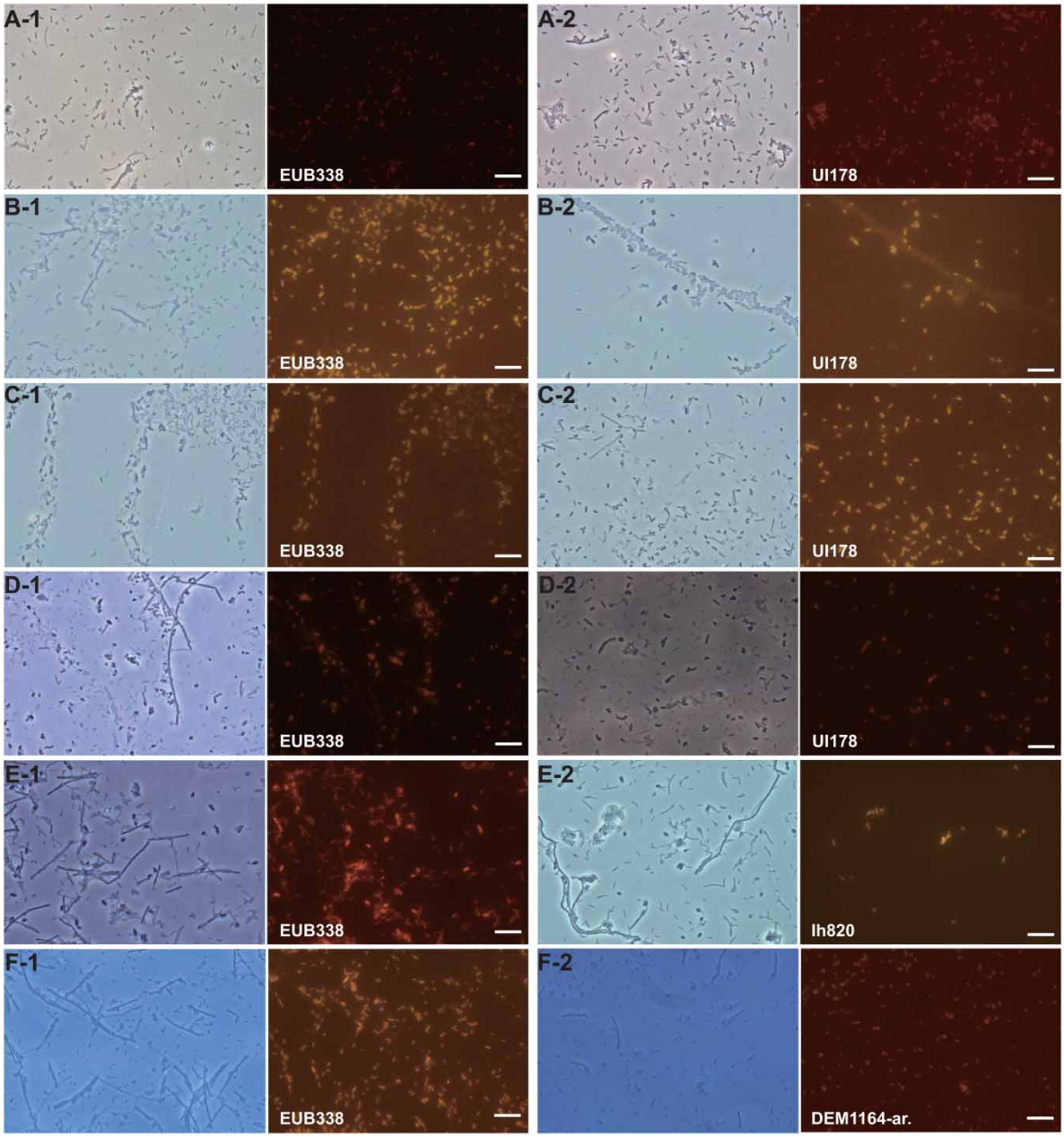


Figure 2 | *In situ* hybridization of phenol and *p*-cresol degrading enrichment cultures with Cy3-labled *Bacteria*-specific probe EUB338 mix (panel 1); *Syntrophorhabdus aromaticivorans*-specific probe UI178, subcluster Ih-specific probe Ih820, and probe DEM1164-ar. specific for clones CPA01 and CPA02 in new subcluster of *Desulfotomaculum* cluster I (panel 2). (a) CP enrichment by phenol with sludge C; (b) CPC enrichment by *p*-cresol with sludge C; (c) NP enrichment by phenol with sludge N; (d) NPC enrichment by *p*-cresol with sludge N; (e) NPA enrichment by phenol-AQDS with sludge N; (f) CPA enrichment by phenol-AQDS with sludge C. Bars represent 10 μm.

Utilization of AQDS by *S. aromaticivorans*, and enrichment cultures CPA and NPA

16S rRNA gene cloning analysis and FISH results revealed the predominant populations were affiliated with *C. phenolicus* (similarity, 87% and 96%, respectively) in CPA and NPA enrichments, and syntrophic phenol degrading bacterium *S. aromaticivorans* were detected in four methanogenic AQDS-free enrichments. To test whether AQDS could be used as an electron acceptor to oxidize phenol by three cultures, batch experiments were conducted. Results revealed that AQDS reduction was not found by three phenol-degrading cultures after four months of incubation, suggesting that neither *C. phenolicus* resembling microbes nor *S. aromaticivorans* could use AQDS (5 mM or 30 mM) as an electron acceptor to

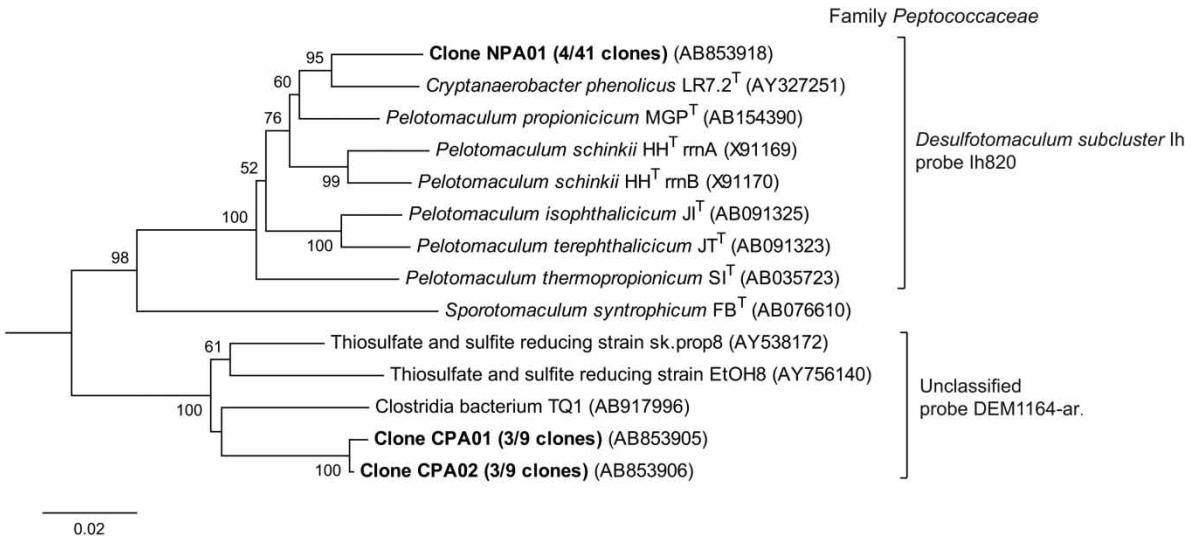


Figure 3 | Phylogenetic tree of *Desulfotomaculum* cluster I in *Firmicutes* based on comparative analysis of 16S rRNA gene sequences, showing the phylogenetic positions of clones obtained from phenol-degrading cultures in this study. The tree was constructed by a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). The sequence of *Arthrobacter globiformis* DSM 20124 (X80736) was used to root the tree (not shown). The bootstrap values that were above 50% are shown at the nodes. The scale bar represents the number of nucleotide changes per sequence position.

oxidize phenol (Table 2). For enrichment cultures CPA and NPA, phenol was completely converted to methane in the absence of AQDS and BES, while phenol was completely converted to benzoate and acetate in the presence of BES. For enrichment culture CPA, the presence of 5 mM AQDS showed significant inhibitory effect on phenol degrading consortia, with only tiny phenol (17–20%) was degraded into benzoate, methane or acetate, however, the addition of 30 mM AQDS completely inhibited the phenol degradation. For enrichment culture NPA, phenol degradation was not observed in the presence of 5 mM or 30 mM AQDS, indicating that AQDS had a strong inhibition to phenol degrading consortia in culture NPA. For *S. aromaticivorans* co-culture, phenol was completely converted to methane and acetate only in the absence of AQDS and BES. However, the degradation of phenol was not observed either with AQDS (5 mM or 30 mM) or BES, indicating that AQDS also had a strong inhibition to *S. aromaticivorans*.

Previous investigations suggest that high concentrations of AQDS (more than 5 mM) have a high redox potential, thus causing the inhibition of methanogens due to the unfavorable environment (Cervantes *et al.* 2000a, 2000b, 2008; Yang *et al.* 2012). Our results provide further support for this suggestion. The AQDS utilization assays showed that *S. aromaticivorans* was more sensitive to AQDS than *C. phenolicus* resembling bacteria of *Desulfotomaculum* cluster I. This may be related to different cell wall structures of these two groups. *S. aromaticivorans* is a gram negative bacterium, whereas *C. phenolicus* resembling microbes are gram positive and heat-resistant bacteria (although spore was not observed) (Juteau *et al.* 2005; Qiu *et al.* 2008). It has been proved that the cell wall of gram-positive bacteria is (20–80 nm) thicker than that of negative bacteria, which provides greater strength and rigidity by multiple cross linked peptidoglycan layers (Yang *et al.* 2020). *C. phenolicus* resembling microbes may be more tolerant to adverse environments. It can be inferred that different tolerance of *Desulfotomaculum* cluster I and *Syntrophorhabdaceae* to AQDS occurring conditions resulted in different distribution of these two clusters in enrichment cultures.

Substrate utilization by two phenol-AQDS enrichment cultures

Phylogenetic analysis results suggested that dominant phenol degrading bacterium in enrichments CPA and NPA was related to phenol degrading *Cryptanaerobacter phenolicus* (similarity, 87% and 96%, respectively), representing new species. Aromatic compounds utilization by two cultures CPA and NPA were studied.

Both enrichment cultures CPA and NPA could degrade phenol, 4-hydroxybenzoate and benzoate completely to methane after one month of incubation, but could not use three cresol isomers (Figure 4, Table 3). Both enrichment cultures CPA and NPA could convert 4-hydroxybenzoate to phenol, and phenol was further degraded to methane. During phenol degradation,

0.28 mM benzoate and 0.01 mM 4-hydroxybenzoate were detected in culture CPA, and 0.26 mM benzoate was detected in culture NPA, indicating that phenol was degraded via 4-hydroxybenzoate and benzoate for two cultures.

Utilization of other aromatic substrates by enrichment cultures CPA and NPA were also tested (Table 3). According to the methane production over four months' incubation, enrichment culture CPA could metabolize 3-hydroxybenzoate, but not catechol, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2-hydroxybenzoate, 3-phenylpropionate, hydroquione and

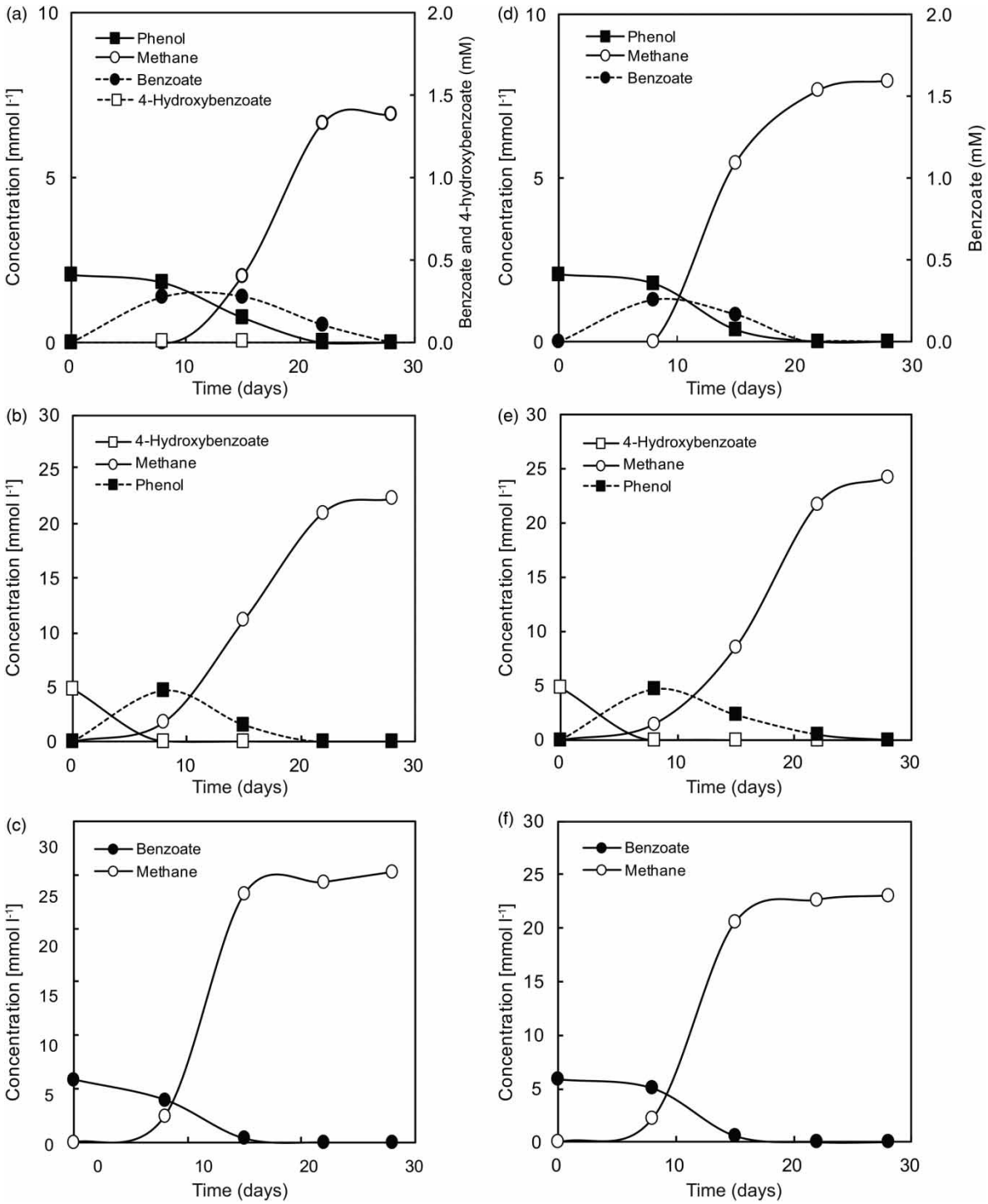


Figure 4 | Degradation of phenol (a, d), 4-hydroxybenzoate (b, e) and benzoate (c, f) by phenol-AQDS enrichment cultures CPA (a–c) and NPA (d–f).

Table 3 | Comparation of enrichment cultures CPA and NPA with described phenol degraders *Cryptanaerobacter phenolicus* and *S. aromaticivorans*

	Desulfotomaculum* cluster I within phylum *Firmicutes			
	Unclassified	**Subcluster Ih**		**Family *Syntrophorhabdaceae* in *Deltaproteobacteria*****
Taxonomy	**Enrichment CPA**	**Enrichment NPA**	***Cryptanaerobacter phenolicus*[a]**	***Syntrophorhabdus aromaticivorans*[a]**
Substrate utilization (mM)[b]				
Phenol (2)	+	+	+	+
o-Cresol (1)	–	–	ND	–
m-Cresol (1)	–	–	ND	–
p-Cresol (1)	–	–	–	+
4-Hydroxybenzoate (5)	+	+	+	+
Benzoate (5)	–[c]	–[c]	–	+
Isophthalate (2)	–	–	ND	+
3-Hydroxybenzoate (2)	+	+	–	–
Catechol (2)	–	+	–	–
2,5-Dihydroxybenzoate (2)	–	+	ND	–
3-Phenylpropionate (2)	–	+	ND	–
Aromatic compounds not utilized (1–2)	phthalate isomers, 2,4-dihydroxybenzoate, 2-hydroxybenzoate, hydroquione	phthalate isomers, 2,4-dihydroxybenzoate, 2-hydroxybenzoate, hydroquione	4-hydroxybenzamide, methyl 4-hydroxybenzoate, 4-hydroxysulphonic acid, 4-hydroxyacetophenone, 4-hydroxybenzoic alcohol, hydroquinone, *o*-, *p*-chlorophenols, 4-hydroxycinnamic acid, 4-hydroxybenzoate hydrazide, 4-hydroxybenzaldehyde, 4-hydroxyphenyl pyruvic acid, 3-(4-hydroxyphenyl) propionic acid, 4-hydroxypyridine, 2-bromophenol, 2-fluorophenol, 2-aminophenol	2-hydroxybenzoate, 2-methylbenzoate, 4-methylbenzoate, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, hydroquinone, resorcinol, aniline, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate
Reference	This study	This study	Juteau *et al.* (2005)	Qiu *et al.* (2008)

[a]The data are from *C. phenolicus* in pure culture and *Syntrophorhabdus aromaticivorans* in co-culture with *M. hungatei*.
[b]The utilization of phenol, benzoate, and 4-hydroxybenzoate by enrichments CPA and NPA was evaluated by substrate depletion and methane production (see Figure 4 for details), the utilization of other substrates was determined by methane production. ND, not determined.
[c]Benzoate could be utilized by *Syntrophus aciditrophicus* or *Sporotomaculum syntrophicum* in enrichment cutures CPA or NPA, but not by *C. phenolicus* resembling bacteria.

phthalate isomers. Enrichment culture NPA could metabolize catechol, 2,5-dihydroxybenzoate, 3-hydroxybenzoate and 3-phenylpropionate, but not 2,4-dihydroxybenzoate, 2-hydroxybenzoate, hydroquione and phthalate isomers.

Metabolic pathways of two phenol-AQDS enrichment cultures

Nowdays, two anaerobic phenol-degrading bacteria have been isolated, pure culture of *Cryptanaerobacter phenolicus* belongs to phylum *Firmicutes* (Juteau *et al.* 2005) and co-culture of *S. aromaticivorans* belongs to *Deltaproteobacteria* (Qiu *et al.* 2008). Physiological traits and metabolic pathways of the two isolates are obviously different: *S. aromaticivorans* transforms phenol, *p*-cresol, 4-hydroxybenzoate, isophthalate and benzoate directly to acetate and methane in syntrophic co-culture with

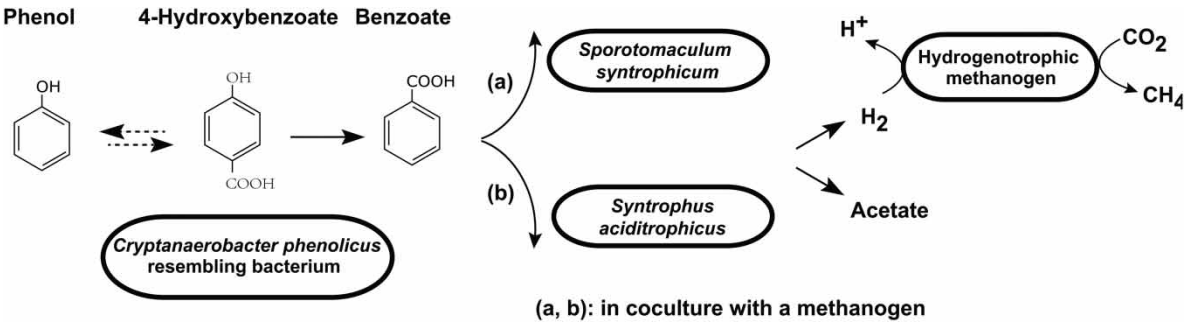


Figure 5 | Proposed model of phenol degradation by *Cryptanaerobacter phenolicus* resembling bacteria in *Desulfotomaculum* cluster I with different partners. Phenol was first converted to benzoate via reversible transformation of 4-hydroxybenzoate by *C. phenolicus* resembling bacteria, benzoate was then degraded into hydrogen and acetate either by *Syntrophus aciditrophicus* or *Sporotomaculum syntrophicum*, and hydrogen was transformed into methane by a hydrogenotrophic methanogen.

hydrogenotrophic methanogens (Qiu *et al.* 2008); however, *Cryptanaerobacter phenolicus* pure culture can only transform phenol to benzoate via 4-hydroxybenzoate (Juteau *et al.* 2005).

Microbes resembling *C. phenolicus* were likely responsible for the conversion of phenol to benzoate, which was further degraded by benzoate-degraders of *Syntrophus aciditrophicus* (Jackson *et al.* 1999) or *Sporotomaculum syntrophicum* (Qiu *et al.* 2003) (Figure 5). In this study, *C. phenolicus* affinities was dominant in two phenol-AQDS enrichments CPA and NPA, representing new species (similarity, 87% and 96%, respectively) (Figure 3). Phylogenetic analysis and further degradation experiments suggested that *C. phenolicus* related microbes in enrichments CPA and NPA were responsible for the conversion of phenol to benzoate. Biodegradation experiment indicated that both enrichment cultures CPA and NPA could degrade phenol completely to methane, benzoate was detected as intermediate (Figure 4, Table 3). Phylogenetic analysis indicated, syntrophic benzoate degrading bacterium *Syntrophus aciditrophicus* (99% similarity) represented 33.3% (3/9 clones) in CPA enrichments, and another type benzoate-degrading bacterium *Sporotomaculum syntrophicum* (100% similarity) represented 4.9% (2/41 clones) in NPA enrichments (Jackson *et al.* 1999; Qiu *et al.* 2003).

The dominant phenol-degrading bacteria in enrichment cultures CPA and NPA show similar metabolic pathways with the cultured *C. phenolicus* (Juteau *et al.* 2005), however, their substrates range for growth are different. Pure culture of *C. phenolicus* can utilize phenol and 4-hydroxybenzoate, while enrichment cultures CPA and NPA could utilize phenol, 4-hydroxybenzoate as well as several other aromatic compounds. Enrichment culture CPA could utilize 3-hydroxybenzoate, and enrichment culture NPA could utilize catechol, 2,5-dihydroxybenzoate, 3-hydroxybenzoate and 3-phenylpropionate. Considering the microbial complexity of enrichment cultures, further isolation attempts of NPA and CPA are in progress to verify the substrate utilization of bacteria in *Desulfotomaculum* cluster I.

CONCLUSIONS

The degradation test using phenol and *p*-cresol as substrate were observed significantly faster than *o*-, and *m*-cresols. 2 mM AQDS mitigated anaerobic phenol degradation and completely inhibited *p*-cresol degradation. 16S rRNA gene analysis revealed that *S. aromaticivorans* (similarity, 100%) was dominant in four AQDS-free enrichments, whereas *C. phenolicus* (similarity, 87% and 96%) was dominant in two phenol-AQDS enrichments. Neither *S. aromaticivorans* nor *C. phenolicus* resembling microbes could metabolize phenol using AQDS as the terminal electron acceptor. The phenol-degrading bacteria in enrichment cultures CPA and NPA show similar metabolic pathways with *C. phenolicus*, however, their substrates range are different.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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