

New DNA Sequences from Bacteria Converting Phenol into Acetate under Strict Anaerobic Conditions

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Abstract—This work reports new fragments of DNA sequences related to microbes able to degrade phenol into acetate under strict anaerobic conditions. For this purpose, anaerobic digesting sludge was acclimatised to degrade phenol, then heat treated and in turn used as fermentative sludge. The resulting microbial community was able to convert phenol into acetate under anaerobic conditions (kinetic constants: 0.396 ± 0.01 and 0.345 ± 0.04 mg of compound $L^{-1} day^{-1}$, respectively). Microscopic, chemical and molecular analyses revealed that only bacteria were present in the final sludge and thus methanogens were eliminated. The bacteria were mainly Gram-negative sporeforming rods, belonging to the Deltaproteobacteria class and had a tendency for aggregation. These are also phenotypically related to organisms thriving at extreme environments. Cloning, temperature gradient gel electrophoresis (TGGE) and probe matching of a short 16S DNA fragment revealed that these new microbes are evolutionary related to, and share 90% of similarities with, *Desulfovibrio sp.*

Index Terms—anaerobic fermentation, bacteria phenol acetate, acetogenesis, SRB

I. INTRODUCTION

Phenol is an industrial commodity and a priority hazardous pollutant [1], [2]. For decades, there has been an interest in reducing the negative environmental impact of phenol bearing streams upon waste treatment processes and biogas production [3].

The fermentative step involved in the breakdown of the aromatic ring into acetate is energetically limited and is carried out by syntrophic microbes [4]. Nevertheless, the vast majority of the microorganisms involved and the pathways used for this process remain unknown.

Historically, attempts to study acetate formation from anaerobic degradation of phenol by acetogens have been carried out with 2-broethane sulphonic acid (BESA),

chloroform or heating at 85 °C. These techniques aimed at the elimination or inactivation of H₂ and acetate consumers [5]–[8].

Sulphate reducing prokaryotes (SRP) have been suspected to be responsible for acetogenesis by aromatic ring degradation under non-methanogenic conditions [9], [10]. Some SRP are able to survive in extreme environments with low redox potential, hard energy conservation limits close to the equilibrium ($\Delta G = 0$), freezing or boiling temperatures, heavy metal reduction and other slow growth conditions [11]–[17].

Until today, complete phylogeny of SRP is unknown and is difficult to decipher. SRP are widely distributed in both Bacteria and Archaea domain [18]. Besides, they are very closely related to non-SRP and therefore difficult to discriminate. This is complicated by the lack of data in public databases, lack of oligonucleotide probes targeting all SRP, evolutionary differences between the 16S rRNA and functional genes due to horizontal gene transfer [11], [19], [18]. Recently, unambiguous identification has been possible only among very close phylogenetically related SRP with the help of a probe database [20].

This work aims at reporting new bacterial sequences related to *Desulfovibrio* microbes that were able to convert phenol into acetate under strict anaerobic conditions. Optic light microscopy and SEM were employed to reveal the effect of experimental procedures on the morphology of the microbial community. Evolutionary relationship was inferred by applying a 16S rDNA based approach. Short gene fragments were amplified and studied by complementing cloning, TGGE and probe matching.

II. MATERIALS AND METHODS

A. Culture Setup

Actively digesting sludge was collected from the Aldwarke waste water treatment plant, Rotherham, UK. Henceforward, all the procedures involving the sludge

were done inside an aerobic chamber containing CO₂ (99%). The sludge was sieved (1 mm²) to remove sand and other particles before use. Subsequently, it was allowed to digest its indigenous organic matter until no biogas production could be measured. Then, it was acclimatised for three months to phenol by increasing its concentration stepwise to produce methane ($3.60 \times 10^{-5} \pm 1.76 \times 10^{-6} \text{ mol CH}_4 \text{ day}^{-1} \text{ gVSS}^{-1}$).

This sludge acclimatised to the conversion of phenol into methane was subsequently heat-treated at $98 \pm 1 \text{ }^\circ\text{C}$ for 15 minutes. Such approach was selected based on successful trials in samples that were certainly different to the present sludge but shared the similarity of having complex microbial communities interacting with mineral and organic materials. For instance, this procedure has been applied to samples from solids waste treatment in order to eliminate non sporeforming microorganisms [21]. It was also applied to suspended cells (not sludge) in aqueous phase to produce an anaerobic consortium capable of degrading phenol into acetate [7].

In this work, the heat treated sludge was inoculated in bottles and was then amended with phenol by following the recommendations of, and using the medium shown in, a standard procedure [22]. This procedure was repeated ten times for six months to increase the number of phenol degraders. Then, a final system was prepared: biomass and phenol concentrations in the initial culture were $6.2 \pm 0.33 \text{ gVSS L}^{-1}$ and $19.54 \pm 0.52 \text{ mg phenol L}^{-1}$, respectively. Two types of control bottles were set up, each missing phenol or sludge. The bottles were incubated at $35 \pm 1 \text{ }^\circ\text{C}$ ($\text{pH} = 7.83 \pm 0.98$) until complete elimination of phenol. After complete non-methanogenic degradation of phenol was achieved, a sample was taken from the bottles for microbial diversity analysis and validation purposes.

B. Microscopy

The procedures of boiling and further enrichment of phenol degrading microbes were coupled to morphology analyses. This was done to verify that the smallest number of similar species were present in the sludge and not only in the suspended fraction of cells as done by [23]. These analyses were done using optic light microscopy (OLM) and scanning electron microscopy (SEM). For OLM analyses, samples were prepared according to the Gram staining technique reported by the kit manufacturer (Pro-Lab Diagnostics, Inc., UK). Images were obtained using a motorized microscope Axioplan 2 Imaging™ by following the procedures of the manufacturer (Zeiss, GmbH, Germany). SEM images were then obtained as described previously by [24]. Potential contamination was verified against blanks.

C. PCR Amplifications of 16S rDNA PCR Amplifications of 16S rDNA

Genomic DNA of sludge samples and blanks was extracted and purified by using a Soil DNA Kit (MoBio Laboratories). A first round PCR was then applied for amplification of a 16S/18S rRNA gene fragment

characteristic of Bacteria, Eukaria and Archaea by using previously reported primers [25]. *E. Coli K12*, *Haloferax volcanii* and red cells were positive blanks for the enzymatic amplification of Bacteria, Archaea and Eukaria domains, respectively. PCR reactions contained 1X REDTaq PCR buffer (Sigma, UK), 200 μM of each dNTP (Sigma), 0.1 μM of each primer, 0.05 units μL⁻¹ of REDTaq polymerase (Sigma) and 200 pg μL⁻¹ of purified template DNA. The reaction mixture was prepared on an ice bath inside of a flow cabinet dedicated to molecular biology work, and then the reaction mixtures were distributed accordingly into 50 μL PCR tubes. These tubes were used for a first round PCR in a thermocycler (Thermo, UK) programmed as reported by [25]. The amplicon was then purified with a QIAquick PCR Purification Kit as instructed by the manufacturer (QIAGEN, Ltd).

D. Production of DNA Amplicons for Cloning and TGGE

The amplicon of the first round PCR was used as a template in a second round PCR. This aimed at amplifying the variable region V3 of 16S rDNA using previously reported set of primers [26]. One set of primers produces an amplicon of 193 bp that was subsequently cloned. The other set produces a -GC clamped 260 bp to increase resolution in TGGE experiments [26]. In this second PCR the conditions were as follows: denaturation was at 96 °C for 5 minutes followed by ten "touchdown" cycles aiming at avoiding spurious by-products generated during the amplification [26]. These cycles consisted in a first step at 94 °C for 1 min and then two cycles were carried out from 65 to 55 °C every 1 °C of temperature decrement. This was followed by a final extension time of 72 °C for 10 minutes. The resulting amplicons (193 bp and 260 bp) were separately purified and concentrated 50 times with a QIAquick PCR Purification Kit (QIAGEN, Ltd). Both DNA templates (193 bp and 260 bp) were stored at 4 °C and then used for cloning and TGGE.

The size of DNA fragments was measured by agarose gel electrophoresis in a gel containing 1.3% agarose, 0.014 μ ml⁻¹ ethidium bromide and 35 ml 1X TAE (40 mM Tris acetate, 20 mM sodium acetate, 1mM Na₂-EDTA). This gel was run at 100 V for 30 min in one electrophoresis equipment (Fisher, U.K.) and subsequently placed on a UV transilluminator (312nm) coupled to a photographic recording system BioDocAnalyse (Biometra, GmH, Germany).

E. Cloning

Subcloning of PCR products was carried out by utilizing the TOPO TA subcloning kit (Invitrogen). Potential bias was reduced by pooling together the purified rDNA from five independent PCR amplifications. These rDNA fragments were subcloned into pCR2.1 cloning vector via TA-cloning and transformed into *E. coli* TOP10 cells as specified by the manufacturer (Invitrogen, U.K.). Recombinant cells were selected

using ampicillin selection and blue/white screening [27]. Bacterial DNA was extracted from positive colonies using the Qiagen mini prep kit (QIAGEN, U.K.), as specified by the manufacturer. Concentrations of plasmid DNAs were estimated via optical density. 200ng of plasmid were used during sequencing.

F. TGGE and Sequencing

16S rDNA from two independent second round PCRs were used in a duplicate TGGE to minimise potential bias. Double stranded rDNA was denatured in single stranded fragments in a polyacrylamide gel [12% wt/vol acrylamide/bisacrylamide 37.5:1, 1XTAE, 2% wt/vol glycerol, 8M urea, 2% vol/vol formamide, 0.07% wt/vol ammonium persulphate, 0.1% vol/vol N,N,N',N'-tetramethylethylene diamine]. The gel was placed on a TGGE equipment (Biometra, Germany) and separation was carried out from 35 to 65 °C, 130 V during 2 hours. TGGE gels were stained with ethidium bromide (0.5 µg ml⁻¹) for 30 minutes, and the pattern was recorded by using the transilluminator-photographic system described above. Once optimal conditions were set up, the separation of the multiple sequence fragments took place in a parallel TGGE. The gel was ethidium stained and photographed. Reproducible profiles were selected to excise fingerprinting bands. rDNA was then eluted by incubating bands in 50 µL TE buffer at 37 °C (30 mM Tris pH 7.6 and 3 mM EDTA). Eluted rDNA from independent bands was reamplified by PCR with previously reported primers in [26]. PCR consisted in one denaturation step (96 °C for 5 min) followed by 25 cycles of annealing (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min). Finally, an extension step was applied (72 °C for 10 min).

G. Sequencing of DNA Extracted from Clones and TGGE Bands

16S rDNA fragments were first reamplified from positive clone colonies or excised TGGE bands were double strand sequenced by employing the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Darmstadt) and the primers M13 forward (TGTAACGACGGCCAGT) and M13 reverse (GGATAACAATTCACACAGG). Sequence reactions were run on an Applied Biosystems 3730 Sequence Analyser following the manufacturer's instruction manual (Applied Biosystems, Darmstadt).

H. Chemical and Physical Analyses

Biomass concentration was assessed as volatile suspended solids (VSS) by applying standard methods [1]. Methane and carbon dioxide were measured to confirm the absence of methanogenic conditions. This was done by using a gas chromatograph (GC, Varian 3400) fitted with a methanizer, flame ionization detector (FID) and injector. The chromatographic column (30m x 0.530mm GS-Q) was packed with 10% nickel nitrate on Chromosorb GAW 100/120. This column operated at 60 °C and inlet pressure of 5.7 psi. The injector and the FID operated at 350 and 280 °C, respectively. In this analysis,

nitrogen gas was used as a carrier gas at a flow rate of 19.0 cm/s.

Liquid samples were filtered through 0.45 µm pore filters. Phenol and acetic acid were analysed by HPLC (Perkin-Elmer, Co., USA). In both cases, the equipment operated at 25 °C, 20 µl of sample injection volume and mobile phase flow of 1 ml min⁻¹. Phenol was separated in a Zorbax column (C18, type zorbax ODS, 4.6 x 250 mm, 5 µm, Agilent Technologies, Co., USA) using H₃PO₄ 0.1 % in water and 70 (V/V) acetonitrile/water as mobile phase. It was then detected by UV absorbance at 230 nm. Acetic was separated in a SB-Aq column (4.6 x 150 mm, 5 µm, Agilent Technologies, Co., USA) using H₃PO₄ 20 mM and 99 (V/V) acetonitrile/water as mobile phase. It was subsequently detected by UV absorbance at 210 nm.

I. Evolutionary Analysis

Each cloned or TGGE excised sequence (193 to 260 bp) was analysed to assess specificity by using BioEdit [28]. The absence of vector and cross contamination was searched with VecScreen (<http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html>). A restriction map was then developed in WebCutter to determine the absence of EcoRI restriction sites (www.firstmarket.com/cutter/cut2.html). Chimera detection was done by performing fractional treeing on the 5' and 3' ends of the fragments and by utilising the CHECK_CHIMERA program in the Ribosomal Data Base Project (RDP-II) [29]. Each sequence was compared with public databases in the National Centre for Biotechnology Information (NCBI) and the RDP-II [29]. More than 100 sequences were retrieved without low complexity regions and without masking DNA sequences. A set of full and partial 16S rDNA sequences having 90% and more similarities was screened and then matched with targeted regions of probe sequences available in ProBase [20]. Clone and database sequences matched 100% by probes were aligned by using the program ClustalW available in BioEdit [28]. Double checking of phylotype affiliation was made by comparing the results from NCBI and RDPII databases. Molecular evolutionary analyses were then conducted using MEGA version 4 [30]. A distance matrix was performed with the Kimura 2-parameter [31] to group closely related sequences (≥99.6 %). The evolutionary tree was constructed with the neighbour-joining method and its reliability was simultaneously assessed by bootstrapping 1000 replicates. Those sequences favouring the best multiple alignment and tree reliability were manually selected. More sequences were searched in NCBI matching 100% of probes and treeing was performed again.

III. RESULTS AND DISCUSSION

A. Degradation of Phenol and Acetic Acid Production

Acclimatization to phenol and subsequent heat treatment of sludge led to an inoculum capable of eliminating phenol and producing acetic acid under anaerobic conditions (test bottles), which is a well known metabolic process [4]. No changes in phenol or acetic

acid were detected in the controls. Contrarily, in the test bottles the lag phase of phenol elimination (20 days) was shorter than the acetate formation (Fig. 1). Phenol was completely eliminated in less than 65 days, producing 0.76 ± 0.13 g of acetic acid per gram of phenol. The kinetic constants of both phenol disappearance and acetate formation were 0.396 ± 0.01 and 0.345 ± 0.04 mg of compound $L^{-1} day^{-1}$, respectively. Certainly, these small values indicate that acetate formation from phenol is not facilitated.

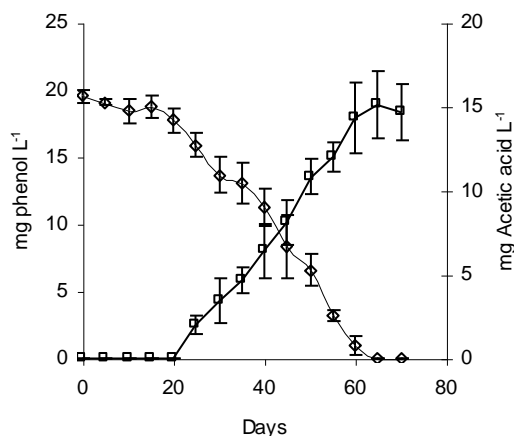


Figure 1. Degradation of phenol (◇) and production of acetic (□) by an anaerobic consortium that survived after a heat treatment of $98 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$. $\text{N}_2\text{S}\cdot 9\text{H}_2\text{O}$ was utilised to favour reductive reactions.

CH_4 was not detected during the incubation period implying the absence of methanogenic activity. This lack of syntrophism was reflected by the accumulation of acetate and long residence time for phenol degradation. However, the presence of other acetate consumers should not be discarded since the concentration of acetate was relatively smaller ($39.58\% \pm 12.97\%$) than its corresponding stoichiometric value ($1.92 \text{ g acetic acid g}^{-1}$ phenol). If methanogens were eliminated then sulphate reducers or denitrifiers were likely to be present in the inoculum. This assumption is valid because anaerobic aromatic ring reduction into acetate by a pure culture, excluding sulphate reducers and denitrifiers, is thermodynamically impossible [10]. Syntrophism is needed to overcome the endergonic energetic barrier ($\Delta G = 5.65 \text{ kJ mol}^{-1}$ phenol) to form acetate and butyrate by acetogens [7], and the elimination of H_2 (i.e. $[\text{H}_2] < 10^{-4} \text{ atm}$) to allow the conversion of butyrate to acetate [32].

The small number of species found in the final inoculum may participate in a complex trophic network preparing the phenol molecule to be finally converted to acetate. Although the role of each species is beyond the scope of this work, it is generally accepted that phenol is carboxylated by microbes utilising CO_2 [9] regardless the formation of acetate [23]. Subsequently, other microbial transformations break down the carboxylated molecule and produce acetate [33], [6].

These results therefore suggest that the formation of acetate from phenol without the syntrophic cooperation of methanogens can be promoted by other microorganisms in the treated sludge. Historically,

acetogenesis from phenol has been observed using different inoculum. Acetate formation from phenol was observed in an experiment using sewage sludge. In such experiment acetogenesis was facilitated due to contamination by sulphate reducers [9], which consume the H_2 produced during acetate formation [34], [35]. Anaerobic degradation of phenol into acetate and butyrate was also possible using suspended cells [7].

B. Optical Light and Scanning Electron Microscopic Analysis

Microbes were not observed in the controls before and after the heating process ($98 \text{ }^{\circ}\text{C}$ for 15 minutes). In the case of the sludge, heating destroyed its natural aggregates and produced a culture of practically isolated microbes, which were most probably interacting with the heat treated minerals and organic matter (Fig. 2, frame 1 and 2). The subsequent acclimatization of such culture to use phenol may have increase the selectivity of phenol-degrading acetate-forming microbes. These microbes have a tendency for aggregation as shown in Fig. 3 frame 3.

Fig. 3 also shows that temperature degraded extracellular polymeric substances (EPS) e.g. polysaccharides, proteins, phospholipids and humic substances [36]. In consequence, this might be another negative factor lowering the degradation of phenol. This is because the destruction of EPS did not allow the retention of exoenzymes and therefore disturbed the functionality of a multicellular synergistic microconsortia [36].

Gram negative bacteria were predominantly present in the produced consortia and remain predominant during 70 days of incubation period (Fig. 2, frame 2). However, scanning electron microscopy confirmed that there were also few sporeforming shaped microbes (Fig. 2, frames 3 to 6), which is a common characteristic of Gram positive cells. The staining method may therefore be insufficient to establish a Gram negative characteristic for the whole phenol-degrading culture. In the past, this method led to misclassification of some *Desulfotomaculum* species that were later demonstrated to have a true Gram positive cell organization [37], [38].

Microbial aggregates were found and they were compacted in bulks, where cracks may be an indication of aggregation (Fig. 2, frame 3). A variety of sporeforming rods were observed. Similar observations were obtained by [7] but their sludge and experimental conditions were different. Although those microbes were also able to degrade phenol, it is not possible to know if they share a genetic relationship to the ones reported in the present work.

Based on molecular studies (discussed in the following sections) coupled to morphology analyses, the assumptions to identify the microbes can be narrowed. One group have rounded ends and seemed to grow as single cells or in pairs resembling *Desulfovibrio sp.* (Fig. 2, A). Thick and long rods with pointed ends forming a central spore (Fig. 2, B) were also observed. They are similar to the long non-motile rods ($0.6 \text{ }\mu\text{m}$ width) found

in pure cultures carboxylating phenol to benzoic acid during the co-digestion of yeast extract and proteose peptone [9], [6].

A third group are thin long rods making short chains and irregular filaments (Fig. 2, C). They are similar to the acetogen *Syntrophobacter* which is commonly associated

with *Desulfovibrio sp.* [39], [40]. Comma shaped rods (Fig. 2, D) and thin short rods individually or in form of chains (Fig. 2, E) were also observed. They resemble *Desulfovibrio sp.* and *Syntrophobacter* previously observed in methanogenic cultures [39].

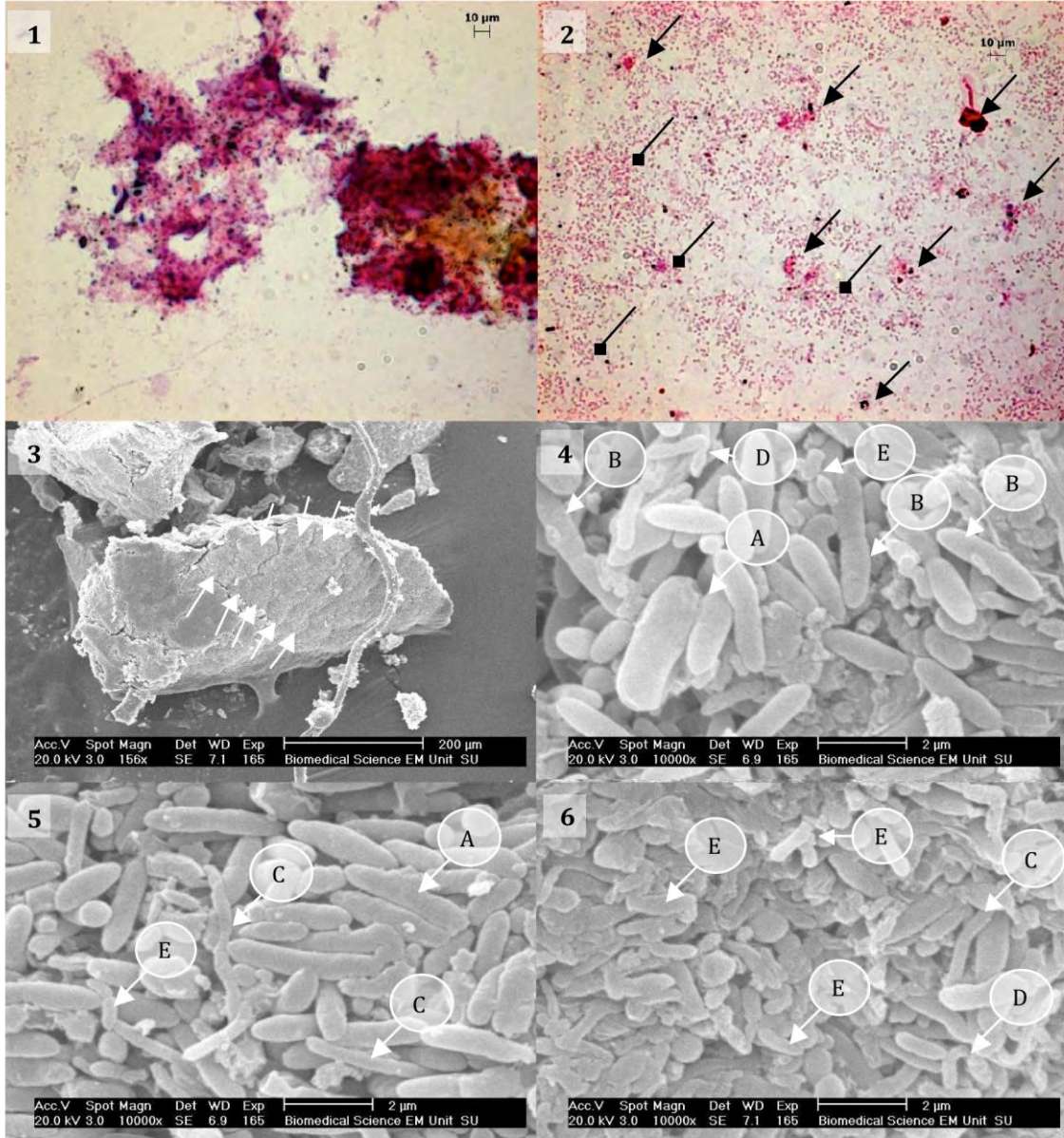


Figure 2. Optic light microscopy image of methanogenic sludge prior (1) and subsequently (2) to heating at $98\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 15 minutes. Gram negative bacteria were mainly found in the destroyed aggregated (2). In frame 2, diamond arrows show colonies in process of aggregation. Pointed arrows show formed aggregates. Frames 3 to 6 are SEM images showing the phenol-degrading acetate-forming consortia obtained after heating. In frame 3, the arrows point at the aggregates forming a bulk. Circled tags: A. Thick and thin long coupled rod with rounded ends forming a spore at the end ($1.87 \pm 0.28 \times 0.59 \pm 0.11\text{ }\mu\text{m}$). B. Thick long rod with pointed ends forming a central spore ($3.01 \pm 0.20 \times 0.65 \pm 0.06\text{ }\mu\text{m}$). C. Thin rods forming chains ($2.48 \pm 0.75 \times 0.35 \pm 0.04\text{ }\mu\text{m}$). D. Comma shaped rods ($1.78 \pm 0.24 \times 0.33 \pm 0.05\text{ }\mu\text{m}$). E. single or forming chain bacillus ($0.98 \pm 0.17 \times 0.30 \pm 0.05\text{ }\mu\text{m}$).

Morphological similarities are shared with *Desulfonema ishimotoei*, *Desulforegula conservatrix*, *Desulfofaba gelida* and the *Desulfovibrio* species *D. desulfuricans*, *D. mexicanus* and *D. vulgaris*. However, their 16S rRNA fragment shares less than 90% of similarities. The microbes observed herein may therefore be *novi* species resembling SRB like *Desulfobacteraceae sp.* and *Desulfovibrio sp.*

C. Molecular Analysis of Sequence Fragments

1) Affiliation

Only the bacteria domain was identified in the culture anaerobically converting phenol into acetate. Methanogenic Archaea are generally eliminated by heating [21] although some might thrive at $100\text{ }^{\circ}\text{C}$ [40]. This was confirmed by three different ways showing: the

lack of methanogenic activity (from GC analysis); sequences of Archaea (from PCR amplifications).
methanogens (from SEM images) and 16S rDNA

TABLE I. PHYLOTYPIC AFFILIATION OF CLONES AS FOUND IN THE RDP AND NCBI DATABASES. NOTES: 1. INFORMATION RETRIEVED THE 25TH OF APRIL 2014; B. PERCENT OF DIVERGENCE.

RDP(II)		NCBI		
Clones (GC%)	Confidence %	94% ≥		% identities 90 to less than 94%
C69 (61.9)	55	Environmental samples	U and Tc bioimmobilization (EF507980)	River sediment contaminated with aromatics (DQ444092)
			PCB dechlorinating consortia (EF393046)	Terephthalate-degrading sludge (AF229789)
			UASB granular sludges (AB291504)	Cold sediments (AB240699)
			Heat treated sludge (EF437217)	Reed bed reactor (AB240344)
				DMP degrading UASB (EF029850)
				Shallow lake freshwater sediment (AB127640)
				Petroleum contaminated aquifer (EU037976)
C19 (63.7)	75		Glacier in the Himalayas (EF434237)	
			wetlands (DQ145163)	
C67 (58.1)	59		Reservoir sediments (AJ518304)	
			Lake sediment (DQ642331)	
			Coal contaminated forested wetland (AF523965)	
			Salt marsh sediment amended with petroleum (EF582552)	
%D ^b 0.4			Salt marsh sediment (AY711819)	
			Acetate-utilizing methanogenic profound sediments (AM181839)	
	Deltaproteobacteria	Trichlorobenzene biodegradation (AJ009465)	Hydrocarbon- and chlorinated-contaminated aquifer (AF050538)	
			Syntrophic propionate oxidation in flooded soil (AY607128)	
			Trichloroethene-contaminated site (AF529129)	
		Proteobacteria		Hydrothermal sediments in the Guaymas Basin (AF420341)
	Thermodesulfobacteriaceae		Hydrothermal sediment (AB175567)	
	Firmicutes		stromatolites in hypersaline marine environment (AY435187)	
C23 (63.3)	93	Environmental samples	PCB dechlorinating consortia (EF393039)	Anoxic zone of a meromictic lake (DQ642340)
			Diethyl phthalate and benzoate degrading UASB (EF053099)	Fatty acid oxidizing syntrophs in granular sludge (AF482443)
			Municipal wastewater treatment plant (CR933142)	Tetrachloroethylene degrading consortium (AF447135)
			Diethyl phthalate and benzoate degrading UASB (EF053105)	UASB reactor degrading trichloroethene (AY446406)
			Wetland sediments (DQ145170)	
			PCB dechlorinating Consortia (EF392922)	
			Lake sediment (DQ067032)	
			Bioreactor pretreating potable water (DQ066685)	
			Mesophilic UASB sludge granules (AB267042)	
				Deltaproteobacteria
		4-Methylbenzoate-degrading consortium (AF254389)	Phototrophic sulfide-removal bioreactor (DQ383313)	
		UASB treating hypersaline tannery wastewater (AM157477)	Anoxic rice field soil (AB293319)	
			Petroleum contaminated aquifer (EF658582)	
			Tidal Sediment (AY254938)	
			Gas hydrates (AY542236)	

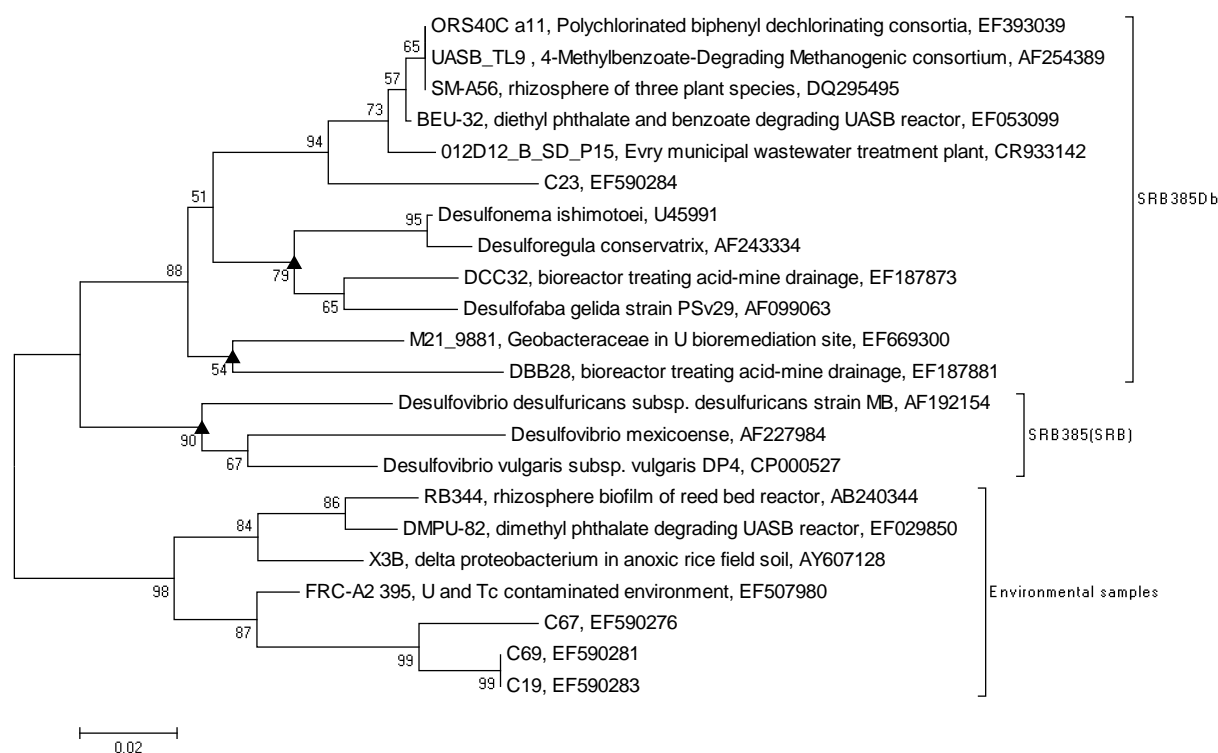


Figure 3. Evolutionary distance tree of closely related 16S rDNA fragments of Deltaproteobacteria. Clones C67, C69 and C19 were closely related to *Desulfovibrio* and C23 was related to *Desulfobacter*. Tree was constructed using MEGA version 4.1 (Kumar, Tamura, Nei 2004). Distances were calculated using the Kimura 2-parameter model. Tree was computed using the Neighbour Joining method and its reliability was tested by bootstrapping 1000 replicates and setting cut-off values higher than 50% (displayed over the branches). Scale bar represents 2% of number of differences between pair sequences. Sequences not retrieved by BLAST are marked with a black triangle (▲).

The clones were associated (55% confidence) to the Deltaproteobacteria subclass by using the RDP(II) tool but false affiliation to other phyla were found by using BLAST (Table I). BLAST retrieved sequences from different phyla such as Thermodesulfobacteria (AB175567) and Firmicutes (AY435187), which have 91% similarity to clones C19, C67 and C69. This association to different phyla indicates therefore the presence of molecular homoplasy (parallel and convergent evolution) in the 16S rDNA data. Homoplasy is a well recognised problem and is the result of the effect of similar environments over a limited combination of four letters in the genetic code [41]. Here again, the 16S rDNA shows that a particular environmental event (heating) may lead to the confusion of relating organisms from different lineages.

The GC content (60%>) indicates a relationship with thermophiles but this is not always the case [42]. The clones reported here are closely related (94%>) to sequences found in UASB reactors treating chemicals; aromatics or heavy metals contaminated sites; and polluted water system sediments (Table I). A lower relationship (90%>) was found with anaerobic microconsortia thriving in extreme environments such as hydrothermal vents, glaciers, sediments, and hypersaline sites. Some of these sequences belong to microbes utilising methylated and carboxylated aromatics; terephthalic acids, petroleum, coal and chlorinated hydrocarbons.

There are also similarities with sequences found in either methanogenic or methanotrophic sediments. C19,

C67 and C69 are similar (90%) to sequences found in acetate-utilizing methanogenic profound sediments (AM181839). C19 and C69 are very similar (91%) to those found in methanotrophic communities in hydrothermal sediments in the Guaymas Basin (AF420341). C23 shows deeper phylogeny association with *Syntrophus* (AY780562, DQ383313) and *Geobacter* (AB293319), and is very similar (98%) to 4-Methylbenzoate-degrading methanogenic consortium (AF254389).

The clones had more than 94% similarities with sequences found in different geographic locations with similar environments. C19, C67 and C69 were similar to those found in Oregon, Ohio, Tsukuba and Berlin. Clone 23 is more than 94% similar to sequences classified as environmental samples (found in Ohio, Hong-Kong, Evry, Washington, Shanghai and several Japanese UASB reactors) and Deltaproteobacteria (found in Jiuduansha, Tainan and Chennai). These findings suggest that similar environmental conditions are therefore related with similar 16S rDNA sequences regardless of geography.

The amplified fragment may be evolutionary related with the capacity of surviving in hardship environments. This kind of adaptation is homologous in functional gene fragments across samples, encoding a potential protein specific for similar environments [43].

Natural selection is valid here because similarities on 16SrDNA fragments indicate that microorganisms may be selectively adapted to the environmental conditions. Neutralism also applies because C19, C67 and C69 and C23 were 90% and 98% similar to sequences in the

rhizosphere of plants (AB240344 and DQ295495). In this case, the microbes were already adapted by chance to respond to the degradation of a deliberately added contaminant (AB240344).

2) Phylogenetic tree and probe matching

The clones reported herein matched, with at most one mismatch, 100% the probes DELTA495, SRB385Db and SRB385(SRB). These short sequences were used to target Deltaproteobacteria, Desulfobacter and Desulfovibrio sequences, respectively. Probe matching allowed the elimination of homoplasious sequences; the selection of sequences from Table I and the search of microorganisms that were not retrieved by BLAST (*Desulfonema ishimotoei*, *Desulforegula conservatrix*, *Desulfobacter gelida* and *Geobacteraceae*). These SRB Deltaproteobacteria (Fig. 3: ▲) have 81 to 86 % similarity to the clones reported herein and produce a robust tree topology (cut off 50%>).

TABLE II. THE PHYLOTYPIC AFFILIATION WAS CONFIRMED BY MATCHING OF CLONE AND TGGE SEQUENCES. THE POSITIVE SYMBOL IS USED WHEN THE SEQUENCE MATCHES 100% THE SEQUENCE OF THE PROBE FOR DELTAPROTEOBACTERIA (DELTA495A), DESULFOBACTER (SRB385DB) AND DESULFOVIBRIO (SRB385). THE NEGATIVE SYMBOL ACCOUNTS FOR ONE MISMATCH. REAMPLIFICATION OF DESULFOBACTER FROM TGGE WAS UNSUCCESSFUL. PROBE SEQUENCES ARE AVAILABLE IN PROBASE (LOY A., 2007).

Clones	DELTA495a	SRB385Db	SRB385	
19	+	-	-	
23	+	+	-	
67	+	-	-	
69	+	-	-	
PCR	TGGE	DELTA495a	SRB385Db	SRB385
	Band			
A	1 (EF373707)	Shorter	-	+
	2	+	-	+
	3	+	-	+
B	1	+	-	+
	2	+	-	+
	3(EF437217)	+	-	-

The close relationship of C23 to Desulfobacter (Fig. 3) and 100% of probe matching (Table II) suggest that C23 is closely related to Desulfobacter genera. C19, C67 and C69 have one mismatch with both probes and a closer affiliation to *Desulfovibrio species*. These three clones also share with *Desulfovibrio species* the common characteristic of being Gram negative (see gram staining above).

In this work, anaerobic fermentation was driven by the use of CO₂ as the sole internal electron acceptor. Thus, these species must be fermentative but also, as the molecular and microscopy study suggest, they are closely related to SRB (Gram negative, see species in the tree (▲)). In the past, using substrates other than phenol, some fermentative species were also closely related to SRB [44], [45]. However, this not necessarily means that those

fermenters are the same, and with the same functions, than the ones found in this work.

3) TGGE fingerprinting

The TGGE profile of two independent PCR mixtures (triplicates of A and B) shows at least five bands (fig. 4), which is an indications that the fermentative culture was integrated by more than one bacterium (Archaea and Eukaria were not found as mentioned). Given the limitation of this technique, only one band was successfully reamplified from TGGE gels (shown in triplicates for each A and B). Here again a probe matching was done and the amplified band matched 100% the probe SRB385 (Table II).

On the other hand, clone 23 matches 100% *Desulfobacter* while the other three only have one mismatch. Fingerprinting techniques and cloning are not exact and have potential sources of biases; they are PCR based; rely on bioinformatics tools and public databases. Nonetheless their complementary utilization allows inferring that the culture may contain at least one species of each *Desulfobacter* and *Desulfovibrio*.

The bacteria found in this work are fermentative and these are capable of carrying out acetogenesis given the conditions of the used medium. It is not possible to say that these bacteria are sulphate reducers and such assumption is beyond the scope of this work, where CO₂ rather than sulphate reduction was the main anaerobic biochemical process. Another reason is that the 16S rDNA segment (used in this work) lack of information on physiology, e.g. sulphate reduction. Nevertheless, it is evident that these bacterial sequences have a close relationship with SRB sequences, which are spread out over distantly related taxas containing organisms with other modes of energy conservation such as fermentation [18].

The sequences found in this work and those from SRB share the similarity of coming from microbes that survive in extreme environments with low redox potential; hard energy conservation and slow growth conditions [11], [14], [15]-[17] or degrade a substrate at thermodynamic limits close to the equilibrium, i.e. $\Delta G = 0$ [12]. All these factors were reunited serially or simultaneously during the whole work. Certainly, such factors should have acted in turn as selective drivers to deliver phenol fermenters. Initially, the acclimatization of the digesting sludge, to convert phenol into acetate, selected a specialized consortia. Then, the thermal treatment destroyed the established synergy between the different components of the sludge. More microbes were evidently eliminated and only bacteria survived till this point. Nonetheless, there is possibility of having dormant bacteria that were not necessarily involved in the degradation of phenol.

D. Relevance

The consortia found in this work is related to bacteria living in extreme environments and some of them are able to degrade aromatic chemicals. These bacteria could be used in remediation works to overcome the limitations of aromatic ring degradation and enhancement of biogas production.

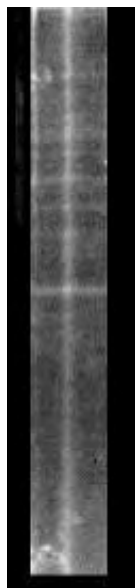


Figure 4. Bacterial 16S rDNA separated by TGGE. The fingerprinting profile was obtained from two independent processes of PCR and TGGE. Denaturing was carried out from 35 to 65 °C, 130 V and 2 hours. Five to seven bands were observed and only the reproducible ones were excised to sequence the 16S rDNA. Only one band (pointed arrow), from each set, was successfully amplified and sequenced. The band matched 100% the probe for *Desulfovibrio* SRB385(SRB).

IV. CONCLUSION

Heating an anaerobic sludge methanizing phenol produced a culture able to convert phenol to acetate anaerobically. The fermentative culture tended to form aggregates and it was mainly integrated by Gram-negative sporeforming rods occurring in single cells or pairs, short chains and filaments. These microbes are related to other microorganisms thriving in extreme environments and limiting growth conditions. Cloning, TGGE and probe matching suggest that this new microbial community is closely related to *Desulfovibrio* sp.

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