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New organic substrates for anoxygenic phototrophic bacteria

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In memory of my beloved father

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Content

List of abbreviations

(Very common abbreviations and units are not listed)

ARB	A software environment for sequence data
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
DGGE	Denaturing gradient gel electrophoresis
DSMZ	Deutsch Samlung von Mikroorganismen und Zellkulturen GmbH
EDTA	Ethylenediaminetetraacetic acid
FID	Flame ionization detector
G + C	Guanine and cytosine
GC	Gas chromatography
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HMN	2,2,4,4,6,8,8-Heptamethylnonane
HPLC	High performance liquid chromatography
MES	2-(4-Morpholino)ethanesulfonic acid
OD	Optical density
PCR	Polymerase chain reaction
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
RAPD	Random amplified polymorphic DNA
SYBR	Nucleic acid gel stain
TAE	Tris-acetate-EDTA buffer
TEM	Transmission electron microscopy
v/v	Volume/volume
UV-VIS	Ultraviolet-visible
w/v	Weight/volume

Summary

Anoxygenic phototrophic bacteria are commonly thought to utilize simple reduced inorganic compounds for photoautotrophic growth and simple organic acids and alcohols for photoheterotrophic growth. In this study, the potentials of (1) humic substances as electron donor for photoautotrophic growth and (2) of hydrocarbons for photoheterotrophic growth were investigated.

1. Humic substances as electron donor for an anoxygenic phototrophic bacterium

a) For the first time, an anoxygenic phototrophic bacterium was isolated with a model compound of humic substances, anthrahydroquinonedisulfonate (AH_2QDS), as electron donor. The isolate, strain E3P, was a purple bacterium, able to utilize a broad range of substrates including H_2, H_2S, Fe^{2+} and organic acids. Analysis of the nearly full length 16S rRNA gene sequence showed that strain E3P is related to the genus *Thiocystis,* the closest relative being *T. violacea* (95.8% sequence similarity). Strain E3P is regarded as a new species of the genus *Thiocystis*.

b) Quantitative growth experiments revealed an oxidation of AH_2QDS coupled to an increase of biomass according to the stoichiometry:

$$17\ AH_2QDS + 8\ HCO_3^- + 2\ NH_4^+ + 6\ H^+ \rightarrow 17\ AQDS + 2\ C_4H_8O_2N + 20\ H_2O$$

c) In addition, strain E3P could oxidize reduced humic acids in light condition.

d) AH_2QDS and humic acids were also shown to serve as electron shuttles between the chemotrophic *Geobacter metallireducens* and the phototrophic *Thiocystis*-like strain E3P. Benzoate was used as electron donor by *G. metallireducens* to reduce AQDS. Strain E3P could not utilize benzoate directly, but grew simultaneously with *G. metallireducens* in the co-culture experiment.

2. Enrichments of anoxygenic phototrophic bacteria with hydrocarbons

Enrichments from marine sediments (Gulf of Mexico) with *n*-hexane and *n*-decane as substrates for anoxygenic phototrophic bacteria were established. Attempts to isolate *n*-hexane or *n*-decane-utilizing phototrophic bacteria as a pure culture failed. Analysis of the enriched microbial communities by DGGE revealed phylotypes

closely related to described phototrophic and sulfate-reducing bacteria. Cultures in dark controls showed an increase in sulfide. Results suggest that sulfate-reducing bacteria in the enrichment culture coupled the degradation of *n*-hexane or *n*-decane to the reduction of sulfate to sulfide. The latter could be used by phototrophic bacteria.

With toluene, a phototrophic strain was enriched and isolated from a freshwater ditch. This strain shared 99% 16S rRNA gene sequence similarity and 92.7% DNA-DNA similarity through hybridization with a described toluene-utilizing phototrophic bacterium, *Blastochloris sulfoviridis* strain ToP1 (Zengler *et al*., 1999). According to genomic DNA fingerprint analysis of the two strains, the newly isolated strain GH1 was another strain of *B. sulfoviridis.* In both strains, the gene which encoded for subunit A of benzylsuccinate synthase was detected (*bssA*). BssA is the large subunit of benzylsuccinate synthase, the enzyme catalyzing the initial reaction of anaerobic toluene degradation. The recovered partial sequences of these genes were completely identical in both strains and grouped together with *bssA* gene of Alphaproteobacteria.

A Introduction

A.1 Anoxygenic phototrophic bacteria

Photosynthesis, which is the most important biological process on Earth, converts light energy to chemical energy (Bryant and Frigaard, 2006). Oxygenic photosynthesis is the principal metabolic process of cyanobacteria and green plants which contain chlorophylls as pigments and use water as electron donor which yields oxygen. Anoxygenic photosynthesis is carried out by diverse purple and green bacteria which contain bacteriochlorophylls as pigments and do not produce oxygen. The study of anoxygenic photosynthesis began at the end of 19th century (Skene, 1914; Gest and Blankenship, 2004; Gorlenko, 2004) and became an important topic in microbiology in the late 1960s (Pfennig, 1967). Anoxygenic phototrophic microorganisms are classified into haloarchaea, filamentous green bacteria, green sulfur bacteria, purple sulfur bacteria, purple non-sulfur bacteria and heliobacteria (Table 1). Based on carbon sources, these microorganisms are classified into photoautotrophs and photoheterotrophs.

Table 1. Major groups of anoxygenic phototrophic prokaryotes (Kondratieva *et al*., 1992; Widdel *et al*., 1993; Overmann and Garcia-Pichel, 2006; and Griffin *et al*., 2007; Budinoff and Hollibaugh, 2008).

Anoxygenic phototrophs	Photosynthetic electron donors	Carbon sources
Archaea		
Haloarchaea	Organic	Organic
Bacteria		
Filamentous green bacteria	Organic, H_2S, $S_2O_3^{2-}$	Organic, CO_2
Green sulfur bacteria	H_2, H_2S, S^0, $S_2O_3^{2-}$	CO_2
Purple bacteria	H_2, H_2S, S^0, $S_2O_3^{2-}$, Fe^{2+}, NO_2^-, H_3AsO_3, organic	CO_2 and/or organic
Purple non-sulfur bacteria	H_2, H_2S, S^0, $S_2O_3^{2-}$, organic	CO_2 and/or organic
Heliobacteria		

Photoautotrophs can use carbon dioxide as the sole carbon source for their biomass (CH_2O) and use inorganic compounds such as sulfide, elemental sulfur, thiosulfate or iron(II) as electron sources (Fig. 1; Pfennig, 1967; Overman and Garcia-Pichel, 2006; Widdel *et al*., 1993; Ehrenreich and Widdel, 1994; Heising and Schink, 1998; Caiazza *et al*., 2007). More recently, it was shown that nitrite or arsenite could serve as electron donors for anoxygenic photosynthesis (Griffin *et al*., 2007; Budinoff and Hollibaugh, 2008).

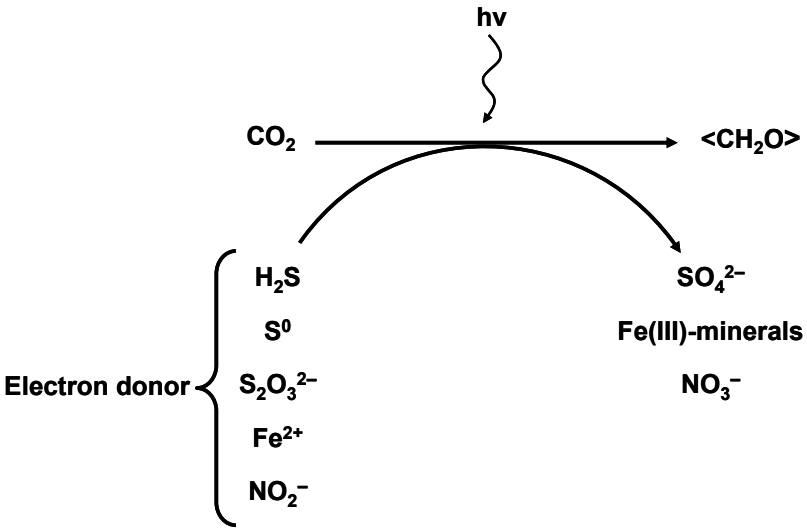

Fig. 1. Diagram of photoautotrophy of anoxygenic phototrophic bacteria.

Photoheterotrophs cannot utilize carbon dioxide as the only carbon source, but depend on organic compounds that are converted by means of energy from light to cell mass (Fig. 2). These organic compounds include fatty acids, alcohols, and even aromatic organic compounds such as benzoate (Proctor and Scher, 1960; Pfennig *et al*., 1965; Dutton and Evans, 1969; Douthit and Pfennig, 1976; Shoreit and Shabeb, 1994; Ramana *et al*., 2006). Few years ago, the aromatic hydrocarbon toluene was shown to be utilized as carbon source by the purple non-sulfur bacterium *Blastochloris sulfoviridis* strain ToP1 (Zengler *et al*., 1999).

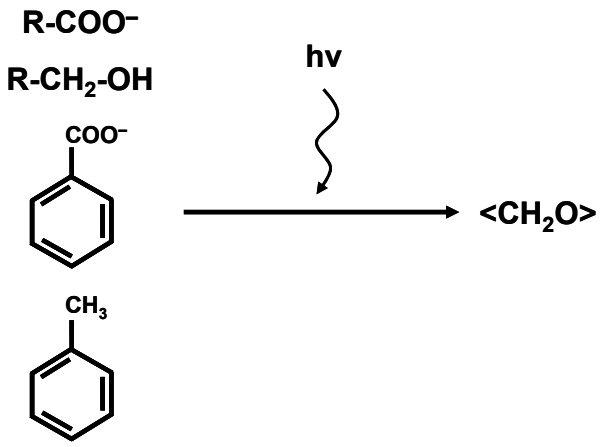

Fig. 2. Diagram of photoheterotrophy of anoxygenic phototrophic bacteria.

In the present work, representatives of two new classes of substrates for phototrophic bacteria were investigated (see A.4). These were (a) humic acids represented by the defined model compound anthrahydroquinonedisulfonate, and (b) hydrocarbons. The relevance of these compounds as substrates for bacteria living under anoxic conditions is described in the following sections (A.2, A.3).

A.2 Humic substances

Humic substances are among the most widely distributed organic compounds on Earth. They can account for as much as 10% by weight of the total content of many soils and sediments (Van Trump *et al*., 2006). They are found not only in soil but also in natural waters, marine and lake (Stevenson, 1994). Humic substances are a complex mixture of partially degraded and transformed organic material. Humic substances are thought to originate from decomposing animal residues, vascular plants, algae and microbial biomass in the environment (Fig. 3). The transformations of the biological compounds to humic substances are supposed to include mainly sugars, polyphenols, amino compounds, lignins and quinones which undergo different reactions that may be partly carried out by microorganisms (Stevenson, 1994).

For many decades, it was thought that humic substances originated from lignin; however, nowadays most scientists support pathways involving quinones (Stevenson, 1994). Humic substances are grouped into three classes, namely humins, humic acids and fulvic acids (Stevenson, 1994; Xie *et al*., 1997; Conzonno and Cirelli, 1998). This classification is based on differences in physical and chemical characteristics such as colors, solubility, molecular mass, specific content of functional groups (carboxyl and phenolic OH-groups) and the extent of polymerization. Fulvic acids have a light yellow to yellow brown color and are soluble in water at any pH value. Humic acids, which are the major extractable fraction of soil humic substances, are dark brown to black and are not soluble at pH values below 2.0. Humins have a black color and are insoluble at any pH value (Stevenson, 1994).

Humic acids are major components of humic substances. It is supposed that humic acids are complex aromatic macromolecules with covalently bound amino acids, amino sugars, peptides and other aliphatic compounds (Grant, 1977; Shulten *et al*., 1991; Stevenson, 1994; Christle *et al*., 2000; Ussiri and Johnson, 2003). The hypothetical structure of humic acids contains free and bound phenolic groups, quinone structures, nitrogen and oxygen as bridging atoms and COOH groups at aromatic rings (Fig. 4). Terrestrial humic acids tend to be more “aromatic” while marine humic acids tend to be more aliphatic in nature (Craig Bingman, http//www.bingman-at-netcom.com). Redox potentials of humic acids are supposed to range from –0.2 to +0.3 V (Table 2).

Because humic acids are diverse and cannot be precisely defined with respect to their chemical structure, defined low-molecular mass compounds with quinone/quinol structure are often used as model compounds in the laboratory to mimic certain properties of humic acids. One of such model compounds is anthraquinone-2,6-disulfonate (AQDS; Table 2; Figs. 5 and 6).

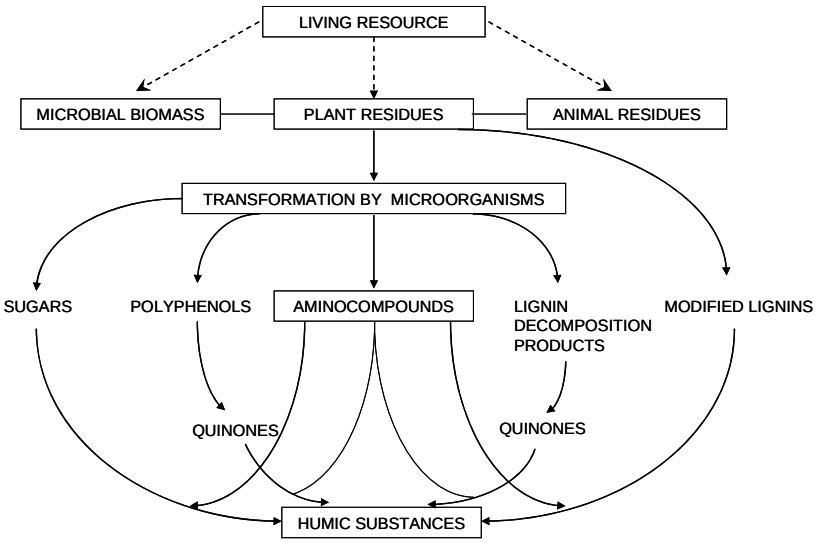

Fig. 3. Scheme of the formation of humic substances (adapted from Stevenson, 1994).

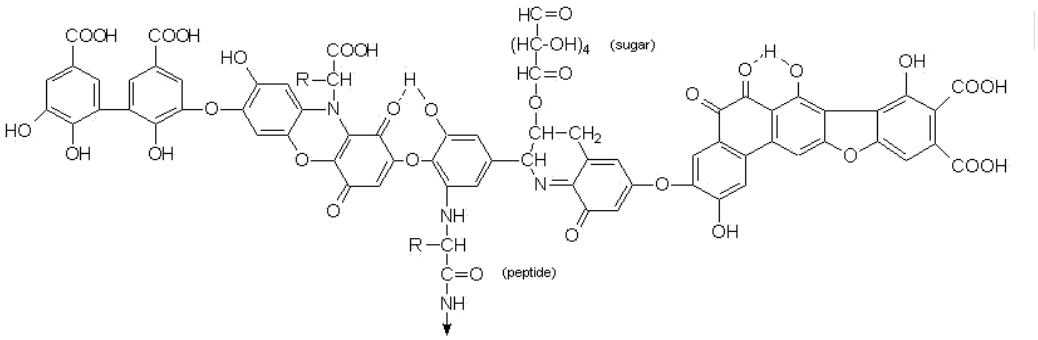

Fig. 4. A hypothetical model structure of humic acids (Stevenson, 1994).

Table 2. Redox potentials of humic acids and anthraquinone-2,6-disulfonate (AQDS).

Substances	E°(V)	References
2,6-AQDS/2,6-AH_2QDS	–0.184	Benz *et al*., 1998
Humic acids ox/red	–0.2 to +0.3	Straub *et al*., 2001

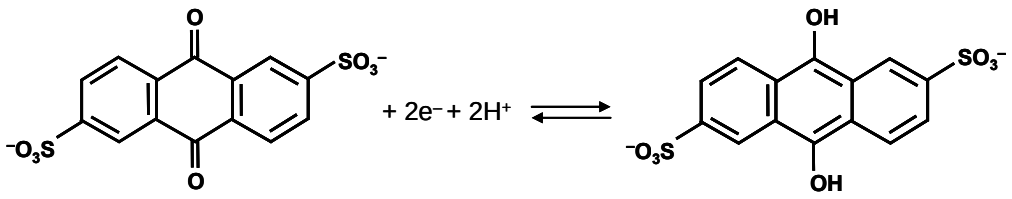

Fig. 5. Anthraquinone-2,6-disulfonate and anthrahydroquinone-2,6-disulfonate.

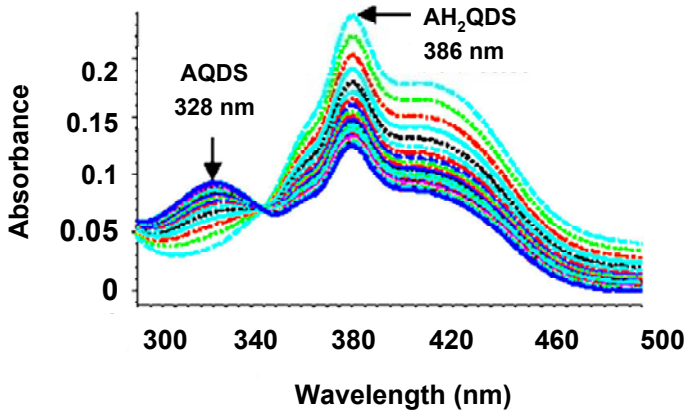

Fig. 6. Spectrum of anthraquinone-2,6-disulfonate in its oxidized (AQDS) and reduced (AH_2QDS) forms (Ainsworth *et al*., 2004).

A.2.1 Interactions between microorganisms and humic substances

The interaction of microrganisms with humic substances has been a subject of research for the last 40 years. Humic compounds were initially studied as potential sources of carbon or micronutrients or as a natural matrix in media for microorganisms. Later on, however, it was discovered that humic acids can also act as redox mediators in the chemical reduction of organic pollutants, such as nitroaromatic or halogenated compounds (Schwarzenbach *et al*., 1990; Dunnivant and Schwarzenbach, 1992; Curtis and Reinhard, 1994). More recently, it was shown that humic substances play an even more important role by serving as electron shuttle in the microbial metabolism and mineralization of carbon compounds in various anoxic environments (Scott *et al*., 1998; Cervantes *et al*., 2001; Coates *et al*., 2001; Cervantes *et al*., 2004).

A.2.2 Humic substances as electron acceptors for microbial respiration

It is generally assumed that the most important electron acceptors for anaerobic bacterial respiration in flooded soils, aquatic sediments and aquifers are nitrate, Mn(IV), Fe(III), and sulfate. However, because of the abundance of humic substances in soils and sediments, electron transfer to humic substances might also be important and diverse microorganisms may exist that are capable of this form of respiration. It was found that humic acids stimulated aromatic hydrocarbon degradation (Lesage *et al.*, 1995). Humic acids were considered as electron acceptors in experiment to study benzene degradation with various Fe(III) chelators (Lovley *et al*., 1996b). Later, this capacity was confirmed in experiments with some species of *Geobacter*, Fe(III)-reducing bacteria (Rooney-Varga *et al.*, 1999).

Because of difficulties to determine the growth of microorganisms on humic acids due to their complex structure, anthraquinonedisulfonate (AQDS) was broadly used as model compound to study microbial respiration with humic acids. AQDS was shown to serve as sole terminal electron acceptor for growth of *Geobacter metallireducens* on acetate (Lovley *et al*., 1996a). Moreover, other quinones such as menadione, lawsone or anthraquinone-2-sulfonate can serve as electron acceptors and support growth of anaerobic bacteria (Lovley *et al*., 1998). The majority of presently known humic substances-reducing microorganisms are also capable of reducing AQDS to AH_2QDS (Lovley *et al*., 1996a; Benz *et al.*, 1998; Coates *et al*., 1998; Lovley *et al*., 1998).

A number of studies have revealed diverse microorganisms that could reduce humic substances or AQDS (Benz *et al.*, 1998; Coates *et al*., 1998; Lovley *et al*., 1998; Lovley *et al*., 2000; Francis *et al*., 2000; Cervantes *et al.*, 2002; Finneran *et al*., 2002). They include Fe(III)-reducers such as *Geobacter metallireducens*, nitrate-reducers such as *Shewanella putrefaciens*, sulfate-reducers such as *Desulfovibrio* sp. G11, fermentative bacteria such as *Propionibacterium freudenreichii*, halo-respiring bacteria such as *Desulfitobacterium dehalogenans* and themophilic archaea such as *Pyrobaculum islandicum* (Table 3).

Table 3. Described microorganisms able to reduce humic acids or AQDS as terminal electron acceptors.

Taxonomic group	Microorganism	Electron donor	Reference
Bacteria			
Acidobacteria	*Geothrix fermentans*	Acetate	Coates *et al*., 1999
Actinobacteria	*Propionibacterium freudenreichii*	Lactate	Benz *et al.*, 1998
Firmicutes	*Thermoanaerobacter siderophilus*	H_2	Slobodkin *et al*., 1999
	Enterococcus cecorum	Glucose	Benz *et al.*, 1998
	Lactococcus lactis	Glucose	Benz *et al.*, 1998
	Desulfitobacterium dehalogenans	Lactate	Cervantes *et al*., 2002
	Desulfitobacterium metallireducens	Lactate	Finneran *et al*., 2002
Betaproteobacteria	Strain CKB	Acetate	Bruce *et al*., 1999
Deltaproteobacteria	*Geobacter metallireducens*	Acetate	Lovley *et al*., 1998
	Geobacter sulfurreducens	Acetate	Lovley *et al*., 1998
	Geobacter humireducens	Acetate	Lovley *et al*., 1998
	Geobacter grbiciae	Acetate	Coates *et al*., 2001
	Geobacter sp. JW-3	Acetate	Coates *et al*., 1998
	Geobacter sp. TC-4	Acetate	Coates *et al*., 1998
	Desulfuromonas acetexigens	Acetate	Lovley *et al*., 1998
	Desulfuromonas sp. SDB-1	Acetate	Coates *et al*., 1998
	Desulfuromonas sp. FD-1	Acetate	Coates *et al*., 1998
	Desulfovibrio sp. G11	H_2	Cervantes *et al*., 2002
Epsilonproteobacteria	*Sulfurospirillum barnesii*	Lactate	Lovley *et al*., 1998; Stolz *et al*., 1999
	Wolinella succinogenes	H_2	Lovley *et al*., 1998
Gammaproteobacteria	*Shewanella algae*	H_2	Lovley *et al*., 1998
	Shewanella putrefaciens	H_2	Lovley *et al*., 1998
	Shewanella sacchrophila	H_2	Lovley *et al*., 1998
	Aeromonas hydrophila	Acetate	Lovley *et al*., 1998
	Pantoea agglomerans	Acetate	Francis *et al*., 2000
Archaea	*Pyrobaculum islandicum*	H_2	Lovley *et al*., 2000
	Pyrodictium abyssi	H_2	Lovley *et al*., 2000
	Pyrococcus furiosus	H_2	Lovley *et al*., 2000
	Archaeoglobus fulgidus	H_2	Lovley *et al*., 2000
	Thermococcus celer	H_2	Lovley *et al*., 2000
	Methanococcus thermolithotrophicus	H_2	Lovley *et al*., 2000
	Methanobacterium thermoautotrophicum	H_2	Lovley *et al*., 2000
	Methanopyrus kandleri	H_2	Lovley *et al*., 2000
	Methanospirillum hungatei	H_2	Cervantes *et al*., 2002

A.2.3 Humic substances as electron donor for microbial respiration

With the increasing number of humic substances-reducing microorganisms discovered, also the potential of humic substances became of interest to serve as electron donors for microbial respiration. Many recent studies reported about the role of reduced humic acids or hydroquinones as electron donors for an anaerobic chemotrophic metabolism (Lovley *et al*., 1999; Coates *et al*., 2001; Coates *et al*., 2002; Luijten *et al*., 2004).

Lovley *et al*. (1999) reported that some nitrate-reducing bacteria such as *Geobacter metallireducens, Geothrix fermentans, Wolinella succinogenes* and *Paracoccus denitrificans* could oxidize AH_2QDS with nitrate or fumarate as electron acceptors to gain energy for growth. *Geobacter sulfurreducens*, even though not able to reduce nitrate, can reduce fumarate with AH_2QDS. Experiments with microbially reduced humic acids demonstrated that reduced humic acids support denitrification and fumarate reduction. *Wolinella succinogenes* and *Shewanella barnessii* can reduce selenate during oxidation of AH_2QDS (Lovley *et al*., 1999). Recently, it was shown that microorganisms which could oxidize reduced humic acids or AH_2QDS are ubiquitous in environment. In some most probable number (MPN) enumeration and isolation studies of AH_2QDS oxidizing microorganisms, it was shown that these microorganisms were present with $2.31 \times 10^1 \pm 1.33 \times 10^1$ cells/g in hydrocarbon-contaminated aquifer sediment and $9.33 \times 10^6 \pm 4.17 \times 10^6$ cells/g in lake sediment; all isolated bacteria affiliated with the Alpha-, Beta-, Gamma- and Deltaproteobacteria (Coates *et al*., 2002; Table 4). In Betaproteobacteria, some perchlorate-reducing microorganisms of the genus *Dechloromonas* were able to grow by AH_2QDS oxidation (Coates *et al*., 2001; Coates *et al*., 2002). More recently, the halorespiring microorganism such as *Desulfitobacterium hafniense*, *Sulfurospirillum deleyianum*, *Sulfurospirillum barnesii* and *Sulfurospirillum arsenophilum* were shown to oxidize AH_2QDS using Se(VI), Fe(III), Mn(IV) or As(V) as electron acceptors (Luijten *et al*., 2004).

Table 4. Identified microorganisms able to oxidize reduced humic acids or AH_2QDS as electron donors.

Taxonomic group	Microorganism	Electron acceptor	Reference
Acidobacteria	*Geothrix fermentans*	NO_3^-, fumarate	Lovley *et al*., 1999
Firmicutes	*Desulfitobacterium hafniense*	Se(VI), Fe(III), Mn(IV)	Luijten *et al*., 2004
Alphaproteobacteria	*"Agrobacterium tumefaciens"* strain PB	NO_3^-	Coates *et al*., 2002
	Paracoccus denitrificans	NO_3^-	Lovley *et al*., 1999
Betaproteobacteria	*"Dechloromonas"* strain RCB	NO_3^-	Coates *et al*., 2001
	"Dechloromonas" strain JJ	NO_3^-	Coates *et al*., 2002
	"Azoarcus evansii" strain HA	NO_3^-	Coates *et al*., 2002
Deltaproteobacteria	*Geobacter metallireducens*	NO_3^-	Lovley *et al*., 1999
	Geobacter sulphurreducens	Fumarate	Lovley *et al*., 1999
	"Stigmatella" strain KC	NO_3^-	Coates *et al*., 2002
Epsilonproteobacteria	*Wolinella succinogenes*	NO_3^-, fumarate, As(V), Se(VI)	Lovley *et al*., 1999
	Sulfurospirillum barnesii	Se(VI), As(V), Mn(IV)	Luijten *et al*., 2004
	Sulfurospirillum deleyianum	As(V), Mn(IV)	Luijten *et al*., 2004
	Sulfurospirillum arsenophilum	Se(VI), As(V), Mn(IV)	Luijten *et al*., 2004
Gammaproteobacteria	*Shewanella algae*	Fumarate	Lovley *et al*., 1999
	Shewanella barnesii	Fumarate	Lovley *et al*., 1999
	"Pseudomonas stutzeri" strain BU	NO_3^-	Coates *et al*., 2002
	"Pseudomonas flavescens" strain NMX	NO_3^-	Coates *et al*., 2002
	"Marinobacter articus" strain SBS	NO_3^-	Coates *et al*., 2002

A.2.4 Humic substances as redox mediators

Because humic substances or AQDS can function as both electron acceptor and electron donor, they can in principle serve as redox mediator shuttles. Their role as redox mediators was shown in reductive biotransformations of pollutants and reduction of solid Fe(III) oxides (Lovley *et al*., 1999; DiChristina *et al*., 2005). Electrons from the anaerobic oxidation of organic substrates were used for the reduction of azo dyes, nitroaromatics, polychlorinated compounds and oxidized radionuclides (Schwarzenbach *et al*., 1990; Curtis and Reinhard, 1994; Keck *et al*., 1997; Collins and Picardal, 1999; Bechtold *et al*., 1999; Field *et al*., 2000; Cervantes *et al*., 2001; Van der Zee *et al*., 2001). AQDS as redox mediator enabled the continuous treatment of an azo dye or mediated the abiotic reduction of nitrobenzenes to the corresponding anilines (Schwarzenbach *et al*., 1990; Dunnivant *et al*., 1992) or the reductive dehalogenation of polychlorinated aromatic compounds (Barkovskii and Adriaens, 1998).

A.2.5 Degradation of humic substances by microorganisms

Humic substances not only serve as redox mediators but also as biodegradable carbon substrates of microorganisms. They were shown to be degraded to lower molecular weight compounds by some aerobic microorganisms (Wetzel, 1993; Wetzel *et al*., 1995) such as white-rot fungi and the basidiomycete *Collybia dryophila* (Wetzel, 1993; Wetzel *et al*., 1995; Steffen *et al*., 2002). Little is known about the possibilities of an anaerobic biodegradation of humic substances. Coates *et al.* (2002) demonstrated that anaerobic chemotrophic microorganisms capable of utilizing humic substances or AQDS as electron shuttles did not degrade their carbon skeletons.

A.3 Hydrocarbons

Biodegradation of saturated and aromatic hydrocarbons was for a long time considered to occur exclusively under oxic conditions (Gibson *et al.*, 1970; Watkinson and Morgan, 1990). But since the late 1980s and early 1990s, more and more enrichments and pure cultures were shown to utilize various hydrocarbons under anoxic conditions. Nearly all of these cultures were chemotrophic (Rueter *et al.*, 1994; Widdel and Rabus, 2001; Van Hamme *et al*., 2003; Widdel *et al*., 2007); however, one phototrophic bacterium growing with toluene has been described (Zengler *et al*., 1999).

A.3.1 Chemotrophic hydrocarbon-utilizing anaerobic microorganisms

Hydrocarbons that can be degraded anaerobically by microorganisms include alkanes (C_1 to C_{20}) and alkenes (C_7 to C_{23}) and aromatic hydrocarbons such as toluene, ethylbenzene, *n*-propylbenzene, *p*-cymene, xylene and ethyltoluene-isomers, benzene and naphthalene (Heider *et al*., 1999; Widdel *et al*., 2004; Widdel *et al*., 2007; Kniemeyer *et al*., 2007). These microorganisms utilize NO_3^-, SO_4^{2-}, Fe(III), Mn(IV) as terminal electron acceptors (Table 5).

Several denitrifying microbial species, which belong to the genera *Thauera* and *Azoarcus* of the Betaproteobacteria can oxidize alkylbenzenes such as toluene, *m*-xylene, ethylbenzene, or *n*-propylbenzene (Rabus and Widdel, 1995; Evans *et al*., 1991; Spormann and Widdel, 2000). Among them, toluene was the most frequently used (Rabus and Widdel, 1995; Ball *et al*., 1996; Hess *et al*., 1997; Harms *et al*., 1999). Some strains of denitrifying bacteria can also utilize alkanes from C_6 to C_{20} (Ehrenreich *et al*., 2000). Anaerobic utilization of hydrocarbons was also demonstrated under Fe(III)- and SO_4^{2-} -reducing conditions (Lovley and Lonergan, 1990; Aeckersberg *et al*., 1991; Widdel *et al*., 2004). Among SO_4^{2-}-reducing bacteria, many different species and strains were isolated that could utilize aromatic hydrocarbons or *n*-alkanes, namely *Desulfobacula toluolica* Tol2, *Desulfosarcina cetonica*, *Desulfotignum toluenicum*, strains TD3, Hxd3, Pnd3, Ak-01, PRTOL1 and most recently BuS5 capable of utilizing propane and butane (Table 5).

Table 5. Anaerobic hydrocarbon-utilizing microorganisms* (modified from Widdel *et al*., 2007).

Taxonomic group	Microorganism (species or strain**)	Hydrocarbons metabolized	Reference
Chemotrophs			
Betaproteobacteria	*Thauera aromatica*	Toluene	Evans *et al*., 1991
	Azoarcus sp. strain EbN1	Ethylbenzene, toluene	Rabus and Widdel, 1995
	Azoarcus sp. strain HxN1	Alkanes (C_6–C_8)	Ehrenreich *et al*., 2000
	Strain OcN1	Alkanes (C_8–C_{12})	Ehrenreich *et al*., 2000
	Strain HdN1	Alkanes (C_{14}–C_{20})	Ehrenreich *et al*., 2000
Deltaproteobacteria	BuS5	Propane, butane	Kniemayer *et al*., 2007
	Desulfoglaeba alkanexedens	*n*-Alkanes C_6–C_{10}, C_6–C_{12}	Davidova *et al*., 2006
	TD3	*n*-Alkanes C_6–C_{16}	Rueter *et al*., 1994
	Hxd3	*n*-Alkanes C_{12}–C_{20}, *n*-1-Alkenes C_{14}–C_{17}	Aeckersberg *et al*., 1991
	AK-01	*n*-Alkanes C_6–C_{18}, *n*-1-Alkenes C_{15}, C_{16}	So and Young, 1999
	Desulfatibacillum aliphaticivorans	*n*-Alkanes C_{13}–C_{18} , *n*-1-Alkenes C_7–C_{23}	Cravo-Laureau *et al*., 2004a
	Desulfatibacillum alkenivorans	*n*-1-Alkenes C_8–C_{23}	Cravo-Laureau *et al*., 2004b
	Pnd3	*n*-Alkanes C_{14}–C_{17}, *n*-1-Hexadecene	Aeckersberg *et al*., 1998
	Desulfatiferula olefinivorans	*n*-1-Alkenes C_{14}–C_{23}	Cravo-Laureau *et al*., 2007
	Desulfobacula toluolica	Toluene	Rabus *et al*., 1993
	Desulfobacula phenolica	Toluene	Rabus *et al*., 1993
	PRTOL1	Toluene	Beller *et al*., 1996
	Desulfosarcina cetonica	Toluene	Harms *et al*., 1999
	mXyS1	Toluene, *m*-xylene	Harms *et al*., 1999
	oXyS1	Toluene, *o*-xylene	Harms *et al*., 1999
	Desulfotomaculum sp. OX39	Toluene, *m*-xylene, *o*-xylene	Morasch *et al*., 2004
	Desulfotignum toluenicum	Toluene	Ommedal and Torsvik, 2007
	EbS7	Ethylbenzene	Kniemeyer *et al*., 2003
	NaphS2	Naphthalene	Galushko *et al*., 1999
Anoxygenic phototroph			
Alphaproteobacteria	*Blastochloris sulfoviridis,* strain ToP1	Toluene	Zengler *et al*., 1999

* Only bacteria isolated in pure cultures are shown.
** If a species and/or genus name has not been proposed yet, only the strain designation is given.

A.3.2 Phototrophic hydrocarbon-utilizing microorganisms

The capacity of anoxygenic phototrophic bacteria to utilize aromatic hydrocarbons was first reported by Zengler *et al.* (1999). The isolate belonged to the purple non-sulfur bacteria, and was classified as *Blastochloris sulfoviridis* strain ToP1. Strain ToP1 assimilated toluene and CO_2 into cell biomass. It was supposed that its pathway of toluene metabolism was the same as that of toluene-degrading chemotrophic bacteria, which form benzylsuccinate by addition of the methyl group to fumarate (Zengler *et al*., 1999). Toluene-utilizing anoxygenic phototropic bacteria were shown to occur in a pond, a river and activated sludge (Zengler *et al*., 1999).

Cyanobacteria were reported to be able to partially oxidize aromatic hydrocarbons in some studies (Cerniglia *et al*., 1980a, 1980b, 1980c; Cerniglia *et al*., 1983; Narro *et al*., 1992). However, a more recent study showed that cyanobacteria which may grow together with aerobic hydrocarbon-degrading heterotophic bacteria were not directly involved in hydrocarbon degradation (Abed and Koester, 2005).

A.4 Objectives of the present work

As explained in the presently given overview, iron(II) (and more recently also nitrite) represented a new type of electron donor and toluene a new type of carbon substrate for phototrophic bacteria. It was therefore of interest to investigate whether there are further electron donors for photoautotrophic growth and further hydrocarbons that can serve as carbon substrates for photoheterotrophic growth. Within the scope of the present work, two types of potential substrates were investigated:

(a) Humic acids were regarded as a naturally important class of electron donors. Humic acids are known to reduce iron(III) to iron(II) chemically; hence, there is a connection to redox-reactions of iron minerals. It was therefore of particular interest whether humic acids can also directly serve as electron donors for anoxygenic photosynthesis. Because of the complexity of humic acids structures, the defined model compound AH_2QDS was used such that quantification of its consumption and cell mass production to enable.

(b) *n*-Alkanes are naturally wide-spread compounds from living organisms, and from petroleum products. In order to investigate whether they can serve as carbon sources for phototrophic bacteria, *n*-hexane and *n*-decane were chosen as representative compounds in this study. For comparison, toluene was also included.

B Materials and methods

B.1 Chemicals and gases

All chemicals used in this study were of analytical grade and obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt), Roth (Karlsruhe) and Sigma-Aldrich (Steinheim). Gases including N_2 (99.99%), CO_2 (99.95%), N_2/CO_2 (90/10, v/v) were supplied by Messer-Griesheim (Osterholz-Scharmbeck).

B.2 Microbiological methods

B.2.1 Samples and microorganisms

Freshwater sediments were taken from Bürgerpark and Kuhgraben (Bremen). Marine sediments were taken at Paraíso and Carmen beaches (Gulf of Mexico; Fig. 7).

A culture of an anthrahydroquinone-oxidizing phototrophic bacterium was obtained from enrichment culture with the same compound, previously established for ferrous iron-oxidizing phototrophic bacteria (Bürgerpark, Bremen). Sediments from Kuhgraben, Paraíso beach and Carmen beach were used as inocula for enrichment cultures of phototrophic microorganisms with toluene, *n*-hexane and *n*-decane as organic carbon substrates.

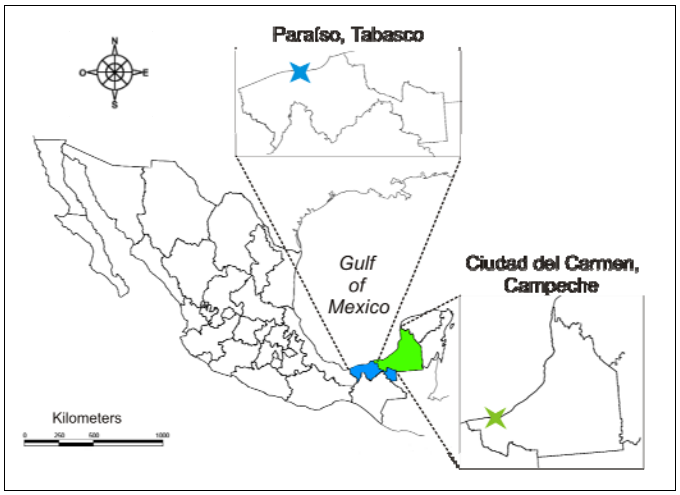

Fig. 7. Sampling sites in Mexico (✕).

The following pure cultures were obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig):

Blastochloris sulfoviridis, DSM 13255

Geobacter metallireducens, DSM 7210

Thiocapsa roseopersicina, DSM 217

Thiocystis gelatinosa, DSM 215

Thiocystis violacea, DSM 207

Thiorhodococcus minus, DSM 11518

B.2.2 Cultivation media

B.2.2.1 Preparation of stock solutions

Trace element solution

Compounds were dissolved in distilled water (ca. 800 ml). The pH was adjusted with NaOH to 6.5. The final volume of 1000 ml was adjusted by addition of distilled water. The solution was sterilized through a nitrocellulose membrane (pore size: 0.2 µm) directly into sterile 50 ml bottles and stored at room temperature.

Na_2-EDTA	5200 mg
$FeSO_4 \cdot 7H_2O$	2100 mg
H_3BO_3	30 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$NiCl_2 \cdot 6H_2O$	4 mg
$CuCl_2 \cdot 2H_2O$	2 mg
$ZnSO_4 \cdot 7H_2O$	44 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg

Thiamine solution

The solution was sterilized through a nitrocellulose membrane (pore size: 0.2 µm) in sterile 50 ml bottles and stored at 4°C in the dark.

Thiamine dihydrochloride	10 mg
Na_2HPO_4/H_3PO_4, 50 mM, pH 3.7	100 ml

Vitamin mixture

The vitamin mixture was dissolved in 50 ml of 10 mM sodium phosphate buffer, pH 7.1, and sterilized through a nitrocellulose membrane (pore size: 0.2 µm) in sterile 50 ml bottles and stored at 4°C in the dark.

4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
Calcium-D(+)-pantothenate	5 mg
Pyridoxine dihydrochloride	15 mg
Na_2HPO_4/NaH_2PO_4, 10 mM, pH 7.1	100 mg

Vitamin B_{12} solution

The solution was sterilized through a nitrocellulose membrane (pore size: 0.2 µm) in sterile 50 ml bottles and stored at 4°C in the dark.

Cyanocobalamine	5 mg
Distilled water	100 ml

Sodium bicarbonate solution

The solution was dispensed in portions of 30 or 60 ml in serum bottles. The head space was exchanged with CO_2 and the solution was saturated with CO_2 by repeated flushing and vigorous shaking. The portions were autoclaved under CO_2 in vials with fixed stoppers and stored at room temperature.

$NaHCO_3$	84 g
Distilled water	1000 ml

Sodium sulfide solution

Crystals of $Na_2S \cdot 9H_2O$ were washed on a plastic sieve by brief rinsing with distilled water. The required weight of clean, colourless crystals was added to distilled water under an N_2 atmosphere. A volume of 30 ml of the solution were filled into 50 ml serum bottle. The head space was exchanged with N_2. The portions were autoclaved under N_2 in vials with fixed stoppers and stored at 4°C.

$Na_2S \cdot 9H_2O$	240 g
Distilled water	1000 ml

B.2.2.2 Preparation of mineral media

Techniques for preparation of media and for cultivation of phototrophic bacteria under anoxic conditions were as described by Widdel and Bak. (1992). Salts were dissolved in H_2O in a special flask (1 or 2 l; Fig. 8). After autoclaving and cooling under an atmosphere of N_2/CO_2 (90/10, v/v), EDTA-chelated trace elements, thiamine, vitamins, B_{12}, the solutions of $NaHCO_3$ (final concentration 30 mM) were added (Table 6). The pH of the medium was adjusted to pH 7.0 – 7.1 with 1 M H_2SO_4 or Na_2CO_3. The medium was dispensed via glass bell into 50 or 100 ml bottles; the bottles were tightly sealed under N_2/CO_2 with rubber stoppers.

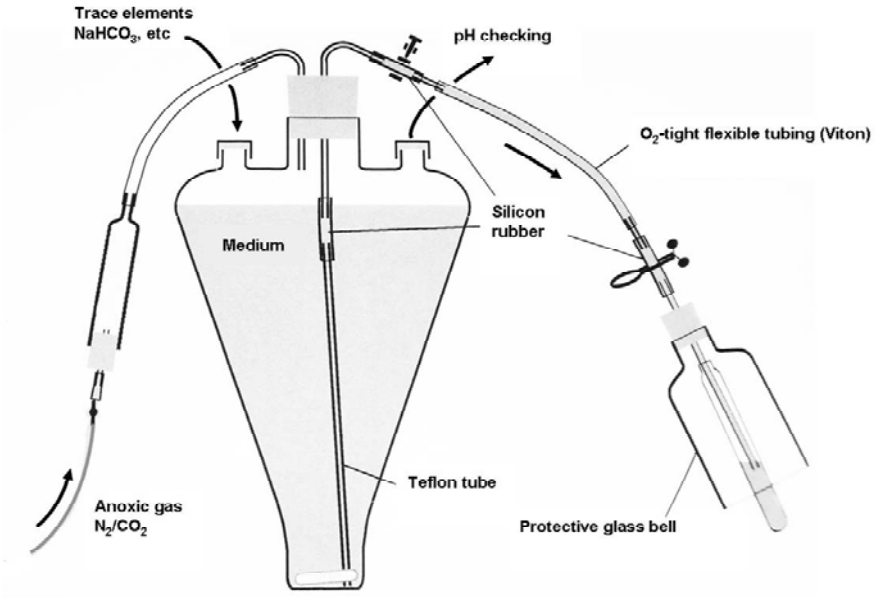

Fig. 8. Preparation of anoxygenic medium for phototrophic bacteria with a special flask (Widdel, pers.commun.).

B.2.2.3 Organic substrates

The main organic substrates used in the present work were summarized in Table 6.

Table 6. Organic substrates

Substrates	Concentration
Non-hydrocarbon compounds	
Anthraquinone-2,6-disulfonate (AQDS/AH_2QDS)	2 mM
Humic acids (reduced and oxidized form)	20 mg/l
Hydrocarbon compounds *	
n-Hexane	1 – 2%
n-Decane	1 – 2%
Toluene	1 – 2%

*Filter-sterilized hydrocarbons were diluted in a carrier phase of sterile, deaerated 2,2,4,4,6,8,8-heptamethylnonane (HMN) to avoid toxic effects of the pure substances (Rabus *et al*., 1993).

B.2.3 Cultivation of anoxygenic phototrophic bacteria

Anoxic sediments were used as inoculum for enrichment cultures. A volume of 5 ml of homogenized sediment was used to inoculate 50 ml of the defined bicarbonate/CO_2-buffered medium in a flat 100 ml bottle (Table 7; Widdel and Bak, 1992; Rabus and Widdel, 1995). In case of enrichment cultures with hydrocarbons, the media were overlaid with 2,2,4,4,6,8,8-heptamethylnonane (HMN) as inert carrier phase containing the hydrocarbons and incubated anoxically under a N_2/CO_2 mixture (9/1, v/v) at room temperature and illuminated with infrared filter-light at a distance of 30 cm (Fig. 9). The bottles were incubated nearly horizontally with orifices below the medium surface so as to yield a large surface, but to avoid contact between the hydrocarbon phase and the stopper (Rabus and Widdel, 1995). Sediment-free subcultures were transferred subsequently into fresh media with 10% (v/v) inoculum. For isolates, cultures were illuminated with tungsten lamps (25 W) at a distance of 30 cm.

Table 7. Cultivation media for anoxygenic phototrophic bacteria.

	Freshwater medium	Artificial seawater medium
Additives before autoclaving		
KH_2PO_4	0.2 g	0.15 g
NH_4Cl	0.3 g	0.2 g
NaCl	1 g	26.4 g
$MgCl_2 \cdot 6H_2O$	–	5.6 g
$MgSO_4 \cdot 7H_2O$	0.5 g	6.8 g
KCl	0.5 g	0.66 g
$CaCl_2 \cdot 2H_2O$	0.1 g	1.47 g
KBr	–	0.09 g
Additives after autoclaving and anoxic cooling		
Trace element solution	1 ml	1 ml
Thiamine solution	1 ml	1 ml
Vitamin solution	1 ml	1 ml
B_{12} solution	1 ml	1 ml
Sodium bicarbonate solution	30 ml	30 ml
Sodium sulfide solution	0.5 ml*	0.5 ml*
pH	7.0 – 7.1	7.0 – 7.3

*Omitted from several media.

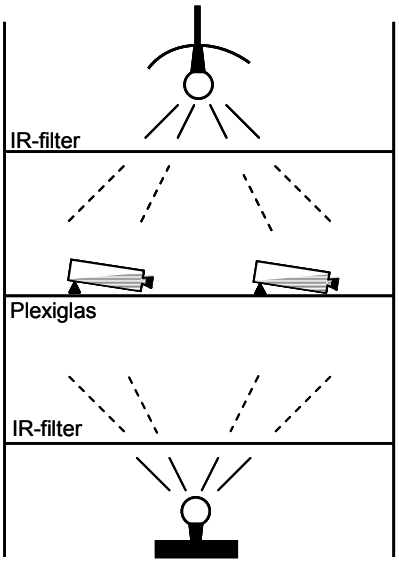

Fig. 9. Diagram of incubation shelf of phototrophic enrichment cultures.

B.2.4 Isolation

For isolation of pure cultures, serial dilutions were carried out in anoxic agar medium. An amount of 3.3 g agar (Difco) was stirred for 15 min in 300 ml of distilled water. Washing was repeated five times. After the final sedimentation, 100 ml of

distilled water were added and the agar was melted. The molten agar was portioned in volumes of 3 ml into Hungate tubes. The tubes were closed with aluminium caps, autoclaved and kept at 4°C until use.

To carry out the agar dilution series, seven tubes with the concentrated agar were heated to melt the agar and kept at 60°C in a water bath. A volume of 6 ml of 41°C-prewarmed medium was added to each tube by means of glass pipettes while contact with air was kept minimal. An inoculum of 0.2 ml was added to the first tube and gently mixed. From this tube, approximately 1 ml was transferred to next new tube and so on until the last tube. All tubes were closed with butyl rubber stoppers and cooled with water. Immediately after gelling, the tubes were gassed with an N_2/CO_2 mixture (9/1, v/v).

B.2.5 Growth of microorganisms

B.2.5.1 Determination of optimal growth temperature

Different temperatures were tested in duplicates for growth of strain E3P with acetate as substrate and toluene as substrate for strain GH1.

B.2.5.2 Determination of optimal growth pH

Different pH values were used to determine the optimal pH (Table 8). The pH of 9 was adjusted after autoclaving. If necessary, the pH-values were adjusted with 1 M NaOH or 1 M HCl.

Table 8. Preparation of different pH-values.

Tested pH	Used buffer
3.0	–
4.0	–
5.0	MES
5.5	MES
6.0	MES
6.5	PIPES
7.0	PIPES
7.5	HEPES
8.0	HEPES
8.5	$NaHCO_3$
9.0	$NaHCO_3$

B.2.5.3 Monitoring of growth

Microbial growth was monitored by measuring optical density at a wavelength 600 nm or 660 nm in a UV-VIS spectrophotometer (Schimadzu TUBE UV) with tubes or UV-VIS spectrophotometer (UV-1240, Shimadzu, Sehnde) with plastic cuvettes.

B.2.5.4 Growth for stoichiometric analyses

Quantitative growth experiments were carried out in 1 l-bottle Pyrex with 500 ml medium under a head space of N_2/CO_2 (9/1, v/v). Different concentrations of AH_2QDS were used (1.6 and 2 mM). Sampling was carried out in an anoxic chamber. For determination of cell mass, a defined culture volume was centrifuged at 14,300 × g for 25 min (Beckman Coulter with JA-10-Rotor). The dry mass of the cells was determined after washing with an ammonium acetate solution (18 mM), removing of the supernatant and the pellet was dried at 60°C constant weight.

B.2.5.5 Co-culture experiment with strain E3P and *Geobacter metallireducens*

A co-culture experiment between strain E3P and *G. metallireducens* strain DSM 7210 was carried out in 1 l-bottle Pyrex with 500 ml medium with 2 mM AQDS, 2 mM benzoate and 5% inoculum. Growth of bacteria was followed by measuring the OD at 600 nm; in addition, the change of AH_2QDS concentration was determined (B.3.1).

B.3 Analytical methods

B.3.1 Quantification of anthrahydroquinone-2,6-disulfonate

A stock solution of AH_2QDS was prepared by reducing anthraquinone-2,6-disulphonate (AQDS; Sigma) to anthrahydroquinone-2,6-disulphonate (AH_2QDS). First, a 50 mM stock solution of AQDS in 30 mM bicarbonate buffer was prepared in a serum bottle and sealed with a butyl rubber stopper. It was autoclaved and gassed with N_2/CO_2 (9/1, v/v). Then, palladium-coated pellets were added as reduction catalyst, and H_2 was bubbled through the solution. Finally, bottles were again flushed with N_2/CO_2 to remove the remaining H_2.

The concentration of AH_2QDS in solution was determined by UV-VIS spectroscopy. Absorbance spectra were recorded with a scanning Lamda 20 spectrophotometer (Perkin Elmer, Germany). The cuvettes (quarzglass Suprasil®, Ochs Laborbedarf) 10 mm light path were used; the reference was an identical cuvette with distilled water. Concentrations of AH_2QDS were determined by monitoring the absorbance at 436 nm and using an extinction coefficient of 3.5 absorbance units per mM (Bayer *et al*., 1996).

B.3.2 Protein quantification

Concentration of protein was determined by the bicinchoninic acid method (Smith *et al*., 1985). Protein was precipitated by trichloroacetic acid (final concentration, 0.5 M). Precipitated protein was centrifuge for 10 min at 30,000 × g, dissolved in 0.1 M NaOH at 60°C for 20 min. The protein concentrations were determined according to the "enhanced protocol" by measurement at 562 nm using UV-VIS spectrophotometer. Albumin was used as standard for calibration (Sigma, Steinheim, Germany).

B.3.3 Humic acids analysis

Humic acids (Aldrich) were dissolved (1 g/l) in 30 mM bicarbonate buffer and incubated under H_2 in the presence of Pd catalyst (COY, USA) on a shaker at 28°C overnight and then flushed with N_2 to remove excess H_2. The electron accepting capacity of humic acids was determined by reaction with Fe(III)-citrate and quantification of the produced Fe(II) with the ferrozine assay (Stookey, 1970; Kappler *et al*., 2004).

B.3.4 Microscopy

Phase-contrast photomicrographs were taken with a Zeiss Axioplan photomicroscope (Zeiss, Germany). Flagella were observed by transmission electron microscopy (TEM). Cells were negatively stained with 2% (w/v) uranyl acetate. Bright-field TEM images were obtained with a Zeiss EM10 transmission electron microscope at an accelerating voltage of 60 kV (Scheffel *et al*., 2006).

B.3.5 Photosynthetic pigments

Absorption spectra of cells were recorded with a scanning Lamda 20 spectrophotometer (Perkin Elmer, Germany). An acetate-grown culture was concentrated 10-fold by centrifugation; a volume of 1 ml of the suspension was added to a solution of 5 g of saccharose in 3 ml H_2O to minimize diffraction (Ehrenreich and Widdel, 1994).

B.3.6 Sulfide analysis

Sulfide was determined according to Cline (1969; Aeckersberg *et al*., 1991).

Solution A **Zinc acetate solution**

$(CH_3COO)_2Zn \cdot 2H_2O$	20 g
Acetic acid (100%)	1 g
Distilled water	1000 ml

Solution B **0.2% N, N Dimethyl-*p*-phenylenediamine dihydrochloride**

A volume of 500 ml of distilled water was cooled in ice bath and 200 ml H_2SO_4 were added under constant stirring. Then 2 g of dimethyl-*p*-phenylenediamine dichloride was added to the cold solution. The final volume was adjusted to 1000 ml and stored at 4°C in dark. Contact with naked skin and eyes had to be avoided because of the acidity and potential carcinogenicity of the solution.

Solution C

$Fe_2(SO_4)_3 \cdot 12H_2O$	10 g
H_2SO_4	2 ml
Distilled water	1000 ml

The assay was carried out in small glass test tubes, with the following components successively added and mixed. The reaction mixture was incubated at room temperature in the dark for 20 min. The absorbance was measured in plastic cuvettes at 670 nm using a UV-VIS spectrophotometer.

Solution A	1 ml
Sample	2 – 20 µl
Distilled water	1 ml
Working solution B (diluted 1:2.5)	1 ml
Working solution C (diluted 1:50)	1 ml

B.3.7 Gas chromatograpy analysis of hydrocarbon

Toluene dissolved in heptamethylnonane was quantified by means of an Auto System gas chromatograph (Perkin Elmer, Überlingen, Germany) equipped with a PVMS 54 column (length, 50 m; inner diameter, 0.32 mm) and a flame ionization detector. The flow rate of H_2 as the carrier gas was 1.7 ml min^{-1}. The temperature program was run from 60°C (2 min isotherm) to 120°C at 5°C min^{-1}, and then from 120°C (0.1 min isotherm) to 220°C at 10°C min^{-1} (5 min isotherm at 220°C). The temperatures at the injection port and the detector were 250°C and 280°C (Zengler *et al*., 1999).

B.4 Molecular methods

B.4.1 Preparation of genomic DNA

Extraction buffer	
Tris-HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	100 mM
Na-Phosphate (pH 8.0)	100 mM
NaCl	1.5 M
CTAB (Hexadecyltrimethylammoniumbromide)	1%
SDS	10% (w/v)
Proteinase K	10 mg ml^{-1}
Chloroform/Isoamylalcohol	24:1 (w/v)
Isopropanol	
Ethanol	70% (v/v)
TE-buffer 0.5×, pH 7.0 – 8.0	
Tris-HCl	5 mM
EDTA	0.5 mM

To prepare genomic DNA for PCR amplification, 2 – 4 ml of culture was centrifuged at 10,000 rpm for 15 min. The supernatant was removed and discarded. The cell pellet was mixed with 1.4 ml of extraction buffer plus 10 µl of proteinase K and incubated at 37°C for 30 min on a shaker. A volume of 300 µl of 10% SDS was added, incubated at 65°C for 1 h under mild shaking. The solution was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was transferred to a fresh Eppendorf tube. An equal volume of chloroform/isoamylalcohol was added, mixed thoroughly and centrifuged at 10,000 rpm for 10 min. The aqueous upper

phase was collected and transferred to a new Eppendorf tube. Isopropanol was added (0.6 ml isopropanol per 1 ml collected aqueous upper phase), and mixed by moderate shaking and incubation at room temperature for 1 h. After centrifugation at 10,000 rpm for 30 min, the supernatant was decanted. The pellet was washed by addition of 1 ml cold 70% ethanol and centrifuged at 10,000 rpm for 5 min. The supernatant was carefully decanted and the pellet was dried for 15 min at room temperature and dissolved in 50 µl TE, and stored at 4°C.

B.4.2 PCR amplification of 16S rRNA genes

PCR amplifications were performed using a Thermocycler Mastercycler (Eppendorf, Hamburg, Germany) in a total volume of 50 µl containing 5 µl BSA 3 mg ml^{-1}, 4 µl of 2.5 mM of each dNTP, 5 µl 10× PCR buffer, 1 µl 50 µM each primer GM3 and GM4 (Muyzer *et al.*, 1995) and 0.15 µl 5 U $µl^{-1}$ Eppendorf Master Taq. The polymerase chain reaction was used for *in vitro* amplification of 16S rRNA gene sequences using general bacterial primers. The amplification was done with PCR program as follows: 5 min denaturising at 96°C followed by 30 cycles of 1 min denaturising at 96°C, 2 min annealing at 48°C and 3 min extension at 72°C, with a final extension step of 72°C for 10 min.

B.4.3 Agarose gel electrophoresis

Agarose	1% or 2% (w/v)
100× TAE, pH 8.0	4 M Tris
	1 M NaAcetate
	0.1 M EDTA
Loading buffer (6×)	Bromphenol-Blue 2.5 g/l
	Xylene cyanol 2.5 g/l
	Sucrose 400 g/l
Ethidiumbromide	0.4 µg ml^{-1} in distilled water

The gel electrophoresis was used for analysis of the fragment size. A volume of 5 µl PCR sample was mixed with 2 µl loading buffer and applied to an 1% agarose gel covered with TAE (1×) in a horizontal electrophoresis chamber (Horizon 58, GIBCO BRL, Life Technologies, Gaithersburg, MD, USA) and run at 75 mV, for 30 min. Afterwards the agarose gel was stained for 15 min in an ethidiumbromide staining

bath. Nucleic acids were visualized with a UV Transilluminator (INTAS, Göttingen, Germany).

B.4.4 Denaturing gradient gel electrophoresis (DGGE)

The universal PCR primer pair GM5F and 907R specific for the bacteria domain was used to amplify 550 bp fragments of the 16S rRNA gene (Muyzer *et al*., 1993). A GC-rich clamp was added to the 5'-end of the forward primer (GM5F) to stabilize the melting PCR products on DGGE gels (Muyzer *et al*., 1993). PCR was carried out in touchdown mode with the annealing temperature decreasing in 20 cycles from 65°C to 55°C; at the latter temperature, PCR was extended for further 16 cycles. Bovine serum albumin (BSA; Sigma, Steinheim, Germany) was added to the PCR solution (final concentration 0.3 mg ml^{-1}) to prevent inhibition of the polymerase by humic substances. DGGE analysis of PCR amplified 16S rRNA gene fragments was performed by using the D-GeneTM system (Bio-Rad Laboratories, Munich, Germany) as described previously (Schäfer and Muyzer, 2001).

PCR products were separated on 6% (w/v) polyacrylamide gels of 1 mm thickness with a 30 – 70% of the denaturing agents urea and formamide (100% denaturant corresponds to 7 M urea and 40% deionized formamide) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3 as listed in Tables 9 and 10). Electrophoresis was performed at constant voltage of 75 V for 16 h at a temperature of 60°C. After electrophoresis, the gels were stained for 30 min with SYBR Gold (1:10000 dilution, Sigma) and visualized on a UV Transilluminator (INTAS, Göttingen, Germany). The resulting bands were excised from the gel and eluted in 50 µl water at 4°C overnight. The eluted DNA was then reamplified using the primers GM5F (without GC-clamp) and 907R.

Table 9. Preparation of denaturant stock solutions of 6% acrylamide/bis-acrylamide (Schäfer and Muyzer, 2001).

Components	Urea and formamide	
	0%	100%
40% Acrylamide/bis-acrylamide (37.5 : 1)	15 ml	15 ml
50× TAE	2 ml	2 ml
7 M Urea	-	42 g
Formamide (deionized)	-	40 ml
Distilled water to	100 ml	100 ml

10% Ammonium persulfate solution

Ammonium persulfate (APS) 1 g

Distilled water to 10 ml

Single-use portions of 10 ml APS were stored at –20°C

TEMED

TEMED was bought as ready-to-use solution (Bio-Rad).

Gradient-Gels

Table 10. Working solutions for gradient gels (12 ml gel; Demba Diallo, pers. commun.)

Denaturant solutions	0%	100%	APS 10%	TEMED
30%	8.4 ml	3.6 ml	100 µl	8 µl
70%	3.6 ml	8.4 ml	100 µl	8 µl

Stacking-Gels

For the stacking-gel, 7.5 ml 0% denaturant solution plus 150 µl APS 10% and 12 µl TEMED were added.

B.4.5 Genomic DNA fingerprints

Genomic DNA of microorganisms was used as template to generate genomic fingerprints with two RAPD primers RCP4 (5'-GGACCAGCGA-3'), SMO3 (5'-CGGGTCGATC3'), and BOX primer BOXA1R (5'CTACGGCAAGGCGACGCT GACG-3'; Versalovic *et al.*, 1994). PCR was carried out in 25 µl reaction mixtures containing PCR buffer 10× (2.5 µl), dNTPs, 0.3 mg BSA ml^{-1}(2.5 µl), and 2 U (1.25 µl) of Taq polymerase (Sigma-Aldrich). The amplification was done with a Thermocycler Mastercycler (Eppendorf, Hamburg, Germany) with PCR program as follows: 5 min denaturising at 95°C followed by 45 cycles of 1 min denaturising at 94°C, 2 min annealing at 36°C and 2 min extension at 72°C, with a final extension step of 72°C for 10 min.

B.4.6 Determination of the G + C content of DNA and DNA-DNA hybridization

The analysis of G + C content of DNA and DNA-DNA hybridization was carried out at German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. DNA was isolated after using a French pressure cell (Thermo Spectronic) and was purified via hydroxyapatite (Cashion *et al*., 1977), hydrolyzed and analyzed by HPLC (Mesbah *et al*., 1989).

DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV-VIS-spectrophotometer equipped with a Peltier-thermostated 6 × 6 multicell changer and a temperature controller with an *in-situ* temperature probe (Varian).

B.4.7 Sequencing and phylogenetic analyses

PCR products were purified with the Quiaquick Spin PCR purification kit (Qiagen). Sequencing was performed by using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, California, USA). Retrieved 16S rRNA gene sequences were added to the rRNA gene sequence database of the Technical University Munich by using the ARB program package (Ludwig *et al*., 2004). A phylogenetic tree was reconstructed by performing maximum parsimony, neighbour joining and maximum likelihood

analyses. Only nearly full-length sequences were used for calculation of the trees (around 1300 bp with GM3/GM4 primers; 500 bp with DGGE primers). Sequences were inserted into the tree according to parsimony criteria without affecting the overall topology.

Degenerate primers specific for the *bssA* gene encoding the large subunit of benzylsuccinate synthase (Galushko, unpublished) were used to get partial sequences of *bss*A gene of strains of toluene-utilizing phototrophic bacteria. Obtained PCR products were sequenced as described. Contigs were assembled and consensus sequences were generated in the SeqMan sub-program of the Lasergene 6.0 computer program package. Related proteins were identified and retrieved from the NCBI protein database after Blast search of translated nucleotides queries of consensus sequences. Protein sequence alignment was calculated with the ClustalW version 1.83 software (Higgins Sharp, 1988; Thompson *et al*., 1994). Phylogenetic analyses were conducted with the MEGA version 3.1 (Kumar *et al*., 2004) using distance-based methods. Confidence limits of branching points were estimated by 1000 bootstrap replications.

C Results

C.1 Phototrophic bacteria enriched and isolated with humic substances

C.1.1 Isolation of an AH_2QDS-oxidizing phototrophic bacterium

From a previously established enrichment culture for ferrous iron-oxidizing phototrophic bacteria, an inoculum was used to enrich phototrophic bacteria able to oxidize anthrahydroquinone-2,6-disulfonate (AH_2QDS). Anthrahydroquinone-2,6-disulfonate is considered as a defined model compound of humic acids. After several subsequent transfers with AH_2QDS as sole electron donor, the culture showed growth of phototrophic bacteria with simultaneous change of the color of the culture from red to colorless. This demonstrated that AH_2QDS (red color) was oxidized to AQDS (colorless). From this enrichment culture, agar dilution series were carried out with acetate as substrate. Two kinds of colonies developed. One of each type colony was picked up and transferred to new liquid media with AH_2QDS as sole electron donor. The isolates were named as strain E3P and FWP1. Both strains grew on AH_2QDS in the beginning. But strain FWP1 did not oxidize AH_2QDS after several transfers. Only strain E3P showed continuous growth with AH_2QDS. Therefore, this strain was chosen for further experiments.

C.1.2 Morphological and physiological characteristics

Cells of strain E3P were oval and 1.6 by 2.2 µm in diameter (Fig. 10a). Cells multiplied by binary fission and were motile by bundles of flagella (Fig. 10b). The bacterium formed purple red colonies in agar medium. Cell suspensions grown in liquid media with acetate in the light were red. Strain E3P developed as single cells in liquid medium.

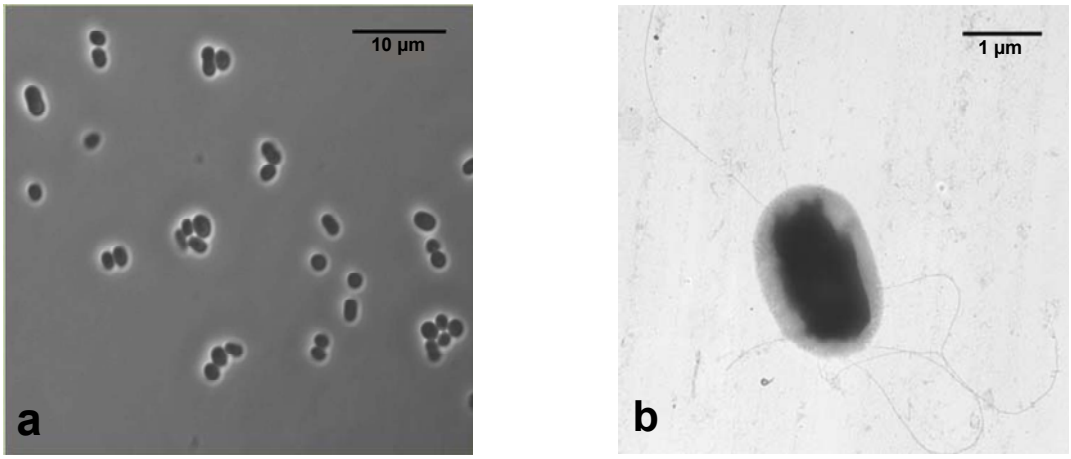

Fig. 10. a, Phase-contrast photomicrograph. **b**, Transmission electron photomicrograph.

The absorption spectra of strain E3P in a suspension in saccharose solution showed maximum at 464, 494, 524, 595, 809 and 859 nm (Fig. 11). The three major absorption peaks at 595, 809, and 859 showed the presence of bacteriochlorophyll *a*, whereas the three peaks at 464, 494 and 524 nm indicated the presence of lycopene and rhodopin (Guyoneaud *et al.*, 1997; Mehrabi *et al*., 2001). This meant that carotenoids of strain E3P belonged to normal spririlloxanthin series. Strain E3P grew at pH values between 5.5 and 8.5, with a pH optimum from 7.0 to 7.5. Growth was possible at temperatures from 14 to 37°C, the optimum being at 28°C – 30°C.

Strain E3P was capable of growing photoautotrophically under anoxic conditions in the light using sulfide, thiosulfate and molecular hydrogen as electron donor, and CO_2 as the sole carbon source (Table 11). Sulfur globules were observed and unevenly distributed within the cells when strain grew with sulfide as electron donor. Oxidation of ferrous iron by strain E3P was also observed. When acetate was added to the culture, oxidation of ferrous sulfide was faster and the black color of FeS disappeared completely within two weeks (Fig. 12). This capability was observed previously in some ferrous iron-oxidizing phototrophic bacteria (Ehrenreich and Widdel, 1994). In addition, strain E3P grew photoheterotrophically with several simple organic compounds (Table 11).

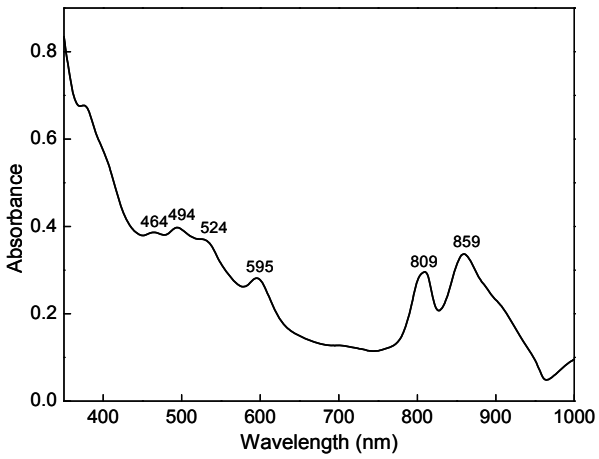

Fig. 11. Absorption spectrum of whole cells of strain E3P.

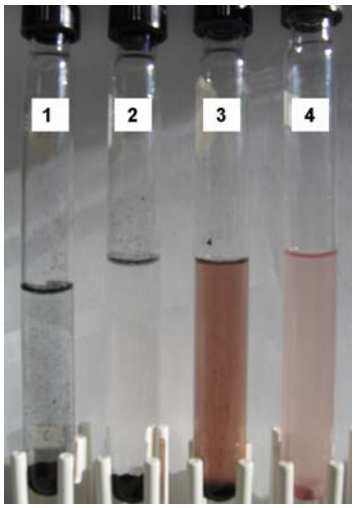

Fig. 12. Progressive oxidation of FeS by train E3P. 1, Sterile FeS medium. 2, Tube after inoculation. 3 and 4, Successive intermediate oxidation of FeS.

The oxidation of AH_2QDS was also tested with some strains of other related purple sulfur bacteria such as *Thiocapsa roseopersicina* DSM 217, *Thiocystis gelatinosa* DSM 215, *Thiocystis violacea* DSM 207, and *Thiorhodococcus minus* DSM 11518. However, none of these strains showed the ability to oxidize AH_2QDS, except only *Thiocapsa roseopersicina* DSM 217 (Table 12).

Table 11. Utilization of various substrates as electron donors or carbon sources by strain E3P. Substrates were added at the concentrations as indicated.

Sources tested (mM)	Growth
Inorganic substrates	
H_2	+
Sulfide (0.5, 1)	+
Thiosulfate (4)	+
FeS	+
Organic acids	
Acetate (4)	+
Propionate (3)	+
Butyrate (2)	+
Lactate (4)	+
Pyruvate (3)	+
Succinate (4)	+
Fumarate (4)	–
Malate (4)	–
Benzoate (2)	–
Alcohos/sugars	
Methanol (2)	–
Ethanol (2)	+
Fructose (2)	+
Glucose (2)	–
Amino acids	
Alanine (4)	–
Aspartate (4)	–
Glutamate (4)	–
AH_2QDS (1.6–2)	+
Reduced humic acids (20 mg/L)	+

Symbols: –, No utilization; +, utilization.

C.1.3 Phylogenetic affiliation

From strain E3P, a nearly complete 16S rRNA gene sequence of 1400 nucleotides was obtained. Phylogenetic analysis based on 16S rRNA gene sequences from the data bank confirmed that strain E3P belonged to the genus *Thiocystis,* family *Chromatiaceae* (Fig. 13). The new isolate is most closely related to *Thiocystis violaceae,* with a similarity level of 95.8%. Other close relative is *Thiocystis gelatinosa* (94.3%).

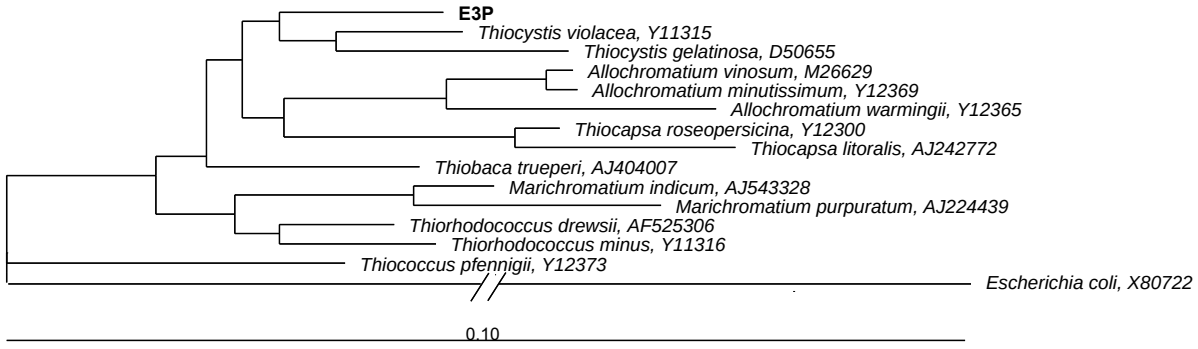

Fig. 13. Position of strain E3P in a phylogenetic tree based on 16S rRNA gene sequence data and maximum likelihood analyses. The scale bar represents 10% estimated sequence divergence.

Table 12. Comparison of strain E3P with its phylogenetic relatives.

Property/substrate	E3P	*Thiocapsa roseopersicina**	*Thiocystis gelatinosa**	*Thiocystis violacea**
Cell shape	Oval	Sphere	Coccus	Coccus
Cell dimensions (µm)	1.6 by 2.2	1.2–3.0	3	2.5–3.0
Motility	+	–	+	+
Color of cell suspensions	Purple-red	Pink/orange-brown	Purple-red	Purple violet
Carotenoid series	Spirilloxanthin	Spirilloxanthin	Okenone	Rhodopinal
G + C DNA mol (%)	41.6	63.3–66.3	61.3	62.8–67.9
Vitamin requirement	–	–	–	–
pH optimum	7.0–7.5	7.3	nd	7.3
pH range	5.5–8.5	6.5–7.5	6.5–7.6	6.5–7.6
Chemoautotrophy	+	+	+	+
Substrates tested				
AH_2QDS	+	+	–	–
Reduced humic acids	+	nd	nd	nd
Hydrogen	+	+	nd	+
Sulfide	+	+	+	+
Thiosulfate	+	+	–	+
Acetate	+	+	+	+
Propionate	+	±	–	±
Butyrate	+	nd	nd	nd
Lactate	+	±	–	–
Pyruvate	+	+	+	+
Succinate	+	+	–	±
Fumarate	–	+	–	+
Malate	–	nd	nd	nd
Benzoate	–	nd	nd	nd
Methanol	–	nd	nd	nd
Ethanol	+	–	–	–
Fructose	+	+	–	±
Glucose	–	–	–	±
Alanine	–	nd	nd	nd
Aspartate	–	nd	nd	nd
Glutamate	–	nd	nd	nd

Symbol: – No utilization, + utilization, ± utilization by some strains, nd not determined. *Data from Guyoneaud *et al*. (1997).

C.1.4 Oxidation of anthrahydroquinone-2,6-disulfonate and reduced humic acids

Anaerobic growth of strain E3P on AH_2QDS was observed only if cultures were incubated in the light (Fig. 14b). In the presence of cells and light, oxidation of AH_2QDS to AQDS was complete (Fig. 15). Analysis of the concentration of AH_2QDS before and after growth of E3P revealed that recovered AH_2QDS (after chemical reduction of AQDS as described in method section) was 106% (2.06 mM) compared to the AH_2QDS concentration in the beginning of experiment (1.93 mM). This result demonstrated that strain E3P did not utilize AH_2QDS as carbon source but only as electron donor for its growth as shown in the simplified scheme (Fig. 16).

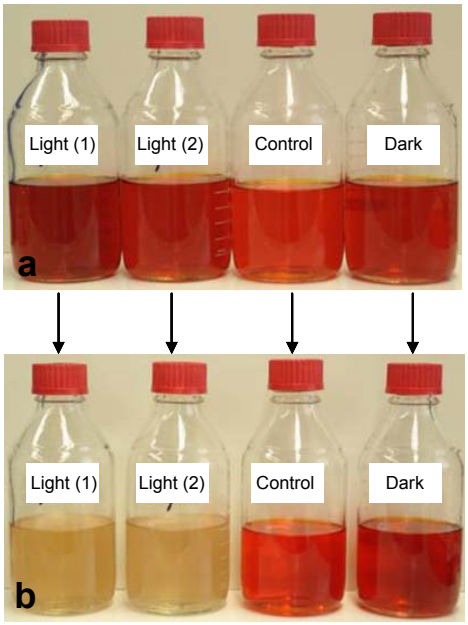

Fig. 14. Oxidation of AH_2QDS by strain E3P. **a**, After inoculation, before incubation. **b**, After 7 days of incubation.

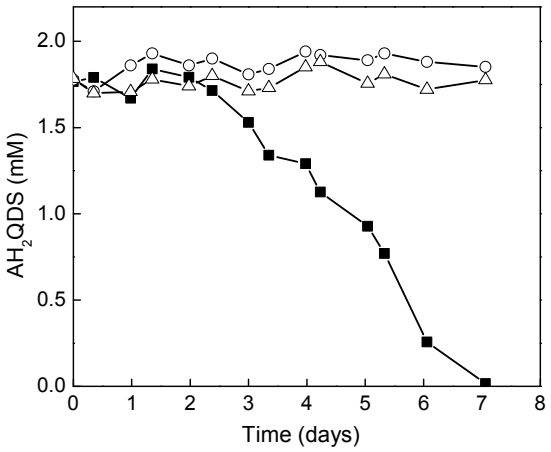

Fig. 15. Incubation of AH_2QDS (■) with strain E3P in the light, (○) with strain E3P in the dark, (Δ) without strain E3P in the light.

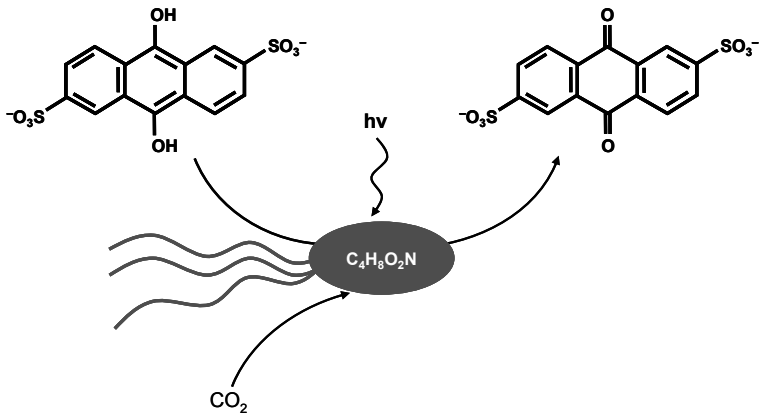

Fig. 16. Scheme of the light-dependent utilization of AH_2QDS by strain E3P.

Phototrophic AH_2QDS oxidation and formation of cell mass were quantified in growth experiments with different AH_2QDS concentrations. Because of the sensitivity of AH_2QDS to oxygen (it was immediately oxidized when exposed to the air), the handling of samples with AH_2QDS were carried out in an anoxic chamber; measurements were performed in butyl rubber-capped quartz cuvettes. Results are summarized in Table 13.

Theoretically, the molar ratio between oxidized AH_2QDS and the formed simplified biomass unit $C_4H_8O_2N$ is 17/2, according to the following equation:

$$17\ AH_2QDS + 8\ HCO_3^- + 2\ NH_4^+ + 6\ H^+ \rightarrow 17\ AQDS + 2\ C_4H_8O_2N + 20\ H_2O$$

This means that 1 mmol of AH_2QDS yields 2/17 mmol of biomass unit $C_4H_8O_2N$ (formula mass, 102), corresponding to 12 mg of cell biomass. In experiment, 52 – 90% of the theoretical cell yields were reached (Table 13). The difference may be due to the loss of cells during harvesting for determination of dried biomass.

Ratio of protein and biomass, experimentally determined, was around 47%. This percentage was nearly the same as that reported for other phototrophic bacterium (Jiao *et al*., 2005). There is 3.53 mg of protein and 7.44 mg of biomass produced per 0.7 mmol AH_2QDS oxidized. No abiotic or biotic oxidization of AH_2QDS in control and in dark bottles were observed respectively (Fig. 14).

Table 13. Quantification of phototrophic growth of strain E3P on AH_2QDS.

	AH_2QDS oxidized	Cell dry mass (theoretical)	Cell dry mass formed	Cell yield	
	(mmol)	(mg)	(mg)	(mg/mmol)	% of expected
Bottle 1	0.80	9.61	5.03	6.27	52.3
Bottle 2	0.86	10.34	7.13	8.27	68.9
Bottle 3	0.69	8.27	7.44	10.7	90.1

C.1.5 Humic acids as electron donor

AH_2QDS does not occur in nature; in the present study, it was used as an artificial model compound of humic acids to simplify isolation and growth experiments. In a next step, capability of strain E3P to oxidize humic acids was examined. The experiment included washed cells of strain E3P and chemically reduced humic acids. Similarly to AH_2QDS, reduced humic acids were oxidized by strain E3P during 3 h of incubation. Whereas the reduced humic acids in dark incubation without cell lost only 79 nmol electrons per mg (corresponding to 18.2% oxidation), reduced humic acids in illuminated cultures lost 434.5 nmol electrons per mg (corresponding to 100% oxidation) within 3 h of incubation with cells at an OD_{600} of approximately 0.28.

C.1.6 Co-culture experiments with *Geobacter metallireducens* and strain E3P based on a humics cycle

To investigate whether humic acids may function as electron shuttle between chemotrophic and phototrophic bacteria (Fig. 17), co-culture experiments were carried out with *G. metallireducens* as humic acids reducer and strain E3P as humic acids oxidizer. Benzoate was chosen as the primary organic substrate, because it is not utilized by strain E3P but only by *G. metallireducens*. Both, AQDS and humic acids were tested as electron shuttles in the mixed culture that was incubated in the light. Cultures incubated in the dark or sterile medium served as controls. Cell growth was monitored by means of optical density.

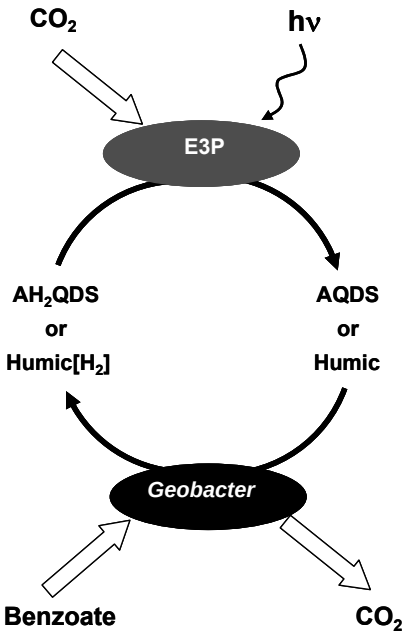

Fig. 17. Expected role of AQDS or humic acids as electron shuttles between *G. metallireducens* and strain E3P.

In the experiment with AQDS, growth indeed occurred only in the light (Fig. 18a). Reduction of AQDS to AH_2QDS was measured. Increase in AH_2QDS concentration was observed only in bottles incubated in the dark (Fig. 18b). With *G. metallireducens* alone or with strain E3P in the dark, the red color of AH_2QDS appeared which could not be reoxidized (Fig. 19b). Also in the experiment with humic acids, growth of the mixed culture occurred only in the light (Figs. 20 and 21), even though growth was much slower than with AQDS.

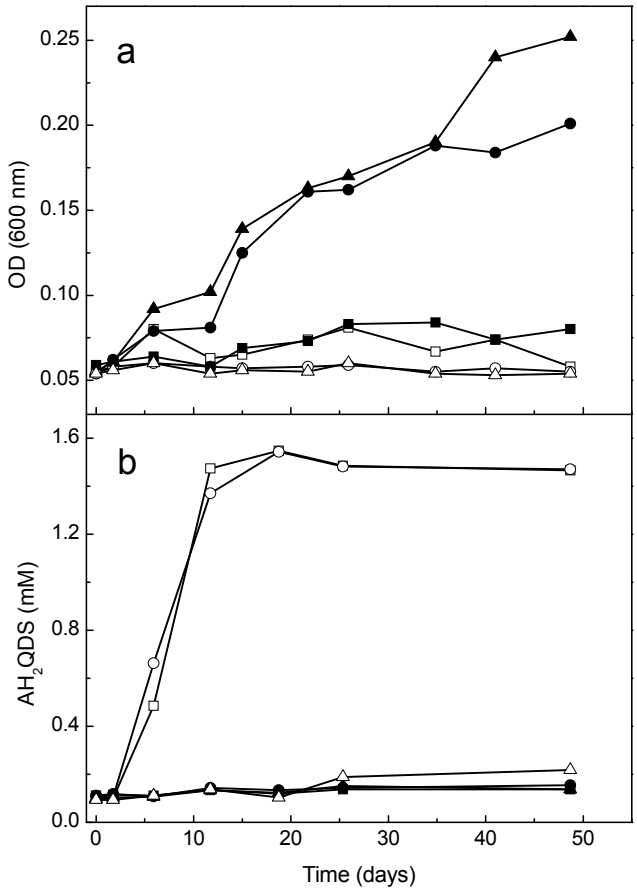

Fig. 18. a, Bacterial cell density (OD) in the co-culture. **b**, Reduction of AQDS in the same co-culture. (□) *G. metallireducens*, dark. (●) Co-culture, light. (▲) Co-culture, light. (○) Co-culture, dark. (■) Strain E3P, light. (△) Without co-culture, light.

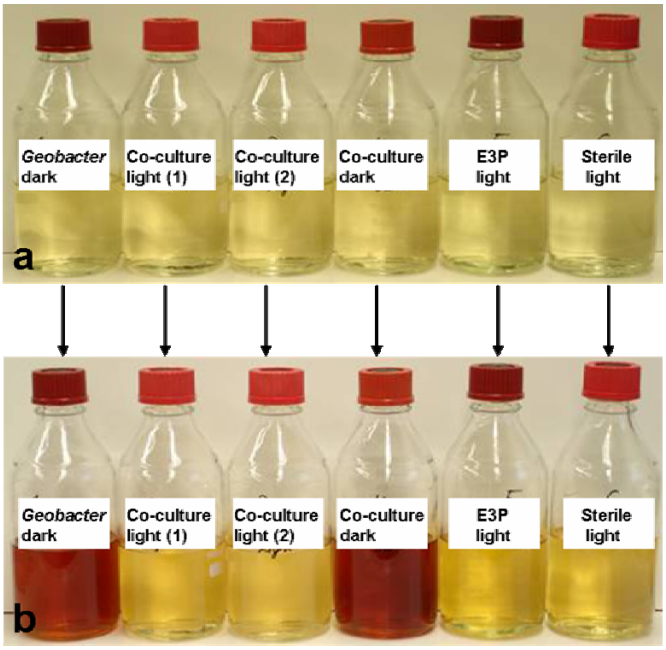

Fig. 19. Incubation of the co-culture of *G. metallireducens* and strain E3P in the presence of AQDS (2mM) and benzoate (2 mM). **a**, After inoculation, before incubation. **b**, After 2 weeks of incubation.

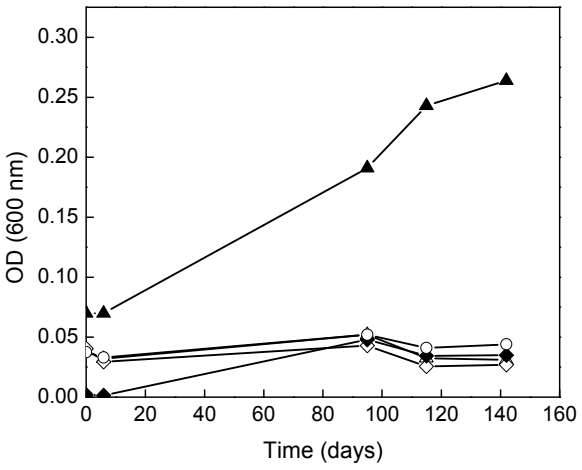

Fig. 20. Bacterial cell density (OD) in the co-culture in the presence of humic acids (20 mg/l) and benzoate (1 mM). (▲) Co-culture, light. (Δ) Without co-culture, light. (◇) No benzoate, light. (□) No humic acids, light. (○) Co-culture, dark.

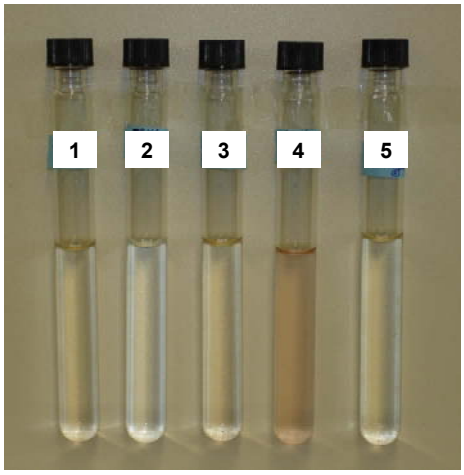

Fig. 21. Incubation of the co-culture of *G. metallireducens* and strain E3P in the presence of humic acids (20 mg/l) and benzoate (1 mM). 1, Without co-culture, light. 2, Without humic acids, light. 3, Without benzoate, light. 4, Co-culture, light. 5, Co-culture, dark.

C.2 Phototrophic bacteria enriched and isolated with hydrocarbons

This section describes enrichment cultures and attempts to isolate pure cultures of anoxygenic phototrophs with hydrocarbons. The initial interest was in the possible utilization of *n*-alkanes, because such a capacity among phototrophs has not been observerd so far. For comparison, enrichment and isolation studies were also carried out with toluene, a compound previously shown to be utilized phototrophically (Zengler *et al*., 1999).

C.2.1 Establishment of enrichment cultures with *n*-alkanes and toluene in the light

Sediments from Paraíso and Carmen beaches (Gulf of Mexico) were collected and used as inocula to enrich *n*-hexane- and *n*-decane-utilizing phototrophic anaerobic bacteria. *n*-Hexane and *n*-decane were dissolved in heptamethylnonane carrier phase (each 2% in HMN, v/v) to avoid the toxicity of the pure substances (Rabus *et al*., 1993). Enrichment cultures were illuminated through infrared filters excluding wavelengths below 750 nm (Zengler *et al*., 1999). With the Paraíso sediment, only *n*-hexane, but not *n*-decane yielded positive growth of microorganisms. The medium turned intensively brown after 2 months of incubation (Fig. 22a) and maintained this color in consecutive subcultures. This color is indicative of green sulfur bacteria, some of which have a pigment composition yielding a brown color. Carmen enrichments showed growth of phototrophic bacteria on both, *n*-hexane and *n*-decane. These bottles turned red and green (Fig. 22a). Subcultures showed the same apparent growth of phototrophic bacteria. Under a phase-contrast microscope, coccoid- and short-, rod-shaped cells were observed in these cultures.

For comparison and as control, toluene containing medium was inoculated with freshwater sediment (Kuhgraben, Bremen). Toluene (2%, v/v) was also dissolved in HMN. The bottle turned intensively green after one month of incubation (Fig. 22b). Subcultures maintained the green color, again indicating growth of anoxygenic phototrophic bacteria.

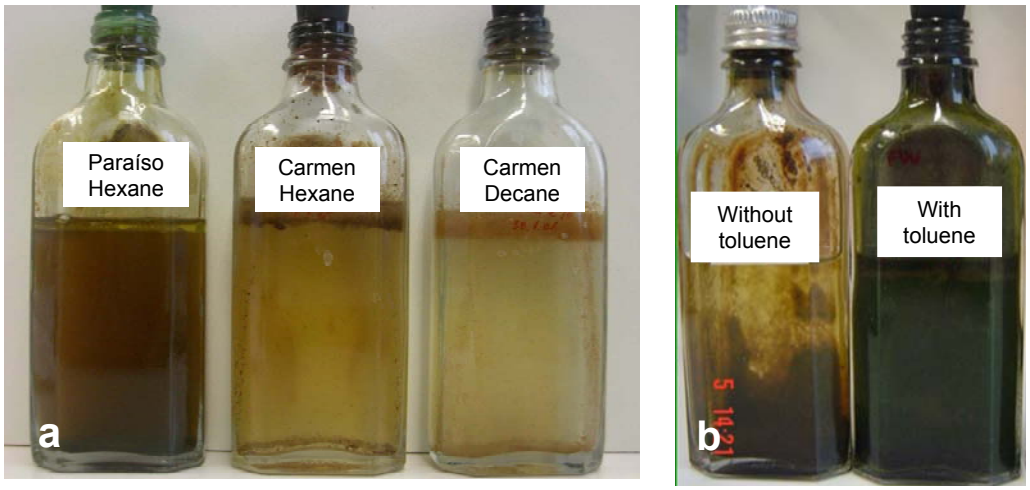

Fig. 22. Enrichment cultures in the light **a**, on hexane and decane; **b**, on toluene.

C.2.2 Attempts to isolate pure cultures with hydrocarbons

Agar dilution series were carried out to isolate potentially hydrocarbon-utilizing phototrophic bacteria. Colonies of phototrophic bacteria able to utilize alkanes were not obtained within the present study. From the toluene enrichment, however, green colonies developed in the agar beneath HMN layer, indicating growth with toluene diffusing into the agar. The green colonies were isolated and indeed showed growth on toluene in liquid media. One isolate, strain GH1, was chosen for further studies (C.2.4).

C.2.3 Characterization of the phototrophically grown enrichment cultures with *n*-alkanes

Since attempts to isolate *n*-hexane- or *n*-decane-utilizing phototrophic bacteria failed until now, the enrichment cultures were therefore characterized on the basis of 16S rRNA genes and activity toward alkanes in the dark so as to gain insights into potentially involved phototrophic and chemotrophic bacteria.

Nucleic acids from the *n*-hexane and *n*-decane enrichment cultures were amplified with a pair of 16S rRNA domain-specific primers for bacteria GM5F and 907R (Muyzer *et al*., 1993). The obtained 16S rRNA gene fragments were separated by DGGE. The DGGE profiles did not reveal significant differences between the enriched communities from Carmen and Paraíso sediments on *n*-hexane and *n*-decane (Fig. 23). Thirteen bands were chosen for analysis. They

were excised from gel, re-amplified and sequenced (Fig. 23). Band 1 and 11 yielded ambiguous sequencing results. Table 14 summarizes the possible affiliation of the 11 sequences. A phylogenetic tree is shown in Fig. 24. In the enrichment cultures on *n*-hexane and *n*-decane from Carmen sediment retrieved phylotypes indicated the presence of green sulfur and purple bacteria (bands 3, 6, 7, 8, 10 and 12). Band 3 has sequence relationship (99%) to *Chlorobium bathyomarinum* (AY627756). This phylotype was apparently present in all samples (Fig. 23). Possible green sulfur bacteria were also found in the Paraíso enrichment, with close relationship (band 12, 99% similarity) to *Prosthecochloris vibrioformis* (AM690796). Sequences from bands 6 and 8 gave close relationships (96% and 98%) to *Rhodobium orientis* (D30792) respectively. Sequence from band 7 gave close relationship to *Marichromatium indicum* (AJ543328) with 97% similarity. Meanwhile, sequence from band 10 gave relative to *Rhodobium pfennigii* (AJ510235) with 94% similarity.

In all bottles, phylotypes closely related to sulfate-reducing bacteria were detected. Band 4 revealed a relationship (91%) to *Desulfatibacillum aliphaticivorans* (AY184360). Bands 5, 11 and 13 had similar electrophoretic mobility, indicating the same phylotype in all samples. Sequences from bands 5 and 13 gave close relationship to uncultured *Desulfosarcina* sp. clone SB4-53 (AY177791), with 95% and 99% similarity respectively. Sequences from bands 2 and 9 gave close relationship to uncultured bacterium clone KM93 (AY216441), 96% and 95% similarity.

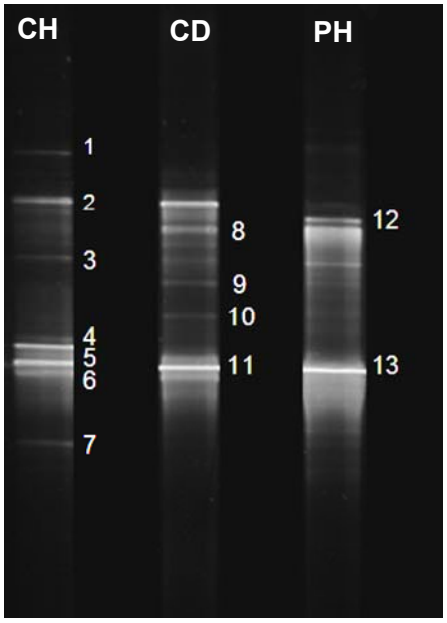

Fig. 23. DGGE community analysis of enrichment cultures grown with alkanes in the light. PCR-amplified 16S rDNA was separated on acrylamide gel and stained with SYBR. CH, Carmen enrichment on hexane. CD, Carmen enrichment on decane. PH, Paraíso enrichment on hexane.

Table 14. Interpretation of the sequence information from the DGGE bands obtained from enrichment cultures with alkanes from Paraíso and Carmen sediments.

Band	Closest relative	Identity (%)	Inferred metabolism
1	(Ambiguous sequence)		
2	Uncultured bacterial clone KM93	96	Chemotrophy?
3	*Chlorobium bathyomarinum*	99	Photosynthesis[1]
4	*Desulfatibacillum aliphaticivorans*	91	Sulfate reduction
5	Uncultured *Desulfosarcina* sp. clone SB4-53	95	Sulfate reduction
6	*Rhodobium orientis*	96	Photosynthesis[1]
7	*Marichromatium indicum*	97	Photosynthesis[1]
8	*Rhodobium orientis*	98	Photosynthesis[1]
9	Uncultured bacterial clone KM93	95	Chemotrophy?
10	*Rhodobium pfennigii*	94	Photosynthesis[1]
11	(Ambiguous sequence)		
12	*Prosthecochloris vibrioformis*	99	Photosynthesis[1]
13	Uncultured *Desulfosarcina* sp. clone SB4-53	100	Sulfate reduction

[1] Always anoxygenic

Fig. 24. Phylogenetic tree (preliminary maximum likelihood) showing the affiliation of the 16S rRNA encoding gene sequences retrieved from DGGE bands from enrichment cultures on *n*-hexane and *n*-decane. Reference sequences obtained from ARB. The scale bar represents 10% estimated sequence divergence.

The presence of sulfate-reducing bacteria indicated by the molecular analysis suggested a role in alkane degradation. To investigate this possibility, inocula from enrichment cultures in light were transferred into new media with 27.6 mM sulfate and incubated in the dark. Indeed, significant concentrations of sulfide were produced during 200 – 300 days of incubations, whereas sulfide production in alkane-free controls was marginal (Fig. 25).

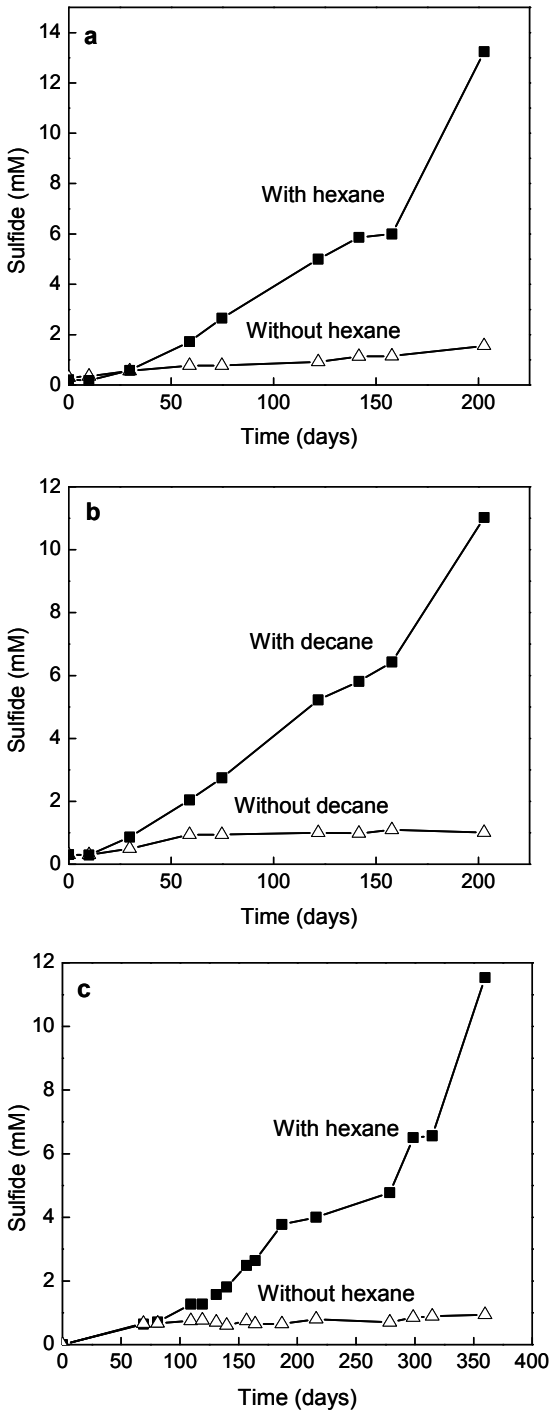

Fig. 25. Alkane-dependent sulfate-reduction to sulfide during subcultivation of phototrophic enrichment cultures in the dark with 27.6 mM sulfate. **a**, Hexane-Carmen. **b**, Decane-Carmen. **c**, Hexane-Paraíso.

C.2.4 Characterization of the phototrophic strain GH1 isolated with toluene

Basic characteristics

The cell shape of strain GH1 is shown in Fig. 26. The cell suspension of strain GH1 on toluene had a green color (Fig. 27). The *in vivo* absorption spectrum of whole cells in aqueous medium showed the presence of bacteriochlorophyll *b* (Fig. 28) with a large peak at 1014 nm, like the toluene-utilizing phototrophic bacterium, *Blastochloris sulfoviridis* strain ToP1 (Zengler *et al.,* 1999). Characteristics are summarized in Table 15. Of the aromatic compounds tested, strain GH1 utilized only benzoate and toluene. Growth with toluene is documented in Fig. 29. Notably, strain GH1 could also grow on crude oil (Table 15).

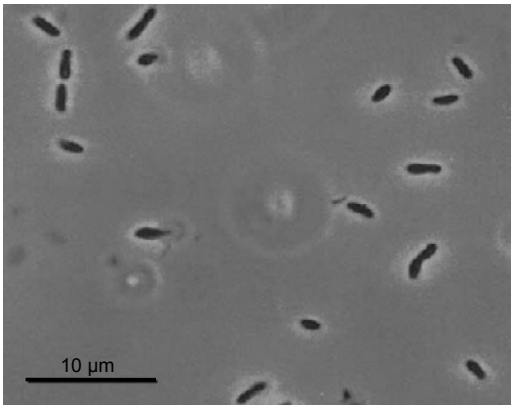

Fig. 26. Phase-contrast photomicrograph of strain GH1.

Fig. 27. Culture of strain GH1 growing on toluene.

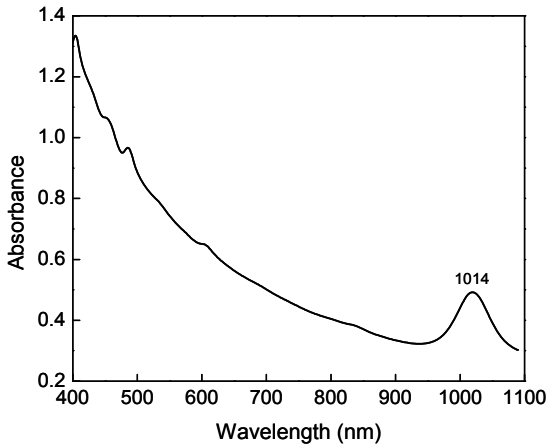

Fig. 28. Absorption spectrum of whole cells in saccharose of strain GH1.

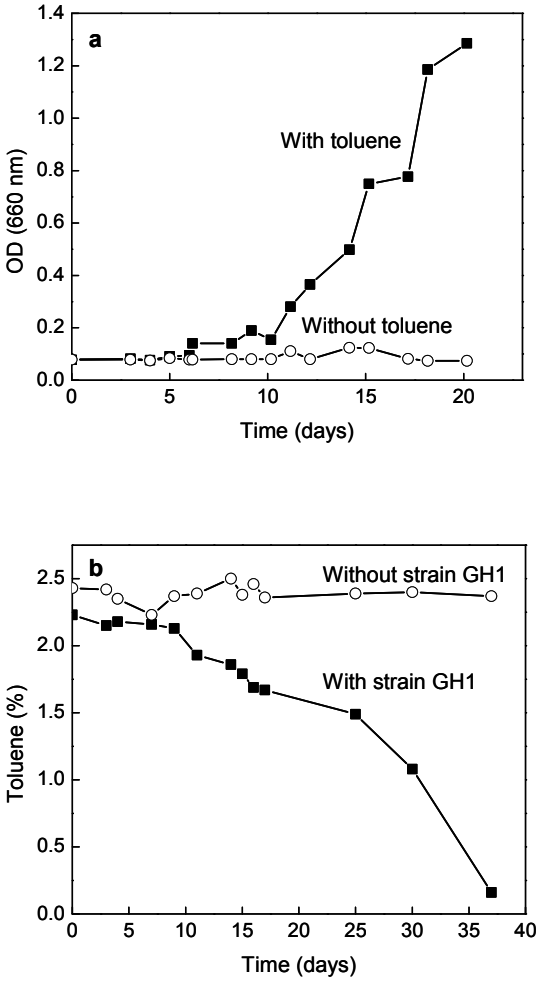

Fig. 29. Growth of strain GH1 on toluene. **a**, OD (660 nm). **b**, Toluene consumption.

Table 15. Characteristics of strain GH1.

Cells
1.4–2 by 0.6 µm in size; motile; bacteriolchlorophyll *b*; bowling cell shapes
pH-Range
6.5–8.5
Temperature range
14–37°C
Substrates[1] utilized
H_2, sulfide (1), thiosulfate (4), acetate (4), propionate (3), lactate (4), pyruvate (3), succinate (4), benzoate (2), fructose (2), crude oil (1–2%)
Substrates[1] tested but not utilized
Caprylate (2), glucose (2), glutamate (4), arginin (4), alanine (4), *o*-xylene (1%), *m*-xylene (1%), *m*-cresol (1%), *p*-cresol (1%)
G + C content of DNA
67%

[1]Added concentration in mM given in parentheses

DNA-based differentiation between strains GH1 and ToP1

The 16S rRNA sequence analysis confirmed that strain GH1 belonged to the genus *Blastochloris* of the α-subclass of Proteobacteria with *B. sulfoviridis* strain ToP1 (Zengler *et al*., 1999) as the closest relative (99% of similarity). Because of this high similarity, DNA-DNA hybridization was carried out for a more refined differentiation. The result showed that strain GH1 shared 92.7% DNA-DNA similarity with type strain, ToP1, of *B. sulfoviridis*. Hence, strains GH1 and ToP1 belong to the same species. The recommended threshold value for the definition of bacterial species is 70% DNA-DNA similarity (Wayne *et al*., 1987).

Genetic differences between strains GH1 and ToP1 were also assessed by using genomic DNA fingerprints as one method to differentiate microbial strains in the same species (Williams *et al*., 1990; Busse *et al*., 1996). The genomic DNA fingerprints were obtained via PCR performed with two RAPD primers RCP4, SMO3, and BOX primer BOXA1R (Versalovic *et al.,* 1994). Results are shown in Fig. 30. Although strains GH1 and ToP1 shared a number of bands, they could be distinguished on the basis of at least 2 polymorphic bands for each primer.

Therefore, strain GH1 maybe was considered as another strain of *B. sulfoviridis*, which could utilize toluene phototrophically as well.

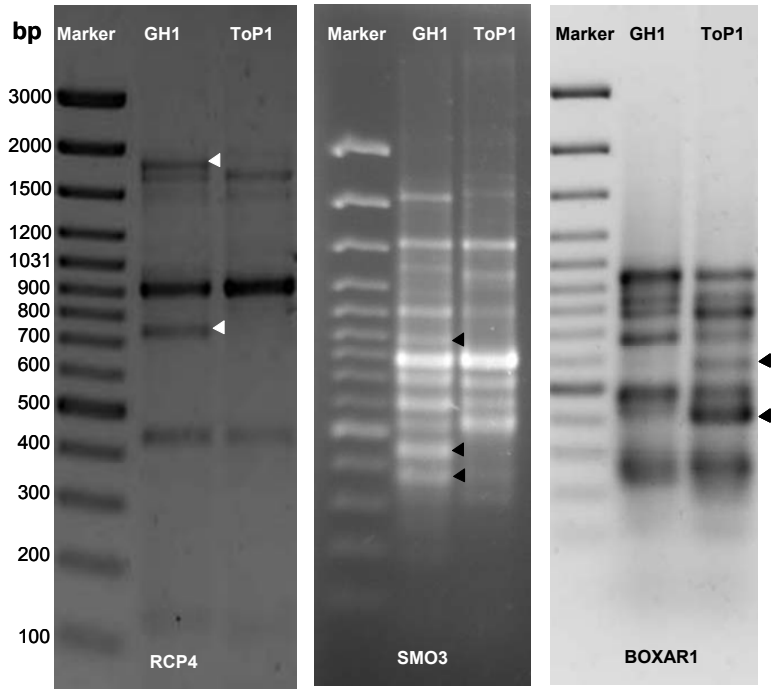

Fig. 30. Agarose gel (2%) electrophoresis showing genomic DNA fingerprint profiles of strains GH1 and ToP1 with three different primers (RCP4, SMO3, and BOXAR1). Marker: GeneRuler 100 bp DNA Lader Plus. Arrow heads (◄): distinct bands.

Benzylsuccinate synthase subunit of toluene-utilizing phototrophic bacteria

Benzylsuccinate synthase (BssAB) is the key enzyme of anaerobic toluene degradation in chemotrophic microorganisms (Achong *et al*., 2001; Shinoda *et al*., 2005; Winderl *et al*., 2007). In a previous study, Zengler *et al.* (1999) reported the presence of benzylsuccinate activity in the toluene-grown cultures of phototrophic strain ToP1 which indicated that toluene utilization in phototrophic bacteria occurs in the same manner as in chemotrophic anaerobes. In this study, we attempted to detect *bssA* genes in phototrophic bacteria. The presence of the gene (*bssA*), encoding the large subunit of benzylsuccinate synthase in the phototropic strains GH1 and ToP1 was confirmed via PCR. Primer design was based on BssA sequences of toluene-utilizing denitrifying bacteria (Galushko, unpublished). The PCR reaction with DNA of the phototrophic strains yielded products of the expected size (strain GH1, 941 bp; ToP1 978 bp). BLASTX protein database search (using a translated query) showed indeed a clear homology to known BssA sequences

(Fig. 31). The derived sequences from both phototrophic bacteria shared 100% similarity. The most closely relative sequence was *Magnetospirillum* sp. strain TS-6 with 94.2% of similarity. Those from *Thauera* sp. strain DNT1 and *Thauera aromatica* strain T-1 were the next relatives, both showing 89.7% of similarity.

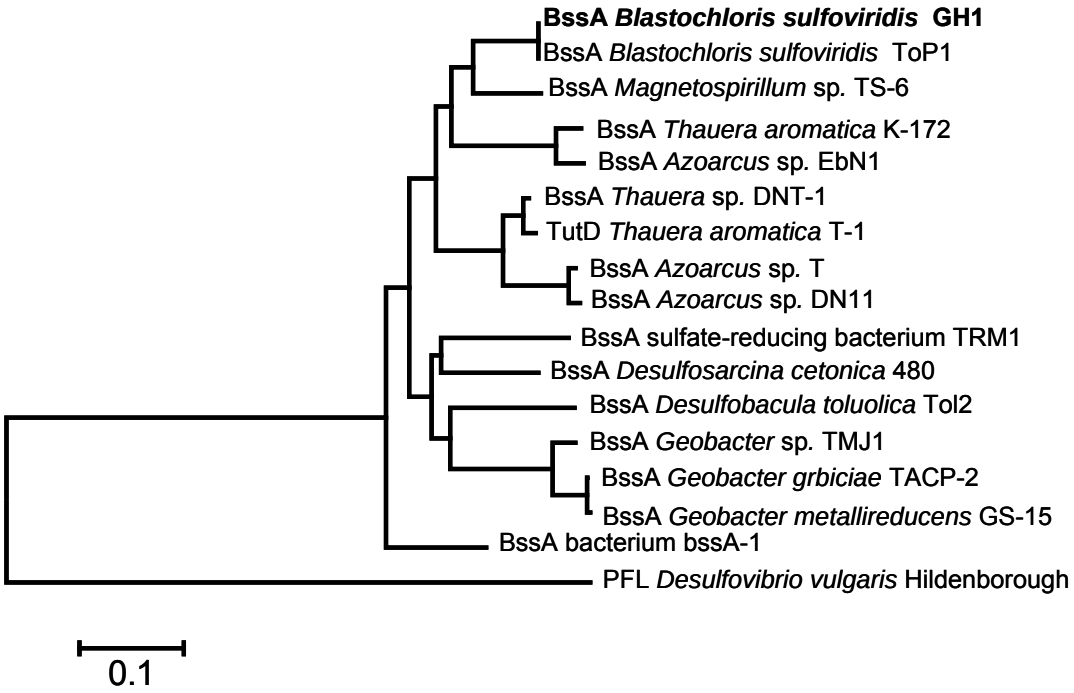

Fig. 31. Phylogenetic tree of BssA subunits of benzylsuccinate synthases from anoxygenic phototrophic bacteria and other chemotrophic bacteria. The scale bar represents 0.1 % sequence divergence. Accession numbers of included sequences: BssA, *Magnetospirillum* sp. TS-6, BAD42366; BssA, *Thauera aromatica* K-172, CAA05052; BssA, *Azoarcus* sp. EbN1, YP_158060; BssA, *Thauera* sp. DNT-1, BAC05501; TutD, *Thauera aromatica* T-1, AAC38454; BssA, *Azoarcus* sp. T, AAK50372; BssA, *Azoarcus* sp. DN11, BAF63031; BssA, Sulfate-reducing bacterium TRM1, ABM92939; BssA, *Desulfosarcina cetonica* 480, not published; BssA, *Desulfobacula toluolica* Tol2, ABM92935; BssA, *Geobacter* sp. TMJ1, ABM92938; BssA, *Geobacter grbiciae* TACP-2, ABM92936; BssA, *Geobacter metallireducens* GS-15, YP_384498; BssA, Bacterium bssA-1, ABO30980; PFL (pyruvate formate-lyase) *Desulfovibrio vulgaris* Hildenborough, YB_011485.

D Discussion

D.1 Phototrophic oxidation of anthrahydroquinone and reduced humic acids

Oxidation of reduced humic acids and the humic acid model compound anthrahydroquinone (AH_2QDS) by anaerobic chemotrophic microorganisms including species of nitrate-reducing bacteria, such as *Geobacter metallireducens, Geothrix fermentans, Wolinella succinogenes*, *Paracoccus denitrificans* (Lovley *et al.,*1999), and *Dechloromonas* (Coates *et al*., 2002) as well as halorespiring microorganisms such as *Desulfitobacterium hafniense* and some species of *Sulfurospirillum* (Luijten *et al*., 2004) were reported previously. The present study shows for the first time that also an anoxygenic phototropic bacterium is capable of utilizing reduced humic acids or AH_2QDS as electron donor. Previously, Bruce *et al.* (1999) reported that strain CKB, a close phylogenetic relative of the phototrophic *Rhodocyclus tenuis*, also oxidized AH_2QDS; however, this oxidation was occurred chemotrophically and strain CKB neither contains bacteriolchlorophyll nor can grow phototrophically (Bruce *et al*., 1999). Because AH_2QDS was only used as electron donor and not as a carbon source, strain E3P represents a novel type of anoxygenic photoautotrophic metabolism that can be of ecological significance.

D.1.1 Ecological significance and physiology of the phototrophic oxidation of humic substances

In previous studies, AH_2QDS served as chemically defined analogue for reduced humics (Lovley *et al.,* 1999, Coates *et al.,* 2002). Indeed, all known AH_2QDS-oxidizing organisms could oxidize reduced humics (Lovley *et al.,* 1999, Coates *et al.,* 2002). Also in the present study, strain E3P not only utilized AH_2QDS but also reduced humics as electron donor. Humic substances are ubiquitous in environments (Stevensons, 1994) and there is increasing evidence for multiply interactions with chemotrophic microorganisms. Several chemotrophic humic substances-reducing as well as humic substances-oxidizing microorganisms have been reported (Tables 3 and 4). The presently investigated phototrophic oxidation of humic substances allows a light-driven redox cycle of humics. This was experimentally demonstrated by combining the chemotrophic bacterium

G. metallireducens and the new phototrophic bacterium strain E3P in the presence of benzoate. Their interaction can be described by the following equations:

Chemotrophic bacterium (*G. metallireducens*)

$$C_7H_5O_2^- + 19\ H_2O + 15\ AQDS \longrightarrow 7\ HCO_3^- + 6\ H^+ + 15\ AH_2QDS \quad (1)$$

Phototrophic bacterium (strain E3P)

$$17\ AH_2QDS + 8\ HCO_3^- + 2\ NH_4^+ + 6\ H^+ \longrightarrow 17\ AQDS + 2\ C_4H_8O_2N + 20\ H_2O \quad (2)$$

Sum:

$$17\ C_7H_5O_2^- + HCO_3^- + 30\ NH_4^+ + 23\ H_2O \longrightarrow 30\ C_4H_8O_2N + 12\ H^+ \quad (3)$$

According to equation (3), 1 mmol of benzoate was converted to 30/17 mmol of the simplified biomass unit, $C_4H_8O_2N$, corresponding to 180 mg biomass. Not only AQDS but also humic acids served as electron shuttle between the chemotrophic and phototrophic bacterium. Because also the phototrophic *Thiocapsa roseopersicina* could oxidize AH_2QDS, one may speculate that even more phototrophic bacteria in the environment can utilize humics and thus play an important role in the natural humics cycle in aquatic habitats.

The demonstrated use of AH_2QDS by strain E3P extends the range of known electron donors for anoxygenic photoautotrophic growth. For a long time, reduced sulphur compounds were known to serve as electron donors for photoautotrophic growth (Overman and Garcia-Pichel, 2006). Later, iron(II) was shown to serve as an electron source for growth of anoxygenic phototrophic bacteria (Widdel *et al*., 1993). Subsequently, there was a speculation whether other reduced inorganic compounds formed via anaerobic respiration by chemoautotrophic microorganisms for example nitrite, would soon be identified as electron donor for photoautotrophic microorganisms (McEwan, 1994). Indeed, more recently, nitrite was shown to support the growth of an anoxygenic phototrophic bacterium (Griffin *et al*., 2007). Redox potentials of the different inorganic electron donors for phototrophic growth are indicated in Fig. 32.

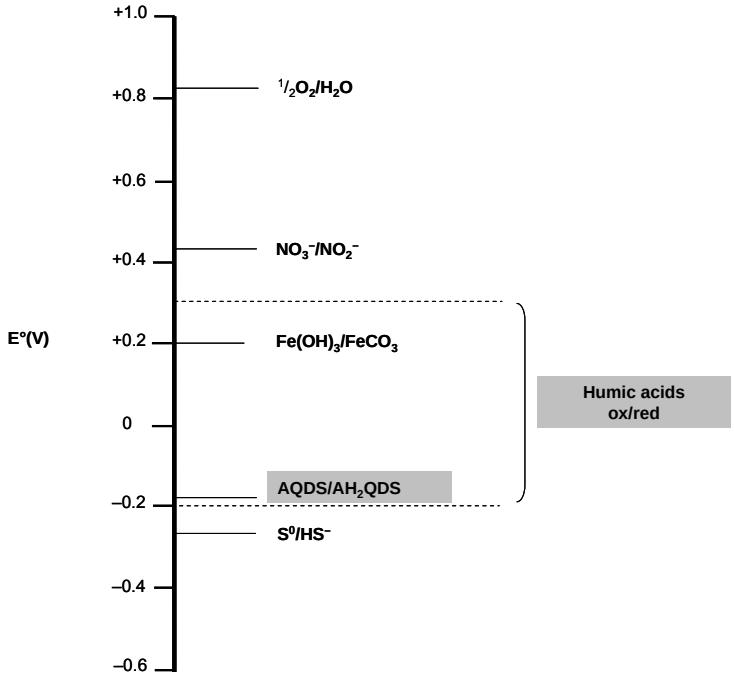

Fig. 32. Reduction-oxidation of redox couples for phototrophic growth. Humic substances do not have a defined structure and therefore a broad range of redox potentials. Data obtained from Sober (1970), Widdel *et al.* (1993), Benz *et al.* (1998), Straub *et al.* (2001), and Griffin *et al*. (2007).

The localization of the site for AH_2QDS and humic acids oxidation in the cell is of particular interest. The possibilities are depicted in Fig. 33. The photosynthetic apparatus and the electron transport system in purple bacteria are localized in the cytoplasmic membrane (McEwan, 1994). Periplasmic cytochrome c_2 serves as electron shuttle between them. In principle, AH_2QDS is small enough to enter the periplasmic space, possibly via porins. Shyu *et al.* (2002) showed that AQDS may even enter cytoplasm of *Shewanella oneidensis* strain MR-1 but electron transfer to AQDS happened in the outer membrane protein (Shyu *et al.*, 2002). Recently, the existence of an extracellular electron transfer has been demonstrated in experiments with *Shewanella putrefaciens*; one of the strains could respire humic acids (Newman and Kolter, 2000). It was shown that menaquinone was microbially excreted and reduced AQDS or humic acids when this strain grew on these substrates as electron acceptors. On the other hand, humic acids as big molecules that can not enter the periplasm may also donate their electrons for photosynthesis to a redox protein in the outer membrane without involvement of an additional external shuttle.

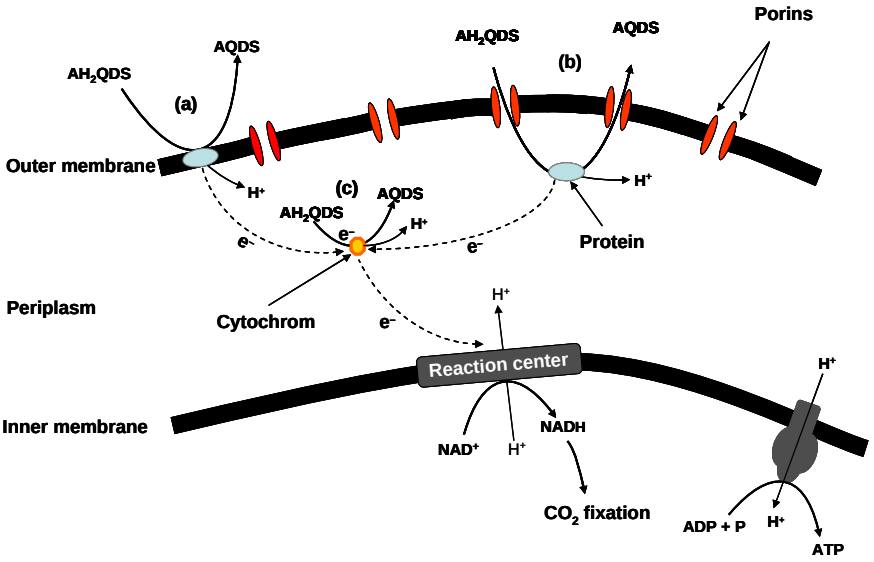

Fig. 33. Proposed mechanisms of electrons transfer during AH_2QDS oxidation (not stoichiometric); (**a**) via protein in outer membrane; (**b**) via protein in periplasm; (**c**) directly to cytochrom in periplasm. Cell wall not shown.

D.1.2 Taxonomy of strain E3P

Like cells of other *Thiocystis* species, also cells of strain E3P were oval, motile by flagella and contain spirilloxanthin carotenoids. A unique characteristic of strain E3P is probably its ability to utilize AH_2QDS and reduced humic acids as electron donors. However, more phototrophic bacteria have to be examined to substantiate that this metabolism is neither unique. In contrast to *Thiocystis violacea* and *Thiocystis gelatinosa*, which can not photoassimilate lactate, ethanol, fructose, propionate, and AH_2QDS, strain E3P can utilize these substrates phototrophically. Phylogenetic analysis based on 16S rRNA gene sequences showed that the new isolate is closely relative to *Thiocystis violacea,* a member of the family *Chromatiaceae* (similarity, 95.8%.). Therefore, strain E3P can be regarded as a new species of the genus *Thiocystis,* and the name *Thiocystis fossae* is proposed.

Description of *Thiocystis fossae* sp. nov

Fos'sae from L.n. *fossa* ditch, referring to the origin from a freshwater ditch.

Cells are oval to spherical, 1.6 by 2.2 µm in diameter. Diplococci are formed during binary fission. Gram negative. Cells are hightly moltile by means of a bundle of

flagella. Dense cultures are purple to pink and contain bacteriochlorophyll *a*. Phototrophic growth occurs under anoxic conditions in the light. Electron donors for photolithoautotrophic growth are H_2, hydrogen sulfide, thiosulfate, AH_2QDS and reduced humic acids. During photolithotrophic growth with reduced sulfur compounds (sulfide, thiosulfate), elemental sulfur globules are formed and stored in the cells. In the presence of bicarbonate, acetate, propionate, butyrate, lactate pyruvate, succinate, fructose or ethanol are photoassimilated.
Optimal pH, 7.0 – 7.5; pH range, 6.5 – 8.5.
Optimal temperature, 28 – 30°C.
DNA base composition of the type strain: 41.6 mol% G + C.
Habitat: Freshwater sediment. The type strain was isolated from a ditch in Bürgerpark, Bremen, Germany.
Deposited with the German Collection of Microorganisms in Brauschweig, Germany, under number DSMZ (to be provided).
The 16S rRNA gene sequence has been deposited in EMBL under accession number (to be provided).

D.2 Phototrophic utilization of hydrocarbons

Whereas control enrichments with toluene led to the isolation of a phototrophic strain able to utilize this hydrocarbon, attempts to isolate alkane-utilizing phototrophic bacteria from enrichment cultures failed within the present study period. Nevertheless, molecular analyses gave hints on important bacteria in the enrichment with alkanes.

D.2.1 Proposed mechanism of *n*-alkane utilization in enrichment cultures

Results of DGGE analyses showed the presence of sulfate-reducing bacteria together with phototrophic green sulfur bacteria, purple sulfur and non-sulfur bacteria in enrichment cultures with *n*-hexane and *n*-decane. Hence, both chemotrophic and phototrophic bacteria may be involved in the utilization of hexane and decane. However, there has been no report of alkane utilization by anaerobic phototrophic bacteria until now; only utilization of the aromatic hydrocarbon, toluene has been demonstrated previously (Zengler *et al.,* 1999). When inocula from

enrichments in the light were transferred into new media and incubated in the dark, a hexane- and decane-dependend increase in sulfide concentration was observed.

In addition, 16S rRNA sequence analyses from DGGE bands (550 bp) showed that sulfate-reducing bacteria related to strains capable of alkane degradation such as *Desulfatibacillum alifativorans* (Cravo-Laureau *et al.,* 2004a) and strain AK-01 (So and Young 1999), both with a similarity of 91% were present. Even though, only a short part 500 bp of 16S rRNA gene was analyzed, the phylogenetic analysis and dark incubation suggested the presence of alkane-oxidizing sulfate-reducing bacteria in the enrichment cultures. The produced sulfide could be used as electron donor and reoxidized by green sulfur bacteria and purple sulfur bacteria (Fig. 34), resulting in an anaerobic sulfur cycle. An interaction between phototrophic bacteria and sulfate-reducing bacteria has been reported previously (Pfennig and Biebl, 1976; Pfennig *et al.*, 1982; Warthmann *et al*., 1992; Caumette, 1993; Overmann and van Gemerden, 2000). Nevertheless, an additional direct utilization of alkanes by phototrophic bacteria cannot be excluded.

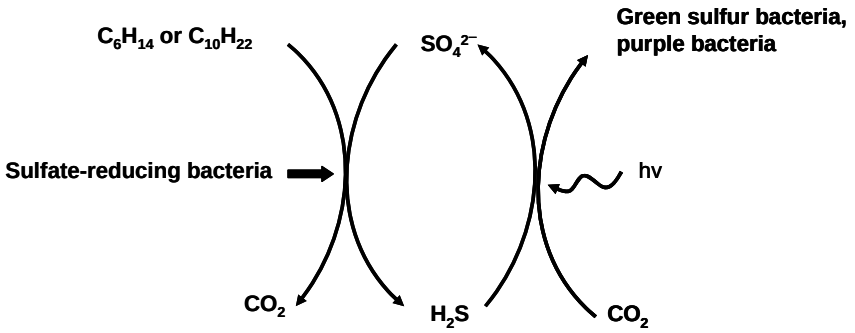

Fig. 34. Supposed hypothesis of *n*-hexane and *n*-decane utilization by sulfate-reducing bacteria and phototrophic bacteria in enrichments.

D.2.2 Toluene-utilizing phototrophic bacteria

The relatively easy enrichment and isolation of strain GH1, another strain of *Blastochloris sulfoviridis* capable of utilizing toluene suggested that this species is well-adapted among phototrophic bacteria to growth with toluene. Toluene not only enters the environment via oil pollution, but also as a natural metabolite from breakdown of phenylalanine (Jüttner, 1991). So these toluene-utilizing phototrophic bacteria may play a role as toluene scavenger in aquatic habitats under anoxic conditions in the light.

In the present study, also the gene encoding the key enzyme for the anaerobic toluene metabolism, benzylsuccinate synthase (Winderl *et al.,* 2007) was investigated. It has been found in chemotrophic bacteria including sulfate-, nitrate- and iron(III)-reducing bacteria (Beller and Sportmann, 1997; Rabus and Heider, 1998; Leuthner *et al.,* 1998; Achong *et al.*, 2001; Kane *et al.,* 2002; Kube *et al.,* 2004). Zengler *et al.* (1999) supposed that the toluene metabolism in the phototrophic strain ToP1 occurs in the same manner as chemotrophic bacteria; this conclusion was based on the detection of benzylsuccinate. In present study, the detection of benzylsuccinate synthase subunit (BssA) in the phototrophic bacteria strain GH1 and ToP1 support the hypothesis that the toluene-metabolism of phototrophic bacteria is essentially the same as in chemotrophic bacteria. The recovered partial sequences of these genes were completely identical in both strains and grouped together with *bssA* gene of alphaproteobacteria. Designed primers for *bss*A could be applied to study diversity of toluene-utilizing microorganisms, especially toluene-metabolizing phototrophic bacteria.

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F Manuscript

A Photoautotrophic Bacterium Utilizing Anthraquinol or Humic Substances as Electron Donor

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Manuscript in preparation

Dedicated to the memory of Norbert Pfennig (deceased 11 February, 2008)

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Many anoxygenic phototrophic bacteria utilize simple inorganic compounds as electron donors for light-driven autotrophic growth. To investigate whether humic substances, which are electron donors or electron acceptors for many chemotrophic bacteria, can also provide electrons for phototrophic bacteria, enrichments were established with anthrahydroquinol-2,6-disulfonate (AH_2QDS) as a defined humic model compound in the light. From these, a novel anoxygenic phototrophic bacterium, strain E3P was isolated and shown to utilize AH_2QDS as electron donor for photosynthesis in the presence of CO_2. Quantitative growth experiments revealed a growth yield of up to 10.7 g of cell dry mass per mol of AH_2QDS oxidized to the corresponding quinone (AQDS), which was up to 90% of the theoretical growth yield. Oxidation of AH_2QDS did not occur in dark or sterile anoxic controls. AH_2QDS did not serve as a source of organic carbon. In addition to AH_2QDS, strain E3P oxidized reduced humic acids in the light. In co-cultures of AQDS-reducing, benzoate-oxidizing *Geobacter metallireducens*, strain E3P exhibited light-dependent growth even though it was not able to photoassimilate benzoate. This result indicates that AH_2QDS/AQDS can act as an electron shuttle system between the chemotrophic and phototrophic bacterium. Analysis of the nearly complete sequences of 16S rRNA genes revealed a close relationship (similarity of 95.8%) of strain E3P to *Thiocystis violacea* (*Chromatiaceae*). Strain E3P is considered as a new species of the genus *Thiocystis* (proposed species, *Thiocystis fossae*).

A unique capability among anoxygenic phototrophic bacteria is the use of simple inorganic compounds such as various sulfur species (23), ferrous iron (3, 12, 30) or nitrite (9) as electron source for CO_2 fixation in biosynthesis. The inorganic electron donors are oxidized to the highest oxidation states of the elements (sulfate, ferric iron, nitrate). Such oxidation can also occur chemotrophically, mainly by aerobic bacteria. The formed highest oxidation states of the elements can vice versa act as electron acceptors for anaerobic chemotrophic bacteria, thus leading to a “redox cycle” of the elements that can be of ecological significance in aqueous habitats.

Complete different classes of naturally important compounds that can undergo biological redox cycling are humic substances. Originating from the biomass of higher organisms and microorganisms, humic substances are wide-spread and abundant in many terrestrial and aquatic habitats (26). Humic substances were shown to serve as electron acceptors for various anaerobic microorganisms that were otherwise known as reducers of iron(III), sulfate or other typical anaerobic electron acceptors, or as fermentative microorganisms (2, 4, 5, 6, 15, 16, 18). Vice versa, reduced humic substances may be also used as electron donors by chemotrophic microorganisms, for instance with nitrate as electron acceptor (7, 17, 20, 28) or oxidized by oxygen chemically. The important structural components in such redox cycling of humic substances are their quinone/quinol moieties (25). In physiological studies, these moieties can be mimicked by the use of simple model compounds such as the anthraquinone/anthraquinol system, for instance by adding the water soluble anthraquinone-2,6-disulfonate (AQDS) as electron acceptor (15). The use of such a defined model compound allows the establishment of reproducible growth conditions and facilitates quantitative measurements of redox reactions.

Whereas the microbial utilization of humic substances as electron donors has been documented with chemotrophic bacteria, photrophic utilization has not been observed so far. On the other hand, phototrophic oxidation of humic substances would in principle be expected from the ecological perspective because phototrophic bacteria constitute the “photooxidative counterpart” also in the cycling of several other substances (see above). Here we describe the isolation and characteristics of an anoxygenic phototrophic bacterium able to utilize AH_2QDS and humic substances as electron donor.

MATERIALS AND METHODS

Sources of organisms. Enrichments of phototrophic AH_2QDS-oxidizing bacteria were obtained from a phototrophic enrichment culture initially established with ferrous iron and originating from an iron-rich ("rusty") forest ditch (Bürgerpark, Bremen, Germany). Two strains, E3P and FWP1, were isolated; strain E3P was studied in detail.

Geobacter metallireducens (DSM 7210), *Thiocapsa roseopersicina* (DSM 217), *Thiocystis gelatinosa* (DSM 215), *Thiocystis violacea* (DSM 207), *Thiorhodococcus minus* (DSM 11518) were obtained from German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany.

Medium and cultivation conditions. Techniques for preparation of media and for cultivation of phototrophic bacteria under anoxic conditions were as described (29). The following salts were dissolved in 1 liter of distilled water: KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; KCl, 0.5 g; $CaCl_2 \cdot 2\ H_2O$, 0.1 g. After autoclaving and cooling under an N_2-CO_2 atmosphere (9/1, v/v), $NaHCO_3$ (final concentration, 30 mM), an EDTA-chelated trace elements mixture and vitamins were added from sterile stock solutions (29). The pH was adjusted to 7.0 – 7.1. Organic substrates were added from sterile stock solutions so as to yield the indicated final concentrations. All cultures were incubated at 20 – 22°C at a distance of 30 cm from a 25 W-tungsten light bulb.

AH_2QDS and reduced humic acids were prepared by reduction of 50 ml stock solutions of AQDS (2 mM; sodium form; Sigma) and commercial humic acids (1 g/l; Aldrich) in the presence of aluminum oxide-palladium pellets (COY, USA) under an atmosphere of H_2.

Isolation of phototrophic bacteria. Pure cultures were isolated via serial dilution in anoxic agar tubes (29) with acetate (5 mM). The isolated colonies were grown in liquid media with AH_2QDS as electron donor. The purity of the isolates was checked by microscopy of the cultures after transfer to various potential substrates (Table 1).

Quantitative growth experiments. Quantitative growth experiments were carried out in 1 l-bottles with 500 ml medium with 1.6 or 2 mM AH_2QDS under an N_2-CO_2 head space (9/1, v/v). All steps for subsequent analysis were carried out in

an anoxic chamber. After withdrawal of small aliquots for chemical analysis (see below), cells were harvested by centrifugation (Beckman Coulter with JA-10-Rotor) at 14,300 × g (25 min), washed with 18 mM ammonium acetate solution and dried to constant weight at 60°C.

Co-culture experiments. Co-culture experiments with AQDS/AH_2QDS as electron shuttle system were carried out in 1 l-bottles with 500 ml medium containing 2 mM AQDS, 2 mM benzoate and 5% (v/v) inoculum of each culture (strain E3P and *G. metallireducens*). Growth of bacteria was followed by measuring the optical density (OD) at 600 nm as well as the change of AH_2QDS (see below). Co-culture experiments with humic acids as electron shuttle system were carried out under similar conditions in 20 ml-tubes with 10 ml medium, 20 mg humic acids per liter and 1 mM benzoate.

Electron microscopy. An Axioplan photomicroscope (Zeiss, Germany) was used for regular microscopic observations and phase-contrast photomicrographs. Presence of flagella was examined by transmission electron microscopy (TEM) of cells negatively stained with uranyl acetate (20 mg/ml) using an EM10 transmission electron microscope (Zeiss, Germany) at an accelerating voltage of 60 kV.

Analytical techniques. The concentration of AH_2QDS was determined photometrically via its absorbance at 436 nm and using an extinction coefficient of 3.5 mM^{-1} cm^{-1} (1). Samples were kept in anoxic quartz cuvettes (10 mm path; Suprasil) while measurements were carried out with a Lamda 20 spectrophotometer (Perkin Elmer, Germany). In addition, the absorption spectrum was recorded to verify the absorption maximum.

The electron accepting capacity of humic acids was determined via reaction with ferric citrate and quantification of the produced ferrous iron using the ferrozine assay (14, 27).

The guanine plus cytosine content of the genomic DNA was kindly determined by Peter Schumann at the Deutsche Sammlung von Mikroorganismen und Zellkutluren (DSMZ, Braunschweig, Germany) using liquid chromatography (22).

Photosynthetic pigments. For measurement of the *in vivo* absorption spectrum of pigments, an acetate-grown culture of strain E3P was first concentrated 10 fold by centrifugation. A volume of 1 ml was mixed with a solution of 5 g saccharose in 3 ml H_2O to minimize diffraction (8). The absorption spectrum was recorded with a Lamda 20 spectrophotometer (Perkin Elmer, Germany).

16S rRNA gene sequencing and analysis. The 16S rRNA gene was amplified by PCR and sequenced using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, California, USA). The 16S rRNA gene sequence was aligned with reference sequences (including those from 13 type strains of *Chromatiaceae*) and analyzed using the ARB phylogeny software package (19). The sequence of *Escherichia coli* served as an out group. Only nearly full-length (1,300 bp) were used. The results from an initial automatic alignment employing the Fast Aligner V1.03 tool were manually corrected. Evolutionary distances between pairs of microorganisms were determined using the Jukes-Cantor equation implemented in ARB (19). A phylogenetic tree was calculated by maximum likelihood, maximum parsimony and ARB neighbor joining implemented in the ARB software. The 16S rRNA gene sequence of strain E3P was submitted to the GenBank database under accession number (to be provided).

RESULTS AND DISCUSSION

Enrichment and isolation. During subcultivation of a phototrophic iron(II)-oxidizing enrichment culture from a ditch rich in humic substances and natural iron, we examined in parallels whether phototrophic growth was also possible with the humic model compound, AH_2QDS instead of added ferrous sulfate. Indeed, a light-dependent change of the color caused by oxidation of AH_2QDS was observed. After some transfers, a sediment-free culture of oval cells was enriched. In comparison to the phototrophic bacteria enriched with ferrous iron (8), the phototrophic bacteria enriched with AH_2QDS had smaller cells and did not contain gas vesicles. One isolate from the purple-red colonies that developed in agar, strain E3P, was studied in more detail.

Morphology and pigmentation. Cells of strain E3P were oval (1.6 by 2.2 μm; Fig. 1) and motile by means of bundles of flagella (not shown).

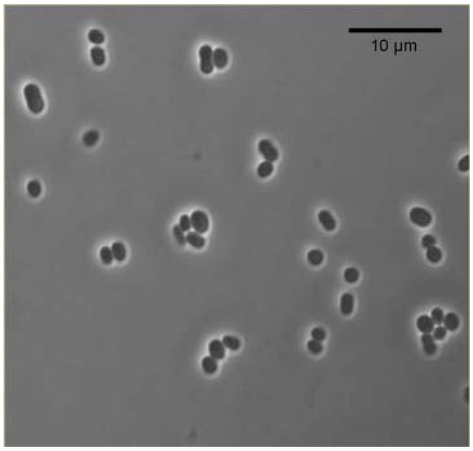

FIG.1. Phase-contrast photomicrographs of strain E3P grown in bicarbonate/CO_2 medium with AH_2QDS as electron donor. *Bar* 10 µm.

Liquid cultures grown in medium with acetate (when the colored AH_2QDS was not present) were reddish. The *in vivo* absorption spectra of E3P suspension exhibited maxima at 464, 494, 524, 595, 809 and 859 nm (Fig. 2). The absorption peaks at 595, 809, and 859 are characteristic of bacteriochlorophyll *a*, whereas the peaks at 464, 494 and 524 nm indicate the presence of the lycopene and rhodopin which belong to the spirilloxanthin series of carotenoids (10, 21).

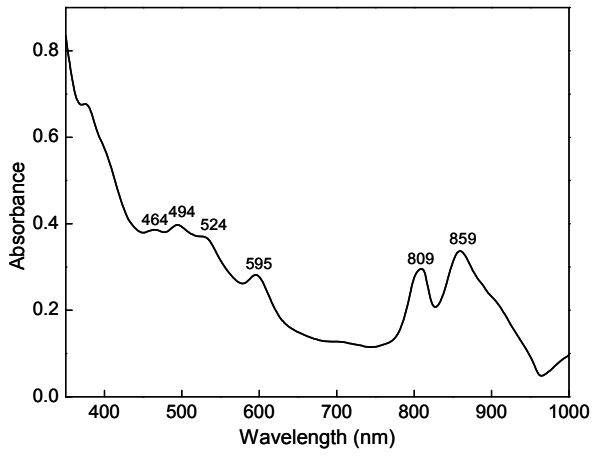

FIG. 2. *In vivo* absorption spectrum of strain E3P after growth with acetate.

Phylogenetic affiliation. Analysis of the nearly complete 16S rRNA gene sequence (1400 nucleotides) indicated an affiliation of strain E3P with the genus *Thyocystis* (family *Chromatiaceae*), the closest relative being *Tcs. violacea* (similarity, 95.8%; Fig. 3).

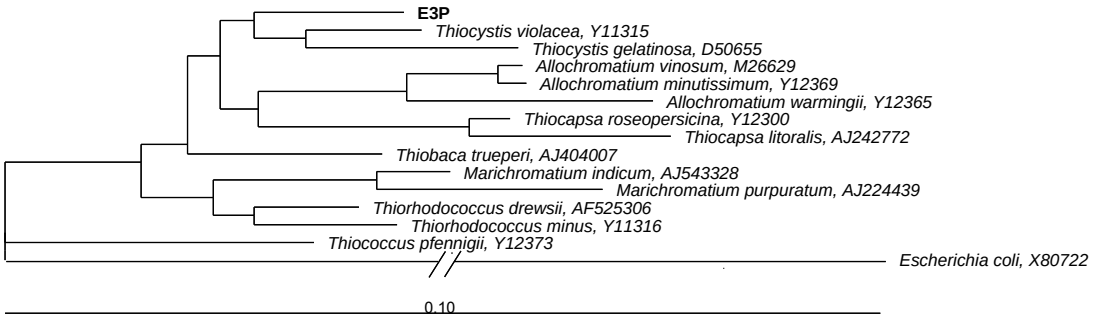

FIG. 3. Position of strain E3P in the phylogenetic tree based on maximum likelihood analyses of 16S rRNA gene sequence data. The scale bar represents 10% estimated sequence divergence.

Physiological properties. In addition to AH_2QDS, strain E3P used sulfide (with formation of sulfur globules in the cells), thiosulfate, molecular hydrogen, free ferrous or ferrous sulfide as electron donor for photoautotrophic growth in mineral medium with CO_2. Furthermore, strain E3P grew photoheterotrophically with a number of aliphatic compounds (Table 1). Fastest growth occurred with AH_2QDS, hydrogen, acetate, and propionate. Oxidation of the ferrous sulfide (as visible by disappearance of the black color) occurred very slowly and was stimulated by acetate, as described elsewhere (8).

In addition to defined compounds, also a commercial humic acids preparation reduced with hydrogen (catalyzed by palladium) was shown to be oxidized by strain E3P in the light. Because the amount of humic acids that could be added to the medium did not allow substantial bacterial growth, the formation of oxidized humic acids was demonstrated by measurement of their electron accepting capacity after incubation. Whereas the reduced humic acids in dark incubation lost only 79 nmol electrons per mg (corresponding to 18.2% oxidation), humic acids in illuminated cultures lost 434 nmol electrons per mg (corresponding to 100% oxidation) within 3 h of incubation with cells at an OD_{600} of approximately 0.28.

Comparison with related phototrophic bacteria. Properties of strain E3P were compared with those of its closest phylogenetic relatives, *Thiocapsa roseopersicina* (93.6) *Thiocystis gelatinosa* (94.3%), *Thiocystis violacea* (95.8%), (Table 1). The only strain able to utilize AH_2QDS phototrophically was *Thiocapsa roseopersicina.*

TABLE 1. Comparison of strain E3P with its phylogenetic relatives.

Property/substrate	E3P	*Thiocapsa roseopersicina**	*Thiocystis gelatinosa**	*Thiocystis violacea**
Cell shape	Oval	Sphere	Coccus	Coccus
Cell dimensions (μm)	1.6 by 2.2	1.2–3.0	3	2.5–3.0
Motility	+	–	+	+
Color of cell suspensions	Purple-red	Pink/orange-brown	Purple-red	Purple violet
Carotenoid series	Spirilloxanthin	Spirilloxanthin	Okenone	Rhodopinal
G+C DNA mol %	41.6	63.3–66.3	61.3	62.8–67.9
pH optimum	7.0–7.5	7.3	nd	7.3
pH range	5.5–8.5	6.5–7.5	6.5–7.6	6.5–7.6
Chemoautotrophy	+	+	+	+
Substrates tested				
AH_2QDS	+	+	–	–
Reduced humic acids	+	nd	nd	nd
Hydrogen	+	+	nd	+
Sulfide	+	+	+	+
Thiosulfate	+	+	–	+
Acetate	+	+	+	+
Propionate	+	±	–	±
Butyrate	+	nd	nd	nd
Lactate	+	±	–	–
Pyruvate	+	+	+	+
Succinate	+	+	–	±
Fumarate	–	+	–	+
Malate	–	nd	nd	nd
Benzoate	–	nd	nd	nd
Methanol	–	nd	nd	nd
Ethanol	+	–	–	–
Fructose	+	+	–	±
Glucose	–	–	–	±
Alanine	–	nd	nd	nd
Aspartate	–	nd	nd	nd
Glutamate	–	nd	nd	nd

Symbol: – No utilization, + utilization, ± utilization by some strains, nd not determined. *Data from ref. 10.

Measurement of cell growth with AH_2QDS. The oxidation of AH_2QDS under strictly anoxic conditions depended on the presence of cells and light (Fig. 4, 5). Quantitative measurements revealed that up to 10.7 mg cell dry mass were formed per mmol of AH_2QDS oxidized (Table 2).

Assuming the simplified sum formula for cell mass, $C_4H_8O_2N$ (11, 13), the results are close to the expected cell yield of 12 mg per mmol AH_2QDS that is calculated from the assimilation equation:

$$17\ AH_2QDS + 8\ HCO_3^- + 2\ NH_4^+ + 6\ H^+ \rightarrow 17\ AQDS + 2\ C_4H_8O_2N + 20\ H_2O$$

The lower cell yields are presently explained by a loss of cell dry mass during washing. If the formed AQDS was chemically re-reduced, photometric quantification of the formed AH_2QDS revealed a complete recovery, showing that AH_2QDS served merely as an electron donor and not as a carbon source. Utilization as a carbon source is also unlikely because AH_2QDS is an artificial sulfonate.

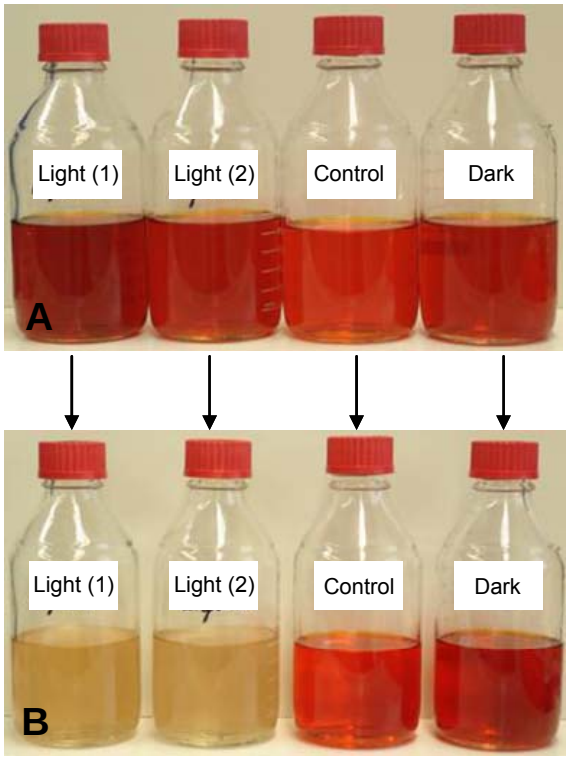

FIG. 4. Dependence of the oxidation of AH_2QDS on cells of strain E3P and light. (A) After inoculation before incubation. (B) After seven days of incubation.

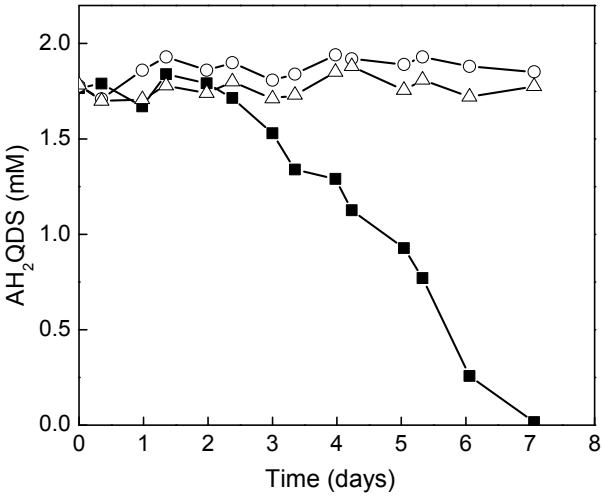

FIG. 5. Incubation of AH_2QDS (■) with strain E3P in the light, (○) with strain E3P in the dark, and (Δ) without strain E3P in the light.

TABLE 2. Quantification of phototrophic growth of strain E3P on AH_2QDS.

	AH_2QDS oxidized	Cell dry mass (theoretical)	Cell dry mass formed	Cell yield	
	(mmol)	(mg)	(mg)	(mg/mmol)	% of expected
Bottle 1	0.80	9.61	5.03	6.27	52.3
Bottle 2	0.86	10.34	7.13	8.27	68.9
Bottle 3	0.69	8.27	7.44	10.7	90.1

Syntrophic electron shuttle-based growth of strain E3P with a chemotrophic bacterium. The capacity of strain E3P to utilize AH_2QDS and humic acids as electron donor for phototrophic growth should in principle allow syntrophic growth with chemotrophic bacteria that reduce the oxidized forms of these electron carriers. To demonstrate such syntrophic growth, *Geobacter metallireducens* was chosen as the chemotrophic bacterium and benzoate as the chemotrophic substrate. Benzoate is not directly utilized by strain E3P. Indeed, significant light-dependent growth with the dominance of the characteristic cells of strain E3P only occurred if both organisms were combined (Fig. 6), confirming that $AQDS/AH_2QDS$ acted as an electron shuttle for syntrophic growth. Incubations with the co-culture in the dark or with *G. metallireducens* alone yielded the brownish-red color of AH_2QDS that was not reoxidized. The indirect photoassimilation of benzoate via the

AQDS/AH_2QDS shuttle can be described with the following equations:

Chemotrophic bacterium (*G. metallireducens*)

$$C_7H_5O_2^- + 19\ H_2O + 15\ AQDS \longrightarrow 7\ HCO_3^- + 6\ H^+ + 15\ AH_2QDS \quad (1)$$

Phototrophic bacterium (strain E3P)

$$17\ AH_2QDS + 8\ HCO_3^- + 2\ NH_4^+ + 6\ H^+ \longrightarrow 17\ AQDS + 2\ C_4H_8O_2N + 20\ H_2O \quad (2)$$

Sum:

$$17\ C_7H_5O_2^- + HCO_3^- + 30\ NH_4^+ + 23\ H_2O \longrightarrow 30\ C_4H_8O_2N + 12\ H^+ \quad (3)$$

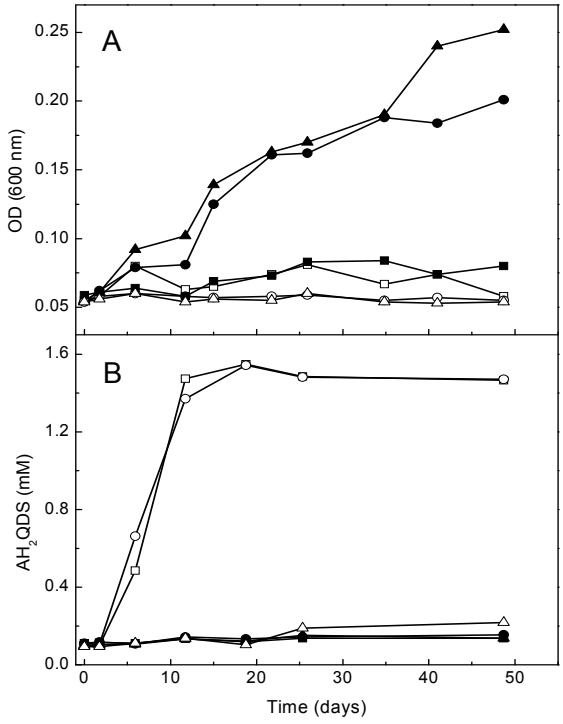

FIG. 6. Growth of co-culture (OD 600nm) (A) and reduction of AQDS (B). (□) Geobacter, dark (●) Co-culture, light (▲) Co-culture, light (○) Co-culture, dark (■) E3P, light (∆) Without co-culture, light.

Similarly, syntrophic growth with humic acids as electron shuttle was demonstrated (Fig. 7). Such mode of phototrophic growth could play a role in anoxic waters that are rich in humic acids. The syntrophic growth demonstrated in these experiments is analogous to that based on sulfur cycling between green phototrophic sulfur bacteria and the sulfur-reducing bacterium, *Desulfuromonas acetoxidans* (24).

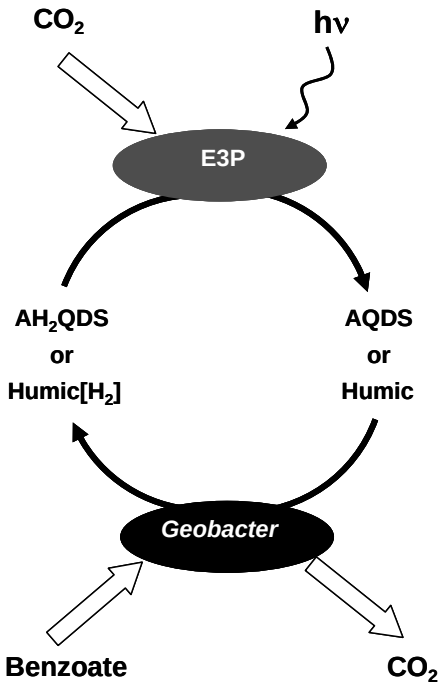

FIG. 7. Scheme of syntrophic growth of a phototrophic bacterium with a chemotrohic bacterium based on a humic acid cycle.

Description of *Thiocystis fossae* sp. nov. Cells are oval to spherical, 2.2 μm in length and 1.6 μm in diameter. Gram negative. Cells are highly moltile by means of a bundle of flagella. Cell suspensions are purple to pink and contains bacteriochlorophyll *a* as well as lycopene and rhodopin (carotenoids of the spririlloxanthin series). Electron donors for anoxygenic photoautotrophic are anthrahydroquinone-2,6-disulfonate, reduced humic acids, hydrogen, hydrogen sulfide, thiosulfate and ferrous iron. During photolithotrophic growth with sulfide, sulfur globules are stored in the cells. Acetate, propionate, butyrate, lactate, pyruvate, succinate, ethanol and fructose (in the presence of CO_2) are used for photoheterotrophic growth.

Optimal pH 7.0 – 7.5; pH range 5.5 – 8.5; optimal temperature 28 – 30°C.

DNA base composition of the type strain: 41.6 mol% G + C.

Habitat: Freshwater sediment. The type strain was isolated from a forest ditch in Bremen (Germany).

The type strain, E3P has been deposited with the German Collection of Microorganisms in Brauschweig, Germany under number DSM (to be provided).

16S rRNA gene sequence was submitted in GenBank database under accession number (to be provided).

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