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Microbial Aspects of Anaerobic BTEX Degradation

LIN BIN [*,#,1], HENK W. VAN VERSEVELD[#], WILFRED F. M. RÖLING[#2]

[*] *Biology Institute, Henan Academy of Sciences, 28 Huayuanlu, Zhengzhou 450008, China;*

[#] *Division of Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, NL-1081HV Amsterdam, The Netherlands*

Combined with conventional methods, developments in both geochemical (delineation of redox processes) and molecular microbial methods (analysis of 16S rDNA genes and functional genes) have allowed us to study in details microorganisms and genes involved in the anaerobic degradation of benzene, toluene, ethylbenzene and xylene (BTEX) under specific redox conditions. This review summarizes recent research in this field. The potential for anaerobic BTEX degradation is widely spread. Specific groups of microorganisms appear to be involved in degradation under different redox conditions. Members of the *Azoarcus/Thauera* cluster perform BTEX degradation under denitrifying conditions, *Geobacteraceae* under Fe (III) reducing conditions and *Desulfobacteriaceae* under sulfate reducing conditions. The information so far obtained on biochemistry and molecular genetics of BTEX degradation indicates that each BTEX compound is funneled into the central benzyol-CoA pathway by a different peripheral pathway. The peripheral pathways of per BTEX compound show similarities among different physiological groups of microorganisms. We also describe how knowledge obtained on the microbial aspects of BTEX degradation can be used to enhance and monitor anaerobic BTEX degradation.

Key words: Benzene; Toluene; BTEX; Degradation; Anaerobic; TEAPs; Molecule.

INTRODUCTION

Pollution with the aromatic benzene, toluene, ethylbenzene and xylene (BTEX) components of petroleum products constitutes a serious environmental and health hazard because of the combination of their high solubility in groundwater and their toxicity. Aerobic microorganisms can degrade all BTEX components and microbial degradation plays an important role in the natural attenuation of BTEX from aerobic groundwater. Supplying oxygen to contaminated sites in order to stimulate aerobic BTEX degradation is a common bioremediable practice. However, aerobic treatment requires a lot of energy, especially for subsurface environments, and often anaerobic conditions with associated

[1] Corresponding author and his current address: Division of Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, NL-1081HV Amsterdam, The Netherlands. E-mail: binlin@bio.vu.nl

[2] Present address: Fossil Fuels and Environmental Geochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, NE17RU, United Kingdom

Biographical note of the first author: Lin Bin, male, born in 1964, Ph.D. candidate in Vrije Universiteit, major in microbiology.

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microbial communities and activities prevail. Also highly reduced anaerobic sediments contain large amounts of reduced products of anaerobic metabolism, such as ferrous iron and sulfide. Supplied oxygen will preferentially react with these compounds and not be available for aerobic BTEX metabolism.

These anaerobic conditions can be due to natural conditions, like the presence of a carbon rich soil (for example in a peat aquifer or in petroleum deposits) or a thick impermeable clay layer limiting the natural oxygen supply. Otherwise, the anaerobic conditions are often the result of pollution (see next section). For a long time it was thought that anaerobic BTEX degradation was not possible. However, over the last decades it has been shown that under every redox condition BTEX compounds can be degraded by microorganisms (Table 1). Here, we review recent research on the microbial populations involved in anaerobic BTEX degradation under different redox conditions and physiology, biochemistry and molecular genetics of BTEX-degraders. This review also indicates how our knowledge on anaerobic BTEX degradation in the future might be used for enhancing and monitoring BTEX degradation in anaerobic environments.

TABLE 1

Known Potential for the Degradation of Each of the BTEX Compounds Under Different TEAPs

	Nitrate	Manganese	Iron	Sulphate	Methanogenesis
Benzene	+	-	+	+	+
Toluene	+	+	+	+	+
Xylene	+	-	+	+	+
Ethylbenzene	+	-	+	+	+

Knowledge on anaerobic degradation of BTEX compounds is also important for Asian countries. In many cities and most of northern China, groundwater is a major source of drinking water. The quality of groundwater directly affects the health of residents. BTEX contaminants in groundwater increase with the development of industry and agriculture.

TERMINAL ELECTRON ACCEPTING PROCESSES AND THE DEVELOPMENT OF ANAEROBIC CONDITIONS

In pure cultures of chemotrophic organisms, reducing equivalents generated during transformation of hydrocarbons to metabolic intermediates, have to be transferred to an external electron acceptor with a more positive redox potential in order to conserve energy for growth. Growth without an external electron acceptor is not possible [1]. In the absence of oxygen, energy conservation can be accomplished by anaerobic respiration with other inorganic chemicals as electron acceptor. These reduction processes are generally referred to as terminal electron accepting processes (TEAPs). Potential natural electron acceptors for anaerobic oxidation of organic matter to carbon dioxide in subsurface environments are nitrate, Mn(IV), Fe(III) and sulfate. Carbon dioxide can also function as an electron-acceptor, yielding methane during methanogenesis. Along with these electron acceptors, BTEX components can be degraded anaerobically (Table 1).

During contamination with organic compounds, such as petroleum or landfill leachate, in both time and space a sequence of redox zones develops downstream of the point of contamination[2,3]. This sequential development relates to the energetics of TEAPs.

Denitrification yields the highest amount of energy, while methanogenesis yields the lowest amount. The preference for electron acceptors decreases with nitrate > Mn(IV) > Fe(III) > sulfate > methanogenesis[2,3]. Thus, most favorable electron acceptors will be depleted first. TEAPs can be determined by measuring the changes in concentration of electron-acceptors and products of reduction along the groundwater flow path. However, the deduction of TEAPs can be hampered by precipitation reactions, such as Fe (II) with sulfide or carbonate, and does not always give information on the present on-going TEAPs, by transporting reduced and oxidized species in the groundwater from another place[2]. Over the recent years a more advanced technique based on hydrogen measurements in groundwater has been developed. Hydrogen is an intermediate in the degradation of organic compounds. Its turn-over time is only a few seconds and its concentration indicates which TEAP is dominantly occurring[2]. Hydrogen concentration decreases with methanogenesis > sulfate > Fe(III) > Mn(IV) > denitrification. By including the concentration of redox species and performing simple thermodynamic calculations even a more precise delineation of TEAPs can be achieved. Thermodynamic calculations have shown that several redox processes can occur simultaneously[4]. Analysis of microbial communities and measurement of redox activities in laboratory cosms have also shown that at one position in a contaminated aquifer microorganisms performing different TEAPs are present and active[5-7]. Such information provides more possibilities for specifically engineered bioremediation, such as addition of electron acceptors.

MICROBIAL COMMUNITIES INVOLVED IN ANAEROBIC BTEX DEGRADATION

Knowledge on TEAPs occurring at a contaminated site provides also a clue for which group of microorganisms to look for. Pollution leads to strong changes in microbial communities and research so far indicates that under each different redox condition other groups of microorganisms are involved in anaerobic BTEX degradation (see below).

In present studies, molecular techniques play an important role in determining which microorganisms are involved in BTEX degradation (e.g. [8]) and will also play a major role in future bioremediation practices[9]. The relatively fast cultivation-independent molecular methods[9], based on analysis of the generally occurring genes encoding 16S ribosomal DNA or genes involved anaerobic BTEX degradation, can theoretically address the whole microbial community present. Due to the limits of cultivation only a small fraction, 0.01 to 10%, will be addressed by using cultivation based studies[9].

BTEX Degradation Coupled to Nitrate Reduction

Nitrate is not typically available in high concentrations in BTEX-contaminated aquifers. However, in some areas high concentrations of nitrate leach from agricultural fields receiving large amounts of fertilizers. All BTEX compounds can be degraded during denitrification (Table 1). So far only beta-proteobacteria belonging to the *Thauera/Azoarcus* cluster have been found to play an important role in degradation under denitrifying conditions. This has been shown by culture-independent molecular analysis of BTEX degrading enrichments[10] and through-flow column experiments[11], as well as by isolation of BTEX degrading bacteria. Up to now, at least 30 pure cultures of denitrifying microorganisms capable of oxidizing toluene, ethylbenzene, and m-, o- and p-xylene have been obtained[12-20] (Table 2). All belong to the *Thauera/Azoarcus* cluster. For a long time it was not clear whether benzene could be degraded with nitrate as electron acceptor[3]. Recently, evidence has been obtained that oxidation of benzene to carbon dioxide can also

be linked to nitrate reduction[21]. In a benzene degrading enrichment culture, nitrite accumulates stoichiometrically as nitrate is reduced. When nitrate is depleted, only nitrite remains and the rate of benzene degradation decreases to almost zero. Benzene is mineralized to CO_2 only in active cultures but not in sterile or uninoculated controls. Other electron acceptors (sulfate, iron, and CO_2) are not involved in the degradation. No molecular studies have yet been performed to establish whether members of the *Azoarcus/Thauera* cluster are also involved in benzene degradation in this enrichment.

TABLE 2

Combinations of BTEX and TEAPs for Which Pure Cultures of Isolates Have Been Obtained

	Nitrate	Manganese	Iron	Sulphate
Benzene	-	-	-	-
Toluene	+	-	+	+
Xylene	+	-	-	+
Ethylbenzene	+	-	-	-

As nitrate dissolves well in water, it can easily be applied to contaminated groundwater to increase the concentration of electron acceptors for anaerobic degradation. Experiments performed by Hutchins *et al.*[22,23] showed that such an approach can be successful. In a fuel-contaminated aquifer, BTEX and JP-4 jet fuel were significantly removed after adding nitrate. A specific probe for *Azoarcus* has been described[10] (Table 3).

TABLE 3

Specific Probes for the Detection of Microorganisms Capable of BTEX Degradation

	TEAP	Sequence	Reference
Azoarcus/Thauera	nitrate	GAATCTCACCGTGGTAAGCGC (AT1458)	[10]
Geobacteraceae	Fe(III)	TACCCGCRACACCTAGT (Geo825R)	[28]
Desulfobacteriaceae	sulfate	CGGCGTTGCTGCGTCAGG (SRB385Db)[a]	[47]

[a] Reacts also with other delta-proteobacteria, like *Pelobacter* and *Desulfuromonas*.

BTEX Oxidation Coupled to Manganese Reduction

Manganese is a widespread transition metal, which occurs at about 5-10 times lower concentrations than iron. Mn(IV) reduction is energetically more favorable than Fe(III) reduction but the rate of degradation with this electron acceptor is low due to its limited availability and solubility[24]. Little knowledge is available on BTEX degradation under Mn(IV) reducing conditions, and only toluene has been found to be degraded in column experiments[24] (Table 1). No pure culture is available (Table 2). However, as most Fe(III) reducting microorganisms can also reduce Mn(IV), results obtained under Fe(III) reduction likely also apply to Mn(IV) reducing conditions[25]. The type of manganese oxide strongly affects the degradation rate of toluene. In a flow-through laboratory column filled with contaminated sediment and sludge, toluene was degraded in the presence of crystalline manganese oxide, amorphous manganese oxide and freeze-dried amorphous manganese oxide under anaerobic conditions. The degradation rate with crystalline manganese oxides was the lowest, with amorphous manganese oxides the highest[24]. The larger specific surface area of amorphous manganese oxides provides better physical contact, which is required to transport the reducing power across the cell envelope and manganese oxide particle

interface[24, 26, 27]. Increasing the solubility of manganese oxide by adding organic ligands such as nitrotriacetic acid (NTA) or EDTA further enhanced the toluene degradation rate[24]. The toluene degrading enrichment culture was identified by phylogenetic analysis of cloned rDNA sequences. At least two major groups of Gram-negative bacteria were present. One group showed a weak similarity with *Bacteroides* and *Cytophaga*, and the other group consisted of members of the beta-proteobacteria, weakly related (91%) to *Azoarcus*[24].

BTEX Degradation Coupled to Fe (III) Reduction

Insoluble iron oxides typically provide the greatest electron-accepting capacity in shallow aquifers, because magnetite (Fe_3O_4) is ubiquitous in natural environments[25]. The zone of Fe (III) reduction can be quite extensive in contaminated aquifers[5]. *Geobacters*, belonging to the delta-proteobacteria appear to be the major iron reducers in iron reducing soils and sediments[28, 29]. Cultivation-dependent and independent studies have showed that they are enriched in petroleum polluted aquifers[8,28,30]. Only members of the *Geobacters* are known to degrade BTEX compounds in pure culture[29, 31]. The first evidence that BTEX can be degraded under anaerobic conditions was obtained by isolating the toluene degrading, Fe (III) reducer *Geobacter metallireducens* from a petroleum fuel contaminated aquifer[31]. In this aquifer, Fe (II) accumulated in groundwater over time and sediment in contaminated portions of the contaminated aquifer contained much less Fe (III) than nearby uncontaminated parts, indicating the *in situ* degradation of toluene with associated Fe (III) reduction[31]. The most recalcitrant BTEX compound, benzene, can also be degraded under Fe (III) reducing conditions, although benzene degradation appears to be found in only a few aquifers as shown in experiments where sediments from anaerobic petroleum-contaminated aquifers were anaerobically incubated without amendments[30]. Only in one case, added radiolabeled benzene was anaerobically oxidized to carbon dioxide without lag period, indicating the *in situ* degradation of benzene. Obvious differences in microbial composition were observed with nearby Fe (III) reducing locations where benzene was present but not degraded, with an uncontaminated part of the aquifer and a part of the aquifer which had turned methanogenic[8]. Both culturing and culture-independent methods (16S rDNA based denaturing gradient gel electrophoresis, most probable number PCR, 16S rDNA sequencing, phospholipid fatty acid analysis) demonstrated that *in situ* anaerobic benzene degradation was associated with much a larger number of *Geobacteraceae*[8,30]. At Fe (III) reducing sites where no benzene degradation occurred, Fe (III) reducing *Geothrix fermentens* dominated. Also benzene-oxidizing Fe (III) reducing enrichments were established[8,30]. After addition of radio-labeled benzene or toluene, monitoring the yield of ^{14}C indicated both aromatic hydrocarbons were mineralized into CO_2 without lag period. Also in the enrichments *Geobacters* dominated, as revealed by culturing-independent methods. Bacteria closely related to *Geothrix fermentens* and *Variovorax paradoxus* were also encountered, but they are not known to be capable of anaerobic BTEX degradation[8]. These studies[8, 30] indicate that organisms closely related to known *Geobacter* species are associated with anaerobic benzene degradation reduction[8]. Attempts to isolate the benzene oxidizer have not yet been successful. Sofar only two toluene degrading isolates have been obtained[8, 29].

Like manganese oxides, the quantity and form of iron oxides are major determinants in the rate and extent of organic contaminant degradation in Fe (III) reducing zones of polluted aquifers. Poorly crystalline Fe (III) oxides are the most readibly available for microbial reduction. The addition of synthetic Fe (III) chelators, such as NTA, can accelerate benzene degradation[32]. Chelators solubilize the highly insoluble Fe (III) oxides and this Fe (III) is much more accessible to Fe (III) reducers than natural Fe (III). Another limiting factor in

degradation is the requirement for Fe (III) reducing microorganisms to establish direct physical contact with insoluble Fe (III) oxides in order to reduce them[33]. *G. metallireducens* is not capable of reducing poorly crystalline Fe (III) oxide through the production of soluble reductants, electron-shuttling compounds or by solublilizing Fe (III) prior to reduction[33]. Fe (III) reduction can be stimulated by the addition of humic acids which act as intermediates in iron reduction[34]. Electrons are donated to quinones in humic acids, then the humic acids non-enzymatically reduce Fe (III) or other metals like Mn (IV), regenerating an oxidized form of humic acid. Fe (III) reducers obtain energy to support cell growth from the process of electron transport to humics[34, 35]. Even low concentration of humics can serve as electron-acceptor for significant amounts of organic-matter oxidation. Contact between humic acids and Fe (III) is much easier than between microorganisms and Fe (III) oxides, also humic acids can react with more types of Fe (III) oxides and reach Fe (III) oxides which can not be reached by microorganisms[35].

As Fe (III) oxides are insoluble, they can not be added to aquifers. However, nitrate addition might be capable of regenerating Fe (III). Anaerobic reoxidation of Fe (II) with associated reduction of nitrate has been observed in a variety of environments[36]. Addition of chelators or humic acids can help to enhance degradation under iron-reducing conditions. A recently developed 16S rDNA-based *Geobacter* primer set (Table 3) allows the specific, culturing-independent monitoring of *Geobacter* populations in iron-reducing environments[8, 30].

BTEX Degradation Linked to Sulfate Reduction

Some anaerobic environments are rich in sulfate, because rainwater provides a recharge of sulfate from fertilized soils and leaches sulfate from landfills and industrial waste. Furthermore, marine environments are by nature rich in sulfate.

Pure cultures of sulfate-reducing bacteria able to degrade toluene, *o*-xylene and *m*-xylene have been isolated from anaerobic sulfate-reducing environments[16, 37-42] (Table 2). All isolates characterized in detail belong to the *Desulfobacteriaceae* cluster of delta-Proteobacteria (*Desulfobacula toluolica*, *Desulfobacterium cetonicum*, a close relative of *Desulfobacterium cetonicum* and *Desulfosarcina variables* and a close relative of *Desulfococcus multivorans*).

All BTEX compounds can be degraded with sulfate as electron acceptor (Table 1). In two thermophilic consortia obtained from two different sulfate reducing environments, radio-labeled toluene, benzene, ethylbenzene and xylene were degraded to $^{14}CO_2$, tightly coupled to H_2S generation by sulfate reduction[43]. Degradation of benzene, the most recalcitrant BTEX compound, was observed with a lag period of 30-60 days in an enrichment established with aquifer sediment[44] and in sediments of a petroleum-contaminated aquifer[45] under sulfate-reducing conditions. The benzene was probably slowly degraded due to preferential utilization of easier consumable toluene and xylene and the requirement for sufficient bacterial biomass capable of benzene degradation.

Another enrichment of a benzene-degrading, sulfate-reducing consortium, obtained from Guaymas basin sediment and with benzene as the sole source of carbon and energy has been molecularly characterized[46]. Phylogenetic analysis of 12 16S rRNA clones showed a broad diversity within the consortium. Clones were related to the gamma-, delta- and epsilon-proteobacteria, Cytophagales and low G+C Gram-positives. Four clones fell within the *Desulfobacteriaea* cluster, one of them was closely related to aromatic degrader *Desulfobacula toluolica* and the remaining three were associated with *Desulfosarcina variabilis.* In addition, one clone was related to sulfide oxidizer *Thiomicrospira*. A bacteria related to *Campylobacter*/*Wolinella* (epsilon-proteobacteria) might be a member of a

commensal relationship to scavenge hydrogen in the consortium. For the other members no possible role could be established. *Desulfobacteriaceae* were also dominant in another alkylbenzene degrading sulfate reducing enrichment, obtained from the water phase of North Sea oil tank. This group constituted 95% of the total bacterial population. When the same source of microorganisms was enriched with lactate as substrate, mainly *Desulfovibrio* were detected[47]. In this study a probe specific for *Desulfobacteriaceae* was developed (Table 3), which is of significance since both culturing and cultivation independent methods show a relation between the presence of *Desulfobacteriaceae* and BTEX degradation.

Stimulation of anaerobic *in situ* bioremediation under sulfate reducing conditions is an interesting option since sulfate can readily be added to a polluted aquifer due to its high solubility[48]. However, this application should be done with care as during sulfate reduction toxic, volatile sulfide is formed. In environments with a high concentration of Fe (II) sulfate addition is a suitable option as the formed sulfide will precipitate with Fe (II), avoiding dangerous situations. Rates of benzene degradation could be stimulated in both batch and column experiments in the presence of added sulfate[48]. These results led to the development of an anaerobic bioremediation field trail based on sulfate injection into the subsurface. Groundwater benzene concentrations as high as 0.1 mmol/L decreased substantially, and this coincided with decreases in supplied sulfate over 84 days of treatment[48].

BTEX Degradation During Methanogenesis

Methanogenesis requires a syntrophic association between proton-reducing bacteria and hydrogen consuming methanogenic *Archeae* in order to obtain energy. Methanogens require hydrogen and acetate for their growth, which are provided by proton-reducing bacteria. The scavenging of hydrogen by methanogens keeps the hydrogen concentration sufficiently low for the proton-reducing or fermentative bacteria, so they can still gain energy from the degradation of BTEX[1].

BTEX degradation has been observed under methanogenic conditions in enrichment cultures containing contaminated aquifers material[49-51] as well as in petroleum-contaminated aquifers[52,53]. In enrichments, benzene degradation associated with methanogenesis normally takes a long lag period (140-400 days)[51]. It has been suggested that the microbial populations need to accumulate to sufficient density to degrade benzene. However, recently the occurrence of benzene degradation without apparent lag period was demonstrated. This was probably due to the ability for the appropriate consortium to develop during long term *in situ* exposure to benzene[53]. Benzene can not always be degraded under methanogenesis[51]. This possibly relates to high toxic benzene concentrations (1.4-4.3mmol/L), which affect the microorganisms adversely. On the other hand, no benzene metabolism is observed at very low concentrations (1.5μM) of benzene, because the concentration of benzene might too low to induce growth of benzene reducers[22].

Two archaeal species related to the genera *Methanosaeta* and *Methanospirillum*, one eubacterium related to the genus *Desulfotomaculum* and a species not related to any previously described genus were responsible for toluene reduction in a methanogenic consortium[54]. *Methanosaeta* species are aceticlastic methanogens that split acetate, oxidizing the carboxylic group to carbon dioxide and reducing the methyl group to methane. No other substrates support growth. *Methanospirillum* species use formate and hydrogen as electron donors. Thus these *Archaea* presumably utilize hydrogen or formate produced by other organisms in the culture. *Desulfotomaculum* is a sulfate reducing bacterium, but grows in the absence of sulfate acetogenically. Upon sulfate addition to the enrichment, toluene

degradation was inhibited, indicating that the initiation of toluene degradation is not performed by this organism. Thus, by elimination it is likely that the unidentified species performs the first step in toluene degradation, resulting in the release of intermediates on which *Desulfomaculum* grows. *Desulfomaculum* in turn produces hydrogen and acetate which are utilized by the *Archaea*[54]. Based on recent biochemical studies on this enrichment, interspecies transfer of the first metabolite in anaerobic toluene degradation, benzylsuccinate, has been proposed[55].

Samples collected from the methanogenic and combined methanogenic/sulfate reducing zone of a hydrocarbon and chlorinate contaminated aquifer undergoing intrinsic bioremediation were analyzed by cloning PCR amplified 16S rDNA, screening, sequencing and phylogenetic analysis[52]. The microbial communities of the methanogenic and methanogenic-sulfate reducing samples had many clones in common, but were very different from the iron or sulfate reducing zone lower in the aquifer. Archaeal and bacterial diversity was high, bacterial clones were spread over 10 well-recognized divisions, as well as four candidate divisions with no cultivated representatives and six novel division level groups. In the methanogenic zones, one archeal clone sequence was highly abundant and comprised 81% of the archeal library, this clone was 97% identical to aceticlastic methanogen *Methanosaeta spp.* In the bacterial clone libraries of the methanogenic site, a sequence representing 7-16% of clones was 96% identical to *Syntrophus gentianae. Syntrophus spp*. obtain energy from the anaerobic oxidation of organic acids to acetate and hydrogen. The authors proposed that the terminal step of hydrocarbon degradation in the methanogenic zone of the aquifer is aceticlastic methanogenesis and that *Methanosaeta sp.* and *Synthrophus spp*. occur in syntrophic association[52]. More detailed information on the microbial participants in methanogenic BTEX degradation is so far not available.

BTEX Degradation in Synthrophic Associations

As described above, methanogenesis requires a synthrophic association of microorganisms. BTEX can not be degraded by disproportion to organic acids and hydrogen under standard conditions because the reaction is endogenic[56]. A negative Gibbs free energy can be obtained for this reaction only if a hydrogen-accepting partner organism reduces the hydrogen concentration[57]. The fermentative oxidation of toluene in a defined synthrophic, non-methanogenic co-culture has recently been established[57]. This syntrophic co-culture consisted of the sulfate reducing strain TRM1 or Fe (III) reducing *Geobacter metallireducens* in cooperation with *Wolinella succinogenes* as electron-accepting partner organism, with fumarate or nitrate as terminal electron acceptor[57]. In pure culture none of these strains can degrade toluene under nitrate or fumarate reducing conditions, while TRM1 can degrade toluene under sulfate reducing conditions and *G. metallireducens* under Fe(III) reducing conditions. Only *W. succinogenes* can grow in pure culture with nitrate and fumarate as electron acceptors. It is proposed that hydrogen and acetate function as electron shuttles between microorganisms in co-cultures. This result indicates that in nature BTEX-fermenting microorganisms might cooperate with hydrogen-consuming organisms that use electron acceptors.

BIOAUGMENTATION

Addition of electron acceptors can improve anaerobic BTEX degradation[22,58,59]. However, the lack of degradation is not always related to environmental conditions which are adverse to microbial degradation, but might also be due to the failure of the appropriate

BTEX-degrader to colonize the polluted environment[59]. Isolated strains and enrichment cultures can be used to seed environments showing no or very slow degradation, to augment microbial degradation (bioaugmentation). Such an approach can be valid as shown by Weiner and Lovley[59]. Laboratory cosms containing sediments from the sulfate-reduction zone of a petroleum-contaminated aquifer, in which benzene persisted, were inoculated with a benzene-oxidizing, sulfate reducing enrichment from aquatic sediments. Benzene was degraded, parallel with growth of the benzene-degrading population. Similar observations have been made for sulfate-reducing marine sediments, where degradation of naphthalene was initiated by the addition of a sulfate reducing naphthalene degrading enrichment[60]. It should be realized that the experiments described were laboratory experiments, in which a relatively large inoculum (10% of total weight) was used. Also, research on bioaugmentation of aerobic sites with aerobic microorganisms has shown that bioaugementation often does not work, often because they are not adapted to the local conditions[61].

BIOCHEMISTRY AND MOLECULAR GENETICS OF BTEX DEGRADATION

Understanding the factors that control the rate and extent of anaerobic BTEX degradation requires information on the microorganisms responsible for the degradation and their metabolic capabilities, and thus requires their isolation. So far, only for some combinations of BTEX and TEAP (a quite limited number of) pure cultures of microorganisms have been obtained that are capable of complete oxidation of some of the BTEX compounds (Table 2). No benzene degrading isolates are known.

Although it is well known that only a small part of the microbial communities can readibly be cultured and that culture-based studies can be biased (e.g. [9,62]), it is interesting to note that pure cultures of BTEX degraders are phylogenetically closely related to microorganisms that are dominantly detected using culture independent molecular analysis of enrichments or samples obtained from sites with on-going biodegradation (see previous sections). Members of the *Thauera/Azoarcus* cluster, *Geobacteraceae* and *Desulfobacteriaceae* have been found to dominate in respectively denitrifying, Fe(III) reducing and sulfate reducing environments as well as are the only groups of microorganism from which till present date BTEX degraders have been isolated in pure culture. Specific 16S rDNA probes to detect these microorganisms in the environment are available (Table 3). It should also be noted that for all isolates close relatives are known which are not capable of BTEX degradation.

The isolates have allowed us to study in more detail the physiology, biochemistry and genetics of BTEX degradation. In the future, this might allow the development of more specific molecular probes, directed at the detection of (the expression of) genes involved in anaerobic degradation. So far the biochemical and especially the molecular studies have mainly be limited to denitrifying *Azoarcus* and *Thauera* species[1].

Anaerobic degradation has to differ considerably from aerobic degradation as under aerobic conditions the initial attack of hydrocarbons involves molecular oxygen as a co-substrate for monoxygenases or dioxygenases[1]. For anaerobic degradation of aromatics several pathways have been described, sometimes occurring in the same strain[63]. So far BTEX compounds appear to be degraded only via benzoyl-CoA in the benzyol-CoA pathway[64]. The individual BTEX compounds, as well as other aromatics[64], are funneled into this central pathway via different peripheral pathways. The enzymes of the peripheral and central pathways of anaerobic BTEX degradation are substrate-induced. The

ethylbenzene degrading strain EbN1 is able to degrade both ethylbenzene and toluene. Cells grown on one of these substrates only exhibit the enzyme activities for metabolism of the respective substrate, but not for the other. This indicates differences in pathways and regulation of expression[1,16].

The Central Benzyol-CoA Pathway

The enzyme benzyol-CoA reductase plays a central role in the benzyol-CoA pathway. This enzyme performs the ATP-driven two-electron reductions of the benzene ring, this reaction uses a ferredoxin as electron. After nitrogenase this is the second enzyme known which overcomes the high activation energy required for reduction of a chemically stable bond by coupling electron transfer to the hydrolysis of ATP (reviewed in [64]). Benzyol-CoA reductase exhibit distinct similarities with 2-hydroxyglutaryl-CoA dehydratase and its ATP-hydolysing activase protein of *Acidaminococcus fermentans.* The resulting cyclohex-1,5 diene-1-carboxyl CoA is oxidized to acetyl-CoA via a modified beta-oxidation pathway.

The genes for the enzymes of the central benzoyl-CoA pathway have been cloned and sequenced from denitrifying *Thauera aromatica* and *Azoarcus evansii*, as well as for the anoxygenic phototroph *Rhodopseudomonas palustris*.[65-67]. Sequence analyses of the genes support observations that phototrophic and denitrifying bacteria use two slightly different pathways to metabolize benzyol-CoA[64]. However, the benzoyl-CoA reductases show a high overall amino acid sequence identity of about 70%.

Anaerobic Catabolism of Toluene and Xylene

Of the four BTEX compounds, most biochemical and molecular genetic information has been obtained for toluene. During the degradation of aromatic compounds, toluene is the most rapidly consumed alkylbenzene[46]. A large number of isolates has been obtained. Analysis of culture supernatants and studies on permeablized cells[16,55,68,69] of denitrifying and sulfate reducing isolates, as well as in methanogenic enrichments shows that toluene degradation is initiated by the benzylsuccinate synthase catalyzed addition of the methyl carbon of toluene to the double bond of fumarate, forming benzylsuccinate[1]. This reaction is recognized as a novel means of enzymatic carbon-carbon bond formation and is performed by a glycyl-radical enzyme. The enzyme is highly oxygen sensitive and contains a redox-active flavin cofactor, but no iron centers. The 220Kda enzyme consists of two subunits each consisting of the 98 kD α , 8.5 kD ß and 6.4 kD ɣ proteins[1]. So far, genes coding the subunits of benzylsuccinate synthase (designated *bssCAB*) of *T. aromatica* K172 have been cloned and sequenced[70]. Highly similar genes have been cloned from *T. aromatica* T1 (designated *tutFDG*) and likely also encode benzylsuccinate synthase[71]. These proteins have considerable similarity to pyruvate formate lyase. Together with gene *bss*D (*T. aromatica* K172), respectively *tutE* (*T. aromatica* T1), they form a toluene-inducible operon. *bssD* and *tutE* are likely benzylsuccinate synthase activating enzymes, as their gene products resemble those of the pyruvate formate lyase activitating enzyme.

Benzylsuccinate derived compounds are also intermediates in the degradation of m-cresol, p-cresol (4-hydroxylbenzylsuccinate, degraded to 4-hydroxylbenzoyl-CoA)[72] and m-xylene (3-methylbenzylsuccinate, degraded to 3-methylbenzoyl-CoA)[73]. Addition of fumarate to methyl groups of these substrates was inferred from enzyme assays. The substrate range of benzylsuccinate of *Azoarcus* strain T1 is wide, fumarate addition occurs with xylenes, nonofluortolulnes, benzalodehyde and 1-methyl-1-cyclohexene. This may indicate a comparable metabolic pathway and associated genes for the degradation of xylene[74].

Benzylsuccinate is further converted to benzoyl-CoA via benzylsuccinate-CoA transferase, benzylsuccinate-CoA dehydrogenase, phenylitaconyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, benzoylsuccinyl-CoA thiolase. Also these genes (*bbs* operon consisting of 9 genes) have been recently cloned and sequenced for the first time, from *T. aromatica* K172[75]. They are induced by toluene.

Anaerobic Catabolism of Ethylbenzene

All ethylbenzene degrading isolates are affiliated with the genus *Azoarcus*, as deduced from 16S rDNA sequence data[13,76]. Benzoate is a transient intermediate in the catabolism of ethylbenzene. The pathway of ethylbenzene is quite different from toluene. Benzylsuccinate synthase is not involved[16]. A study under denitrifying conditions demonstrated that the first initial reaction is the oxidation of ethylbenzene to 1-phenylethanol, catalyzed by ethylbenzene dehydrogenase. The oxygen atom of the hydroxyl group of 1-phenylethanol is derived from water. Subsequent enzymatic reactions are catalyzed by 1-phenylethanol dehydrogenase, acetophenone carboxylase, benzoylacetyl-CoA forming enzyme and benzoylacetyl-CoA CoA thiolase and result in the formation of benzoyl-CoA. No genes have been isolated so far.

Anaerobic Degradation of Benzene

No pure culture is available which is capable of anaerobic benzene degradation. Little is known about how anaerobic benzene degradation proceeds. A sulfate reducing bacterial enrichment from a petroleum contaminated aquifer anaerobically degrading benzene accumulated phenol and benzoate as putative intermediates[77]. Benzoate was also an intermediate under methanogenic conditions or in iron reducing enrichments. Therefore, benzoate appears to be of importance during anaerobic benzene degradation[77].

THE FUTURE FOR ANAEROBIC BIOREMEDIATION

In the past, oxygen has often been supplied for active remediation of aerobic sites, but can cause environmental problems and is expensive. In environments in which anaerobic processes already prevail, stimulating the anaerobic communities has the advantage of enhancing an already established, active microbial population. Knowledge on anaerobic degradation has rapidly gathered during the recent years and starts to open ways to monitor or enhance anaerobic BTEX degradation.

It is not surprising that under each different TEAP other groups of anaerobic microorganisms are involved in BTEX degradation. Far more surprising is that the TEAP-specific BTEX degraders are limited to only one phylogenetic cluster and closely related to culturable species. Members of the *Azoarcus/Thauera* cluster perform BTEX degradation under denitrifying conditions, *Geobacteraceae* with Fe(III) reduction and *Desulfobacteriaceae* with sulfate reduction. As 16S rDNA probes are available for this microorganisms (Table 3), this allows the simple and rapid detection and monitoring of their presence in polluted environments. Based on measurement of dominantly occurring TEAPs in the particular environment as well observed BTEX compounds, one can then decide to engineer the environment, by the addition of electron acceptors or by seeding with more appropriate BTEX degraders, or to continue monitoring to follow the process. However, the dominance of *Azoarcus/Thauera*, *Geobacteraceae* and *Desulfobacteriaceae* in cultivation-independent, molecular studies and cultivation-based studies might be caused by the fact

that most cultivation-dependent studies have been performed on enrichment cultures, and only a few samples were directly taken from environments where *in situ* degradation occurred. Enrichment conditions can be biased as they often do not resemble the environmental conditions[62]. This can cause the enrichment of organisms which are not relevant for the environment under study. Therefore, more cultivation independent studies on environmental samples or on enrichments mimicking better natural conditions are required to obtain more detailed information on microbial communities involved in anaerobic BTEX degradation.

The phylogenetic clusters in which the BTEX degraders fall also contain non-BTEX degraders, thus a high level of a particular group in the environment might not necessarily indicate that degradation is occurring. However the increasing number of bacterial isolates, as well as results regarding biochemistry and molecular genetics of BTEX degradation, like similarities in enzymes and genes among different physiological groups of BTEX degraders, make us hopeful that probes specific for functional genes will be developed. Besides that used for the detection of the potential of BTEX degradation, such probes can also be used for the determination of *in situ* BTEX degradation activity (by directing probes at mRNA), as the gene-expression is induced by BTEX.

Interpretation of results from molecular methods will help to diminish the number of sites which have to be actively remediated. For strongly polluted sites, it provides information about which path should be followed for successful bioremediation. All this will help to substantially reduce the costs of bioremediation. This is especially of importance for developing countries, like China, as the development often causes environmental and health-affecting problems.

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