Adaptation of anaerobically grown *Thauera aromatica*, *Geobacter sulfurreducens* and *Desulfococcus multivorans* to organic solvents on the level of membrane fatty acid composition

Ilka Duldhardt,[1] Julia Gaebel,[1] Lukasz Chrzanowski,[2] Ivonne Nijenhuis,[3] Claus Härtig,[4] Frieder Schauer[5] and Hermann J. Heipieper[1]*

Departments of [1]Environmental Biotechnology, [3]Isotope Biogeochemistry and [4]Environmental Microbiology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany.
[2]*Institute of Chemical Technology and Engineering, Department of Organic Chemistry, Poznan University of Technology, Pl. M Curie 2, 60965 Poznań, Poland.*
[5]*Department of Applied Microbiology, Institute of Microbiology, Ernst-Moritz-Arndt-University Greifswald, F.-L.-Jahn-Str. 15, 17487 Greifswald, Germany.*

Summary

The effect of different solvents and pollutants on the cellular fatty acid composition of three bacterial strains: *Thauera aromatica*, *Geobacter sulfurreducens* and *Desulfococcus multivorans*, representatives of diverse predominant anaerobic metabolisms was investigated. As the prevailing adaptive mechanism in cells of *T. aromatica* and *G. sulfurreducens* whose cellular fatty acids patterns were dominated by palmitic acid (C16:0) and palmitoleic acid (C16:1*cis*), the cells reacted by an increase in the degree of saturation of their membrane fatty acids when grown in the presence of sublethal concentrations of the chemicals. Next to palmitic acid C16:0, the fatty acid pattern of *D. multivorans* was dominated by *anteiso*-branched fatty acids which are characteristic for several sulfate-reducing bacteria. The cells responded to the solvents with an increase in the ratio of straight-chain saturated (C14:0, C16:0, C18:0) to *anteiso*-branched fatty acids (C15:0*anteiso*, C17:0*anteiso*, C17:1*anteiso*Δ9*cis*). The results show that anaerobic bacteria react with similar mechanisms like aerobic bacteria in order to adapt their membrane to toxic organic solvents. The observed adaptive modifications on the level of membrane fatty acid composition can only be carried out with *de novo* synthesis of the fatty acids which is strictly related to cell growth. As the growth rates of anaerobic bacteria are generally much lower than in the so far investigated aerobic bacteria, this adaptive response needs more time in anaerobic bacteria. This might be one explanation for the previously observed higher sensitivity of anaerobic bacteria when compared with aerobic ones.

Introduction

Since oxygen is scarce in many subsurface environments contaminated with organic pollutants, the study of the biodegradation potential of anaerobic microorganisms has become of great interest in the last decades (Lovley, 2001; 2003). Many bacteria with the capability to degrade various organic solvents and compounds in the absence of oxygen have been isolated. Nevertheless, one potential limitation for the biodegradation of chemical contamination in natural environments is the toxicity of many compounds to the degrading microorganisms. The problem of the high toxicity of chemicals is especially significant in highly contaminated aquifers polluted with aromatic compounds, such as benzene, toluene, ethylbenzene and xylenes (BTEX), present in phase and overall concentrations of more than 1 g l⁻¹ can occur (Griebler *et al.*, 2004). For many years, our knowledge about the toxicity of organic compounds on anaerobic bacteria was limited to only a few reports (Ennik-Maarsen *et al.*, 1998; van Beelen, 2003). However, already in one of the first descriptions of a toluene-degrading nitrate-reducing bacterium, a concentration-dependent lag phase of the bacteria was observed (Evans *et al.*, 1991). Recently, a first systematic approach could demonstrate that anaerobic bacteria are about three times more sensitive towards a series of different organic compounds when compared with aerobic bacteria (Duldhardt *et al.*, 2007).

Thus far, it is not known how anaerobic bacteria adapt to different organic substances and other forms of

environmental stress. Most adaptive mechanisms were only described for Gram-negative aerobic bacteria (for review see Sikkema *et al.*, 1995; Heipieper *et al.*, 2007). Only very recently, the nitrate-reducing stain *Aromatoleum aromaticum* stain EbN1 was investigated regarding its stress response especially using an proteomic approach (Trautwein *et al.*, 2008).

The toxicity of most hydrophobic organic hydrocarbons is caused by general, non-specific effects on membrane fluidity due to their accumulation in the lipid bilayer. The property of an organic solvents to accumulate in the membrane correlates with its toxicity and hydrophobicity (logP). Since membranes constitute the main target for solvents effects, most adaptive mechanisms are concerned with maintenance of the membrane fluidity and lipid-phase stability (Weber and de Bont, 1996). In aerobic bacteria, alterations in the membrane composition are known to play a crucial role in adaptation to the presence of high concentrations of contaminants. These adaptation mechanisms, relying on a modification of the membrane to keep it in the same fluidity condition, have been described extensively. Changes in the fatty acid composition of membrane lipids are the most important reactions of bacteria against membrane active substances. This mechanism is called 'homeoviscous adaptation' (Sinensky, 1974).

In anaerobic bacteria especially in sulfate-reducing bacteria the analysis of cellular fatty acids has been performed mostly with the intention to use them as biomarkers for the description of microbial communities or for the chemotaxonomical classification of bacterial isolates (Taylor and Parkes, 1983). Within several studies on sulfate-reducing bacteria the effect of the growth substrate, the growth state and the growth temperature on the fatty acid pattern has been investigated (Taylor and Parkes, 1983; Dowling *et al.*, 1986; Aeckersberg *et al.*, 1998; Konneke and Widdel, 2003).

However, except a few studies to some solventogenic *Clostridium* species (Herrero *et al.*, 1982; Baer *et al.*, 1987; Lepage *et al.*, 1987; Wang *et al.*, 2005), less information is available about the modification of cellular fatty acid pattern of anaerobic bacteria responding to organic solvents.

In this contribution, we investigated the adaptive response of the reference strains for nitrate-reducing bacteria *Thauera aromatica* K172 (Anders *et al.*, 1995), for sulfate-reducing bacteria *Desulfococcus multivorans* (Stieb and Schink, 1989) and for iron-(III)-reducing bacteria *Geobacter sulfurreducens* (Caccavo *et al.*, 1994) that had previously been studied in general solvent toxicity tests (Duldhardt *et al.*, 2007) to several important pollutants and representative organic solvents on the level of the membrane lipid fatty acid composition.

Results

Adaptation of T. aromatica, G. sulfurreducens *and* D. multivorans *to the presence of different organic solvents on the level of membrane fatty acid composition*

All three examined bacteria were grown anaerobically under optimal growth condition in mineral media. The growth-inhibiting effect of several organic solvents such as BTEX compounds as well as chlorinated phenols and aliphatic alcohols to three different anaerobic bacteria already has been investigated (Duldhardt *et al.*, 2007). After application of the test compounds to the microbial cultures in the middle of the exponential growth phase, bacterial cells continued growing, but in comparison with cells not treated with solvents, at reduced growth rates. When the estimated results were presented as a plot of the relative growth rates, against the concentration of the compounds, a direct relation between the concentration of the test compounds and their growth-inhibiting effect was visible. All bacterial cultures were harvested and prepared for cellular fatty acids analysis, when the cells were still in the state of exponential growth.

Alteration in the cellular fatty acid composition of T. aromatica *cells in the absence and presence of solvents*

Cells of *T. aromatica*, grown with sodium benzoate (5 mM) and nitrate (20 mM), showed a growth rate μ of about 0.21 h^{-1} and a doubling time t_D of 4.8 h. Predominant fatty acids of *T. aromatica* were C12:0, C16:0, C16:1cis, C18:0, C18:1Δ11cis which were already shown for this strain (Anders *et al.*, 1995). Among those phospholipid fatty acids, palmitic acid (C16:0), palmitoleic acid (C16:1cis) and *cis*-vaccenic acid (C18:1Δ11cis) are predominant and make out more than 95% of the fatty acid content of the strain. The occurrence of *cis*-vaccenic acid (18:1Δ11cis) and corresponding absence of oleic acid (18:1Δ9cis) in this bacterium indicates that it only contains the anaerobic pathway of fatty acid biosynthesis (Keweloh and Heipieper, 1996). Cells of *T. aromatica* reacted towards the application of the selected organic compounds with an increase of saturated fatty acids and a relative decrease in unsaturated fatty acids compounds (Fig. 1). Therefore, the degree of saturation of fatty acids was chosen as the major parameter to describe membrane adaptation. In a first attempt, the time dependence of the modification of fatty acid patterns of the cells grown in the presence of phenol at the previously calculated EC50 of 2 mM was investigated (Fig. 2). Immediately after exposure to the toxic compound, the cells started to decrease the fluidity of the membrane by increasing the degree of saturation of their fatty acids from 0.5 to about 0.65. This process
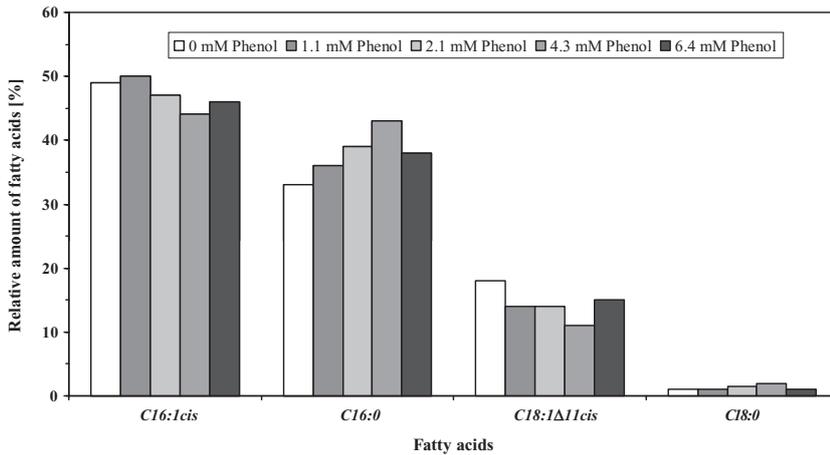
Fig. 1. Effect of phenol on the fatty acid composition of *T. aromatica*.

was finished after about 15 h in the presence of phenol. Therefore, we decided to investigate the concentration-dependent effect of the toxic chemicals to the membrane modifications by analysing samples from cells incubated for 15 h in the presence of the toxic compounds.

Figure 3 shows the concentration-dependent changes in the degree of saturation of fatty acids of cells grown in the presence of phenol. Similar results were obtained for all investigated toxic compounds in their corresponding toxic concentration ranges (data not shown). A direct relation between sublethal concentration of phenol and the decrease in degree of saturation from about 0.5 to 0.8 was observed. The highest response occurred at a concentration that inhibited cell growth around 75%. At concentrations, which totally inhibit cell growth, the fatty acid saturation degree was not influenced by any of the tested compounds.

Cells of G. sulfurreducens *showed an increase in degree of saturation depending on the used solvent concentration*

Cells of *G. sulfurreducens*, which were cultivated with acetate (5 mM) and fumarate (25 mM), showed a growth rate μ of about 0.15 h^{-1} and a doubling time of 6.7 h. The fatty acid pattern of *G. sulfurreducens* mainly consisted of C16:1Δ9*cis* and C16:0, which accounted for more than 80% of the total fatty acids. Next to these also small amounts of C14:0, C15:0*iso*, C16:1Δ11, C18:1Δ11*cis*, C18:0 and traces of C15:0*anteiso* and C15:0 were detected. No 10-methyl-hexadecanoic acid was found as described for *Geobacter metallireducens* (Lovley *et al.*, 1993).

Cells of *G. sulfurreducens* decreased their relative content of unsaturated fatty acids (particularly C16:1Δ9*cis*) and correspondingly increased the content of
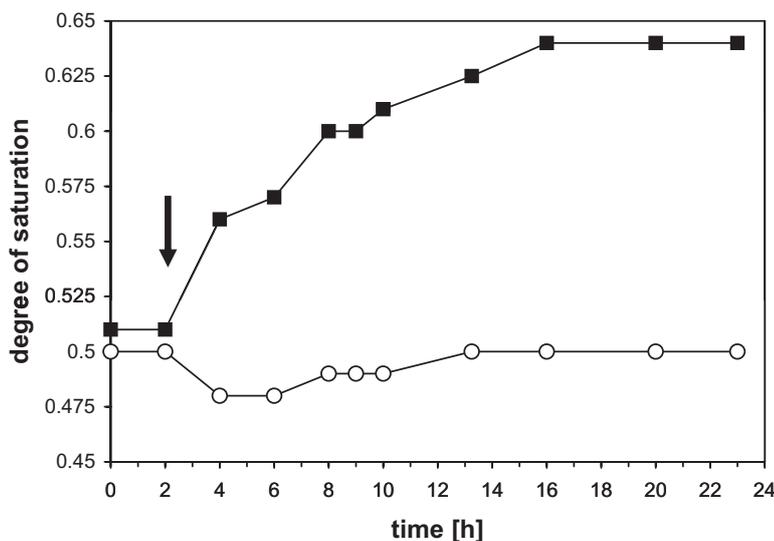

Fig. 2. Time-course of the effect of 200 mg l^{-1} phenol (■) on the degree of saturation of membrane fatty acids of *T. aromatica*. Control cells (○) were cultivated in the absence of the toxic chemical. The arrow indicates the addition of the phenol.
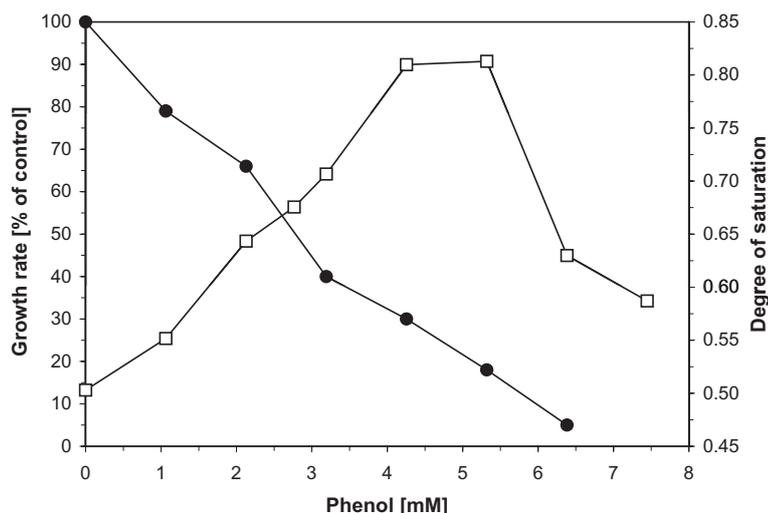
Fig. 3. Effect of phenol on growth rates (●) and on the degree of saturation of membrane fatty acids (□) of *T. aromatica*.

the saturated fatty acids (mainly C16:0) (Fig. 4). Accordingly, *G. sulfurreducens* cells treated with solvents showed a dose-dependent increase in the degree of saturation as shown for 2,4-dichlorophenol (Fig. 5). The strongest response to 2,4-dichlorophenol was examined after 8 h in the presence of the toxic chemical at concentrations similar to the calculated EC50 concentration. At this concentration the cells almost doubled their degree of saturation from 0.7 to 1.2 (Fig. 5). Comparable concentration-dependent responses were observed for all tested solvents (data not shown). At higher concentrations of the tested compounds which totally inhibit cell growth the degree of saturation was not influenced.

Changes in the cellular fatty acid composition of D. multivorans

Cells of *D. multivorans* were grown on sodium benzoate (4 mM) and sulfate (25 mM). Corresponding to a growth

rate μ of about 0.028 h^{-1}, a doubling time t_D of about 35.7 h for cells of *D. multivorans* was calculated.

The fatty acids routinely detected in *D. multivorans* DSM 2059 were under preliminary conditions: C14:0, C15:0, C15:0-*iso*, C15:0*anteiso*, C16:0, C16:1Δ9*cis*, C17:0*anteiso*, C17:1*anteiso*Δ9*cis*, C18:0, C18:1Δ11*cis*. Although 10 different fatty acids were identified, four of them: the *anteiso*-fatty acids (C15:0*anteiso*, C17:0*anteiso*, C17:1*anteiso*Δ9*cis*) and C16:0, accounted for more than 80% of the total. The observed fatty acid pattern was in agreement with reported results (Taylor and Parkes, 1983; Dowling *et al.*, 1986).

Since branched-chain fatty acids, especially that of the *anteiso* type, were present in high amounts in this strain and as unsaturated fatty acids are proposed to have a minor role in such bacteria (Kaneda, 1991), the alteration in the proportion of saturated straight-chain to *anteiso*-branched fatty acids was observed to be the major adaptive response of these bacteria causing much
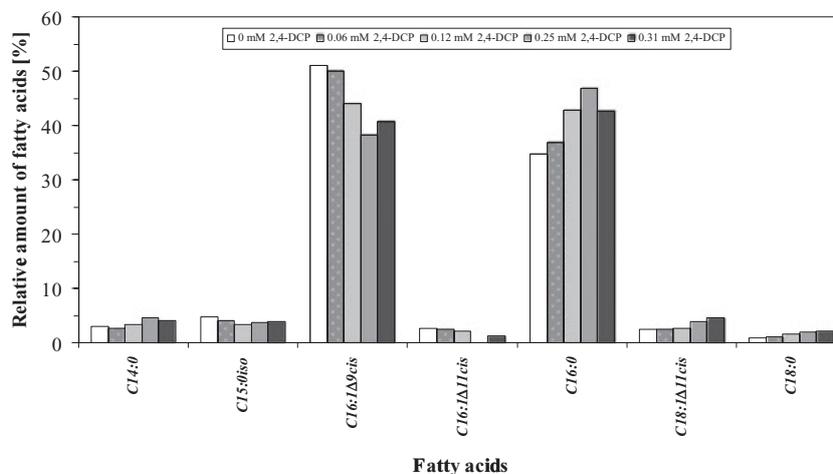

Fig. 4. Effect of 2,4-dichlorophenol on the fatty acid composition of *G. sulfurreducens*.
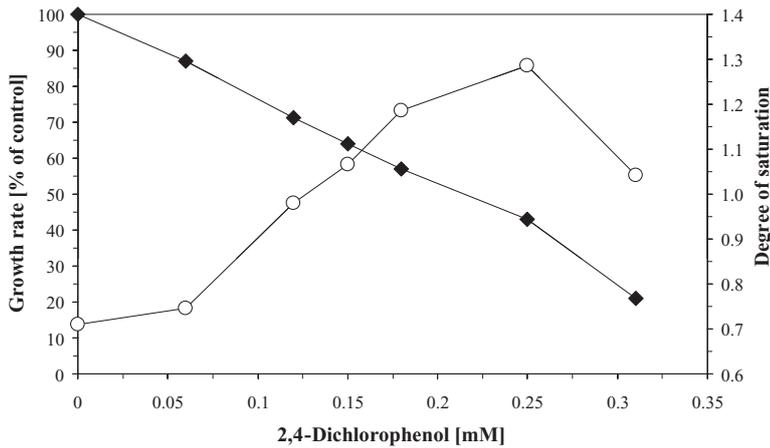
bigger changes than the degree of saturation (Fig. 6). As a consequence, the ratio of saturated straight-chain to *anteiso*-branched fatty acids is shown for cells of *D. multivorans* exposed to toxic concentrations of organic compounds.

Desulfococcus multivorans reacted to the presence of sublethal concentrations of the tested organic compounds with an increasing ratio of the three non-branched saturated (C14:0, C16:0, C18:0) to the three *anteiso*-branched (C15:0*anteiso*, C17:0*anteiso*, C17:1*anteiso*Δ9*cis*) fatty acids as shown for cells treated with benzene (Fig. 7). The highest response was reached after about 6 days in the presence of the toxic compounds. After reaching a 75% growth inhibition, *D. multivorans* showed, independent of the used test compound, a drop of *anteiso*-branched fatty acids while the relative content of saturated straight-chain fatty acids increased.

Discussion

In contaminated sites, including anaerobic environments such as aquifers or saturated zones of soils, bacteria are exposed to high concentrations of toxic organic compounds that are known to mainly act on the cell envelopes causing an increase in the fluidity of the membrane. The ability to control the biophysical properties of their membrane allows bacteria to survive under various environmental conditions. Consequently, membrane alterations are regarded as one of the most important adaptive mechanisms in bacteria because active restructuring of membrane's lipid composition preserves a suitable dynamic state of the bilayer and restore membrane functions (Hazel and Williams, 1990; Sikkema *et al.*, 1995; Weber and de Bont, 1996; Segura *et al.*, 1999; Bernal *et al.*, 2007). Thereby, modifications in the composition of
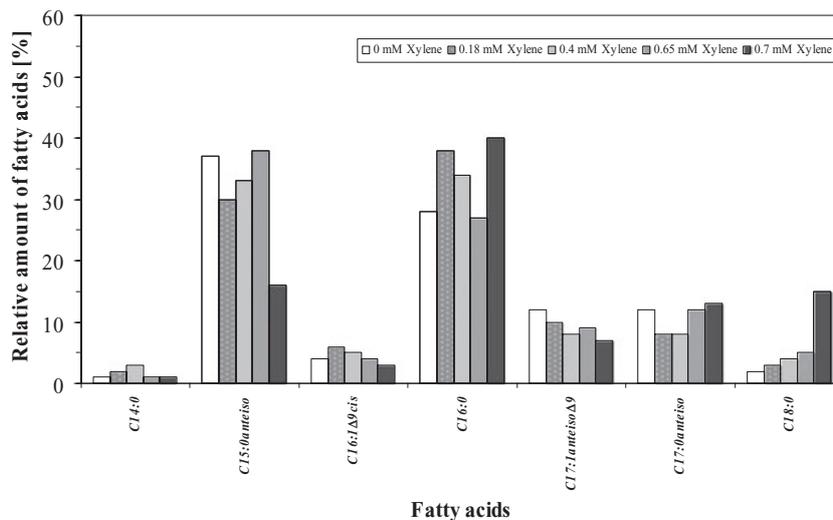

Fig. 6. Effect of xylene on the fatty acid composition of *D. multivorans*.
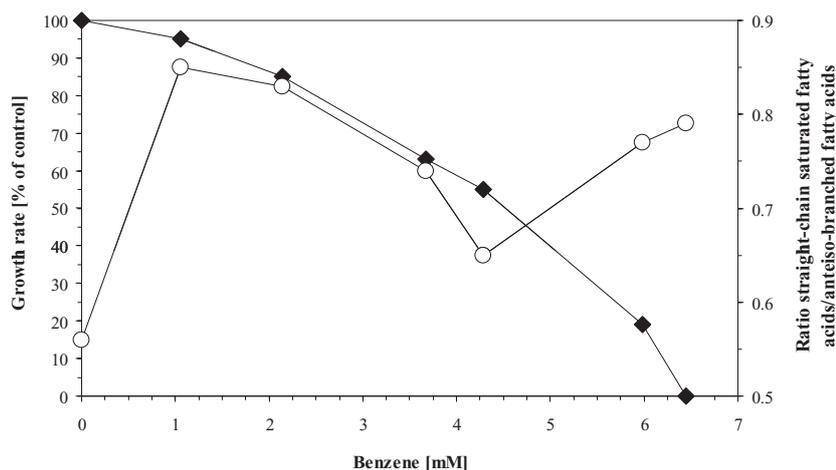
Fig. 7. Effect of benzene on growth rates (◆) and on the degree of saturation of membrane fatty acids (○) of *D. multivorans*.

membrane phospholipid fatty acids play the most important role. In the present work, a first systematic approach on these adaptive response mechanisms to organic solvents on the major groups of anaerobic bacteria using different terminal electron acceptors is presented.

In cells of *T. aromatica* and *G. sulfurreducens* the major adaptive membrane modification to the presence of the tested solvents was a concentration-dependent increase in the degree of saturation which is known to be the major adaptive response of most Gram-negative bacteria. The transition temperatures of saturated fatty acids are very high (e.g. for 16:0: 63°C), whereas those for the corresponding mono-unsaturated fatty acids with the *cis*-configurated double bond are far lower (e.g. 16:1*cis*: 0°C). Therefore, the linear acyl chains of saturated fatty acids that can be tightly packed leads to a membrane of low fluidity that counteracts the fluidizing effects caused by the presence of organic compounds as well as high temperatures (Ingram, 1976; 1977; Hazel and Williams, 1990; Heipieper and de Bont, 1994; Cronan, 2002; Kabelitz *et al.*, 2003). For both bacteria, a correlation between the added solvent concentration, their effect on growth inhibition and the corresponding adaptive reactions were observed.

Regarding membrane adaptation, *D. multivorans* showed a completely different strategy. The fatty acid pattern of most sulfate-reducing bacteria consists only in lower amounts of unsaturated fatty acids and instead of *anteiso*-branched fatty acids. Therefore, these bacteria modify their membrane fluidity by increasing the relative ratio between saturated and *anteiso*-branched fatty acids. Also these fatty acids have a lower transition temperature (e.g. 15:0*anteiso*: 23°C) than saturated fatty acids. Thus, like *cis*-unsaturated fatty acids, high proportions of *anteiso*-branched fatty acids lead to membranes with high fluidity (Kaneda, 1991). At growth inhibition of more than 75%, *D. multivorans* cells showed, independent of the used solvent, a dramatic drop of *anteiso*-branched fatty

acids and parallel strong increase in the proportion of saturated fatty acids in relation to the total fatty acids. Branched fatty acids can only be created by *de novo* synthesis under consumption of energy equivalents. Therefore, also in these Gram-positive bacteria the maintenance of fluidity and phase stability depends on the energetic status of the cells as well as on *de novo* synthesis of the precursors valine, leucine (*iso*-branched fatty acids) and isoleucine (*anteiso*-branched fatty acids) (Kaneda, 1991; Unell *et al.*, 2007).

Also for cells of *T. aromatica* and *G. sulfurreducens* the highest response in the degree of saturation occurred at concentrations of the solvents that inhibited the cell growth around 50–75%. Thus, changes in the proportions of fatty acids in the membrane phospholipids in the presence of solvents are limited, admittedly. Such relationships have already been observed also for several aerobic bacteria and yeast (Ingram, 1977; Heipieper and de Bont, 1994; Heipieper *et al.*, 1995; 2007; Kabelitz *et al.*, 2003). Changing the degree of saturation needs *de novo* synthesis of membrane lipids, which is a growth- and energy-dependent process. Dependent on the solvent concentration in the aqueous phase, the solvent hydrophobicity, and membrane composition, solvents partition and accumulate in the bacterial membrane. This causes a disturbance of membranes fluidity and permeability. The leakage of protons (and potassium ions), stimulated by the accumulation of solvent molecules in membrane interior, causes a lowering in the proton (potassium) motive force and leads to an impairment in the energy conservation (Heipieper *et al.*, 1992; Sikkema *et al.*, 1994). In bacteria, only the energy-dependent *de novo* biosynthesis of saturated fatty acids allows the increase in the degree of saturation, which may also be the reason why alteration in the degree of saturation was only observed in growing cells (Heipieper and de Bont, 1994). Therefore, under growth-inhibiting conditions, lipid biosynthesis is stopped due to stringent-response regula-

tion and that is why only growing cells can perform such kind of membrane adaptation.

In *T. aromatica* T1 a closely related strain of *T. aromatica* K172, with capability for toluene degradation, a concentration-dependent lag phase was observed (Evans *et al.*, 1991). Obviously, the bacteria needed a certain time to adapt to the presence of high, toxic concentrations of the compounds. As growth rates of anaerobic bacteria are far lower than aerobic bacteria, these processes also take longer time. This might be one explanation of the higher sensitivity of anaerobic bacteria to toxic organic compounds that was previously described (Duldhardt *et al.*, 2007). However, as we already know that whole cascades of different mechanisms are necessary for the bacterial adaptation to hazardous chemicals (Heipieper *et al.*, 2007), further research will be necessary in order to explain the higher sensitivity of anaerobic bacteria. Such progress in understanding adaptation of anaerobic bacteria to chemical pollutants is potentially important for many biotechnological applications of these microorganisms.

Experimental procedures

Strains and culture conditions

All investigated microorganisms were obtained from the DSMZ (Braunschweig, Germany). *Thauera aromatica* K172 (DSM 6984; Tschech and Fuchs, 1987; Anders *et al.*, 1995) was grown anaerobically at 30°C in mineral salt medium. Benzoate (5 mM) and KNO_3 (20 mM) served as C-source and electron acceptor respectively. *G. sulfurreducens* (DSM 12127; Caccavo *et al.*, 1994; Methe *et al.*, 2003) was grown anaerobically at 34°C in a bicarbonate-buffered mineral salt medium containing acetate (5 mM) and fumarate (25 mM) as C-source and electron acceptor respectively. *Desulfococcus multivorans* (DSM 2059; Stieb and Schink, 1989) was cultivated under sulfate-reducing conditions (25 mM sulfate) in a bicarbonate-buffered mineral medium at 34°C. Benzoate (4 mM) was added as source of energy and cell carbon. Cells were cultivated in 120 ml vials with 50 ml of medium in rotary shaking incubator with 180 r.p.m. (*T. aromatica*) or 100 r.p.m. (*G. sulfurreducens*, *D. multivorans*). All chemicals were reagent grade and obtained from commercial sources.

Investigation of the toxic effects of organic compounds on growing cells of anaerobic bacteria

For the determination of organic compound toxic effects, test compounds were added to non-adapted, exponentially grown cells as described by Heipieper and colleagues (1995). Bacterial growth was monitored by measuring the optical density at 560 nm. The impact of organic compounds on the anaerobic grown cells was ascertained by comparing the differences in the growth rates of cultures treated with organic compounds (μ_1) with that of a growth controls (μ_0) not treated with the test compound. The growth inhibition of various concentrations of the investigated organic compound was defined as the percentage of the growth rates of bacteria in the presence of the organic compounds with that of the growth cultures (Eqn 1).

$$\text{Inhibition growth (\%)}: \frac{\mu_{1,\text{toxin}}}{\mu_{0,\text{control}}} \times 100 \qquad (1)$$

Lipid extraction and analysis of fatty acid composition with gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analysis

Cells were harvested in the late exponential phase by centrifugation, washed two times with potassium phosphate buffer (50 mM, pH 7.0) and stored at −20°C prior to the lipid extraction. Lipids were extracted with a chloroform–methanol–water mixture according to a method described by Bligh and Dyer (1959). Fatty acid methyl esters (FAMEs) were prepared by using extracted lipids and boron trifluorid-methanol (15 min at 80°C) applying the method of Morrison and Smith (1964) and extracted with hexane. The methylesters were analysed by fatty acid composition with gas chromatography (GC-FID) (HP 6890 N, Hewlett Packard, USA) on a CP-SIL 88 column (50 m length × 0.25 mm inner diameter × 0.2 μm film thickness, Varian, Germany). Gas chromatography (GC) conditions were: injector temperature 240°C, detector temperature 270°C, splitless injection. The carrier gas was He at a constant flow rate of 2 ml min^{-1}. The oven temperature programme was run 2 min isothermal at 40°C, from 40°C to 220°C (8°C min^{-1}) and 15 min isothermal at 220°C. The peak areas of the FAME were used to determine their relative amounts. The fatty acids were identified by GC and co-injection of authentic reference compounds obtained from Supelco (Bellefonte, USA).

Additionally, the acid composition the FAMEs were analysed by gas chromatography-mass spectrometry (GC-MS) (HP6890N-HP5973, Hewlett Packard, USA) on a BPX-5 column (30 m length × 0.32 mm inner diameter × 0.25 μm film thickness, SGE, Germany). The carrier gas was He at a constant flow of 2 ml min^{-1}. Injector temperature was 280°C. The oven temperature programme was run 1 min isothermal at 70°C, from 70°C to 150°C (8°C min^{-1}), from 150°C to 220°C (2°C min^{-1}) and then for 1 min isothermal at 220°C, from 220°C to 300°C (20°C min^{-1}). Mass spectra were collect in a full scan mode (m/z 30–400) at a transfer line temperature of 280°C, a source temperature of 230 °C. Mass spectra and locked retention times were used for confirmation of FAME identity; the positions of the double bounds of unsaturated FAMEs were determined by DMDS adducts.

The degree of saturation of the membrane fatty acids was defined as the ratio between the saturated fatty acids and the unsaturated fatty acids present in the bacteria. The ratio of the non-branched to *anteiso*-branched fatty acids for *D. multivorans* was calculated as the ratio of the predominant saturated non-branched fatty acids (14:0, 16:0, 18:0) and the three *anteiso*-branched fatty acids (15:0*anteiso*, 17:0*anteiso*, C17:1*anteiso*Δ9*cis*).

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