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EXPERIMENTAL ARTICLES

Mutualistic Growth of the Sulfate-Reducer *Desulfovibrio vulgaris* Hildenborough with Different Carbohydrates[1]

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Abstract—*Desulfovibrio vulgaris* Hildenborough genome presents a phosphotransferase system putatively involved in the transport of carbohydrates. However, utilization of sugars by this sulfate-reducing bacterium has never been reported. Herein, we have observed proliferation of *D. vulgaris* Hildenborough with some carbohydrates, in mutualism with *Stenotrophomonas maltophilia,* a non-fermentative, gram-negative gammaproteobacterium, or *Microbacterium*, a gram-positive actinobacterium. These results suggest the importance of feedback interactions between different heterotrophic bacterial species including the alternative for *D. vulgaris* of exploiting additional organic resources and novel habitats. Thus, *D. vulgaris* strongly participates in the mineralization of carbohydrates both in complex natural and artificial systems.

Keywords: mutualism, *Desulfovibrio vulgaris* Hildenborough, *Stenotrophomonas maltophilia*, Microbacterium sp.

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Desulfovibrio vulgaris subspecies *vulgaris* strain Hildenborough, a member of the delta subdivision of the *Proteobacteria* group, is a typical example of sulfate-reducing bacteria, i.e., it uses sulfate as terminal electron acceptor for the heterotrophic oxidation of a few organic compounds including lactate, pyruvate, formate, and some primary alcohols [1]. Hydrogen increases the growth yield on lactate and growth on acetate plus CO_2, with H_2 as sole energy source, is also possible [2]. *D. vulgaris* Hildenborough fermented pyruvate in the absence of sulfate [3]. Due to the presence of an incomplete tricarboxylic acid cycle [4] carbon sources are generally oxidized down to acetate [5]. To date, utilization of sugars by *D. vulgaris* Hildenborough has not been reported. Utilization of carbohydrates has been reported for *Desulfovibrio* fructosovorans [6], *D. zosterae* [7] and *D. cavernae* [8].

The putative phosphotransferase system (PTS) genes present in *D. vulgaris* Hildenborough genome [9] have been scarcely studied. Sequence homology analysis suggests that *D. vulgaris* Hildenborough could potentially transport PTS-sugars of the mannose family [10]. The additional presence in *D. vulgaris* Hildenborough of genes encoding enzymes of the Embden–Meyerhof–Parnas pathway [9, 10], the degradation pathway of PTS-transported sugars, indicates that mannose class sugars may be degraded by this pathway to pyruvate, producing ATP by substrate level phosphorylation [10]. *D. vulgaris* is a common member of diverse microbial communities from soils [11] and a variety of environments and actively participates in the complex process of organic matter degradation. PTS-transported sugars are common carbohydrates likely to be found in natural habitats where *D. vulgaris* has been detected [12].

In order to define conditions for potential growth of *D. vulgaris* Hildenborough on several carbon sources, including several mannose-PTS sugars, this strain's growth was examined under different conditions including interactions with other bacteria.

MATERIALS AND METHODS

Growth conditions. A volume of 100 μL of a *Desulfovibrio vulgaris* Hildenborough culture in exponential phase of growth (OD at 600 nm 0.37), measured with a Hitachi U-180 spectrophotometer, was inoculated on modified A1 medium [13] plates with the following composition per liter: 2 g NH_4Cl, 2 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 0.035 g $CaCl_2 \cdot 2H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.125 g L-cysteine hydrochloride, 0.1 g of yeast extract and 0.002 g EDTA. The pH was adjusted to 7.5 by addition of NaOH and 15 g/L bacto-agar were added. After autoclaving, 1 mL of an anoxic supplement solution (per liter: 2 g $CaCO_3$, 6 g iron(III) citrate, 1.44 g $ZnSO_4 \cdot 7H_2O$, 0.9 g $CoSO_4 \cdot 7H_2O$, 0.06 g H_3BO_3, 0.1 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g $NiCl_2 \cdot 6H_2O$, 50 mg

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Na_2SeO_3 and 51.3 mL of 37% HCl) was added. Modified A1 medium plates containing either 20 mM of sodium sulfate or the alternate electron acceptor 2,6-anthraquinon-disulphonate (AQDS) were also prepared. Sterile circular pieces of Whatman 3 MM filter paper embedded with different carbon sources (prepared at 1 M) were placed on top of the medium in petri dishes over the *D. vulgaris* inoculum. These plates were incubated at 30°C, in anaerobic jars, under two distinct atmospheres (nitrogen or a hydrogen/nitrogen mixture (2.5% H_2/97.5% N_2)). Colonies with white-gray coloration were growing next to the filters (see "Results and discussion" section) and were collected with a sterile pipette tip. Afterwards they were suspended in microfuge tubes with 500 μL of anaerobic PBS buffer.

Tip-collected colonies were grown in Postdate's medium C [11], which contained per liter: 0.5 g KH_2PO_4, 1 g NH_4Cl, 4.5 g Na_2SO_4, 0.06 g $CaCl_2 \cdot 6H_2O$, 0.06 g $MgSO_4 \cdot 7H_2O$, 6 g sodium lactate, 1 g yeast extract, 0.004 g $FeSO_4 \cdot 7H_2O$ and 0.3 g sodium citrate $2H_2O$. The pH was adjusted to 7.5.

PCR amplification and analysis. The extracted DNA [14] was used as a template for a PCR amplification of the 16S rRNA gene sequences. The PCR products were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) [15]. PCR reactions were carried out in a total volume of 25 μL containing 3 μL of the above suspensions (previously heated for about 5 min at 95°C for cell lysis), 1× PCR buffer (TaKaRa Bio. Inc.), 200 μM dNTPs, 1 μM of each primer 341FGC and 518R [15] and 0.125 μL of Ex *Tag* (TaKaRa Bio. Inc.) (5 U μL^{-1}). The PCR cycling reactions included an initial denaturation step at 95°C for 2 min, followed by 35 cycles at 95°C for 20 s, annealing at 55°C for 20 s, extension at 72°C for 40 s. The last cycle was followed by 10 min at 72°C. Bacterial 16S rRNA gene fragments from known strains were used as migration markers for gel comparison: *Escherichia coli* K12 (CECT 433), *Paenibacillus* sp. DSM 34 and *Streptomyces caviscabies* ATCC 21619. Relative quantification of DNA band intensity after DGGE analysis was carried out as previously described [14].

RESULTS AND DISCUSSION

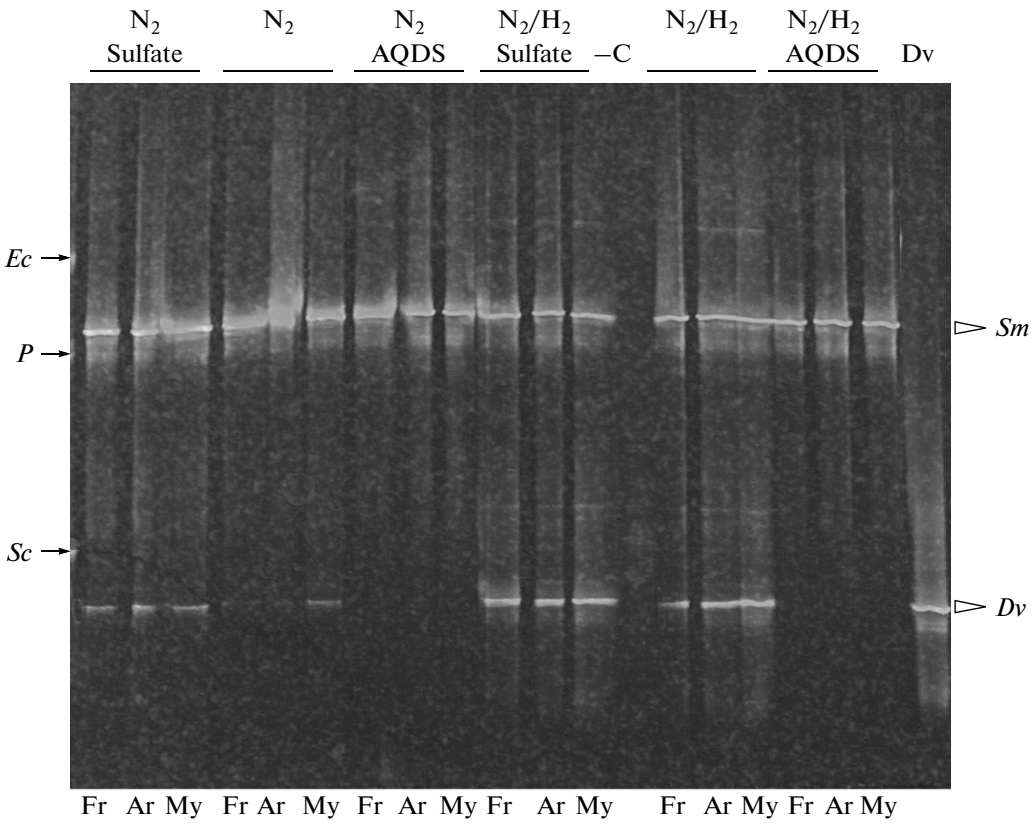
In the growth experiments, no colonies were observed next to the filters containing mannose or glucose, but colonies presenting a white-gray coloration were found next to filters with fructose, arabinose and xylose. Growth was also observed for mannitol and myo-inositol. DGGE analysis (Fig. 1) showed additional bands to the ones corresponding to *Desulfovibrio vulgaris* Hildenborough indicating that those colonies were formed by cells of different microorganisms, and therefore that white-gray colonies contained contaminant microorganisms, which were growing during the incubation procedure. Recovery of the bands followed by sequencing allowed the identification of the microorganisms developing in the cultures: *Stenotrophomonas maltophilia* (Blast E value $4e^{-69}$) and *Microbacterium* sp. (Blast E-value $3e^{-56}$). Suspensions of the colonies were observed under a phase-contrast microscope. *Desulfovibrio* motile cells were observed and many cells were larger than the ones observed in lactate/sulfate cultures. Slightly curved and straight motile rods were also present. One hundred microliters of suspensions of tip-collected colonies grown under a nitrogen atmosphere on medium lacking the addition of sulfate were re-inoculated in a lactate/sulfate Postgate's medium C [11]. As shown in the DGGE analyses of Fig. 2, *D. vulgaris* cells outcompete the cocultured microorganism during growth in medium containing lactate and sulfate, showing sulfate reduction during its growth in this medium.

Albeit dependent carbohydrate growth of *D. vulgaris* Hildenborough has never been reported, the *D. vulgaris* Miyazaki F strain, similar in physiology to the Hildenborough strain, can show poor growth on glucose [16]. We were not able to reproduce such results with *D. vulgaris* Hildenborough strain, either with glucose or mannose. However, here it is shown that both *S. maltophilia* and *Microbacterium* interact with *D. vulgaris* Hildenborough allowing the sulfate-reducer to proliferate in a medium with carbohydrates where it is unable to grow by itself. Both microorganisms found in coculture with *Desulfovibrio* have been isolated from both clinical and environmental sources, noteworthy *S. maltophilia*, is known to be a plant-root associated bacterium [17, 18], often being a dominant member of the rhizosphere microbial community [19]. *Microbacterium* rhizosphere isolates have also been reported [20, 21]. The reported utilization of carbon sources by *Microbacterium* sp. and *S. maltophilia* is heterogeneous, for instance, growth on fructose or mannose is only possible for some *S. maltophilia* strains and most strains are unable to use arabinose [18, 22]. The type strain *S. maltophilia* LMG 958^T is unable to utilize arabinose and xylose [23].

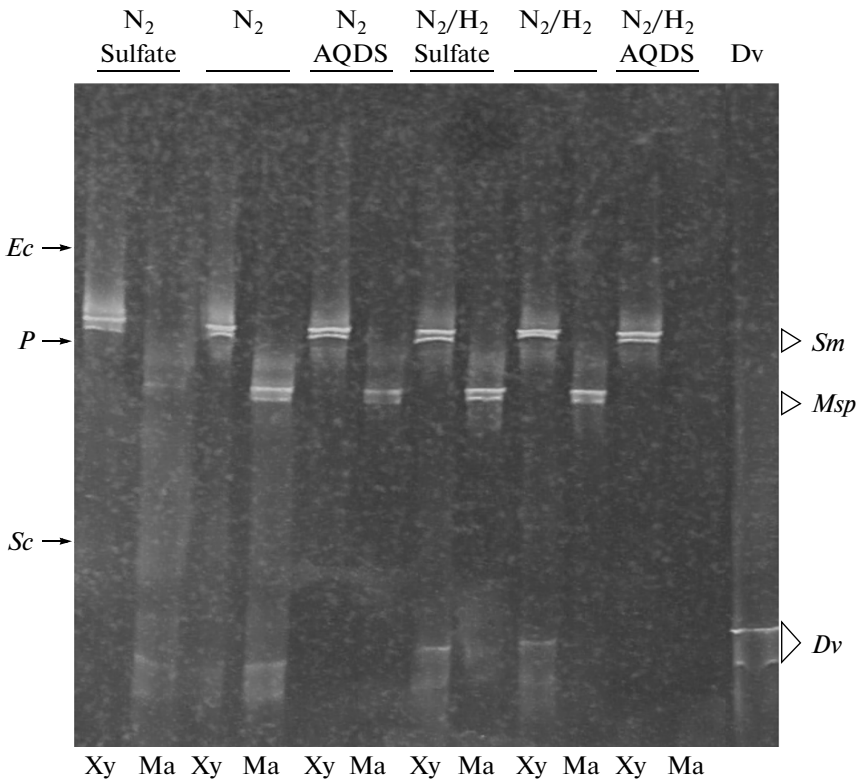
Both *S. maltophilia* and *Microbacterium* are aerobic bacteria. However, persistence under suboxic or anox-

Fig. 1. DGGE fingerprints of *D. vulgaris* and a cocultivated bacterial species in the presence of different sugars, presence and absence of sulfate or AQDS, and exposed to an atmosphere of nitrogen or a nitrogen/hydrogen mixture. The migration of *D. vulgaris* Hildenborough is shown (*Dv*). The migration of *S. maltophilia* (*Sm*) (a) and (b) and *Microbacterium* sp. (*Msp*) (b) is also indicated. Supplemented sugars are labeled as follows; in panel (a): Fr, fructose; Ar, arabinose; My, myoinositol; and in panel (b): Xy, xylose; Ma, mannitol. A negative amplification control is shown (−C). Migration markers are indicated on the left: *Ec*, *Escherichia coli*; *P*, *Paenibacillus* sp.; *Sc*, *Streptomyces caviscabies*.

(a)


N2 Sulfate
N2
N2 AQDS
N2/H2 Sulfate
–C
N2/H2
N2/H2 AQDS
Dv
Ec
P
Sc
Sm
Dv
Fr Ar My Fr Ar My Fr Ar My Fr Ar My Fr Ar My Fr Ar My

(b)


N2 Sulfate
N2
N2 AQDS
N2/H2 Sulfate
N2/H2
N2/H2 AQDS
Dv
Ec
P
Sc
Sm
Msp
Dv
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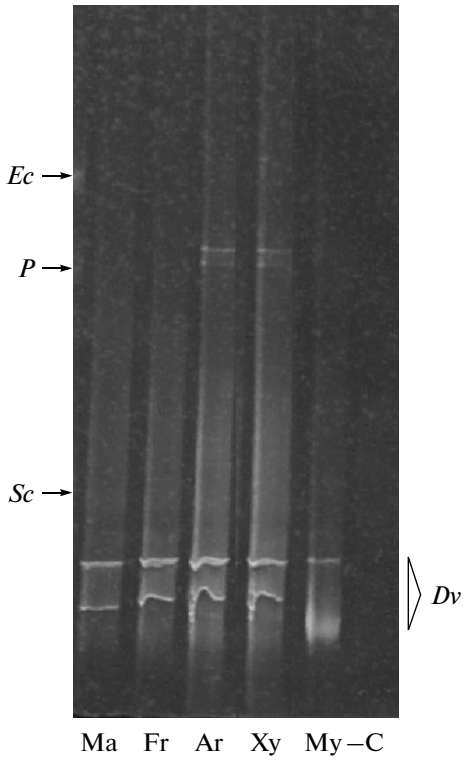

Fig. 2. DGGE fingerprints performed from cultures in Postgate medium C. The inocula of these cultures were colonies previously grown under a nitrogen atmosphere, on different carbon sources and lacking sulfate. The letters at the bottom of the figure indicate the carbon source supplementing the medium where the inoculum was grown: Ma, mannitol; Fr, fructose; Ar, arabinose; Xy, xylose; My, myoinositol. The migration of *D. vulgaris* Hildenborough is shown (*Dv*). A negative amplification control is shown (−C). Migration markers are similar to those indicated on Fig. 1.

ic conditions has been reported for *Microbacterium* spp. [24]. *S. maltophilia* is a non-fermentative aerobic bacteria and a potential for growth under microaerophilic conditions has been previously mentioned [25]. Trace amounts of oxygen could be present in our assays, despite anaerobic indicators used denoted the existence of an anoxic atmosphere throughout the experiment. Interestingly, the anaerobic bacterium *D. vulgaris* Hildenborough contains two membrane-bound oxygen reductases, a quinol oxidase *bd* and a cytochrome *c* oxidase [9, 26]. Moreover, *D. vulgaris* Hildenborough can show aerobic respiratory activity comparable to aerobic microorganisms [13 and related references within]. The interactions between the preferentially aerobic growth of a cocultured bacterium and the oxygen respiration survival strategy mechanism of the sulfate-reducer have probably resulted in adequate feedback mechanisms allowing *D. vulgaris* proliferation on carbohydrates. The growth of *Desulfovibrio* observed in coculrure with *S. maltophilia* or *Microbacterium* might have been the consequence of the production of one or more metabolites which were used by *Desulfovibrio*. A *S. maltophilia* strain growing on the aforementioned plates could produce pyruvate and acetate to be used by *D. vulgaris*, as inferred from the metabolic pathways noted for several strains of *S. maltophilia* in the Kyoto Encyclopedia of Genes and Genomes. For instance, strain Hildenborough could grow under N_2 atmosphere in the absence of sulfate due to fermentation of pyruvate.

Our results suggest a role for hydrogen in the mutualistic associations with *D. vulgaris* Hildenborough; relative quantitative analysis by DGGE of the experimental communities forming colonies on media supplemented with specific sugars (except in the case of mannitol) showed a higher percentage of *D. vulgaris* cells in the presence than in the absence of hydrogen (Fig. 3). Considering the ability of *D. vulgaris* to grown on hydrogen, it is possible that the use of metabolites produced by the co-cultured bacteria is incremented. Hydrogen uptake could also be associated with the increase of a proton motive force and a proton symport of several metabolites or primary substrate, such as the one previously described for myo-inositol [27]. At this respect, we have observed proliferation of *D. vulgaris* on maltose only when sulfate and hydrogen were present.

An inhibitory effect of AQDS on *D. vulgaris* Hildenborough has been observed because growth remained undetected and no band through DGGE analysis was observed in the media supplemented with ADQS (see Figs, la and b). Shyu et al. [28] have provided evidence that AQDS enters *Shewanella oneidensis* and causes cell dead if it accumulates past a critical concentration. A similar effect could also explain the inhibition of *D. vulgaris* Hildenborough by ADQS during this study.

In conclusion, we have identified two microorganisms allowing cell growth of *D. vulgaris* strain Hildenborough in the presence of several carbon sources which are substrates that can not be utilized by this sulfate-reducer. Further experiments are required to understand the nature of these mutualistic associations and the implications for the metabolism and microbial interactions of these bacterial species. Nevertheless, the presented results can explain the growth of the sulfate-reducer *D. vulgaris* in complex systems with abundant organic matter where sulfate-reducing bacteria usually play a critical role in the decomposition of organic compounds. Beyond their obvious function in the sulfur cycle, sulfate-reducing bacteria play an important role in global cycling of elements [29]. For example, in the carbon cycle, the sulfate-reducing bacteria have been hypothesized to form part of microbial consortia that completely mineralize organic carbon in anaerobic environments; polymeric materials (e.g., cellulose) should be first depolymerized and then metabolized by fermentative microorganisms [30, 31]. Previous studies have reported that *D. vulgaris* uses lactate, pyruvate, ethanol, malate, and fumarate, but

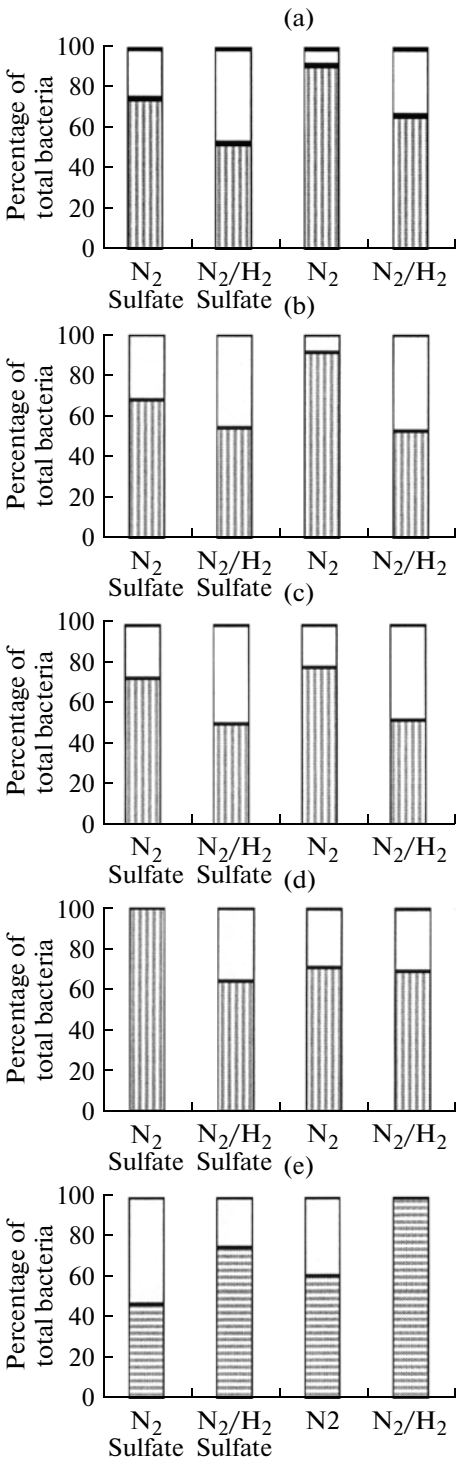

Fig. 3. Results of relative quantitative DGGE analysis of band intensity obtained from cultures of *D. vulgaris* and a cocultivated bacterial species in the presence of different sugars, presence and absence of sulfate, and under an atmosphere of nitrogen or a nitrogen/hydrogen mixture. The fractions represented by *D. vulgaris* Hildeborough (white portions) and *S. maltophilia* (portions with vertical lines) and *Microbacterium* sp. (portions with horizontal lines) are shown. The results obtained for growth on different sugars are presented: (a), fructose; (b), arabinose; (c), myoinositol; (d), xylose; (e), mannitol.

not sugars [9, 32]. The evidence shown in this study corroborates the role of sulfate-reducing bacteria, in particular of *D. vulgaris* Hildenborough, in microbial mineralization and degradation processes of organic matter and carbon cycling in nature through interactions with other heterotrophic bacteria.

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