Biodegradation **3**: 387–398, 1992.

Microbial degradation of pollutants at high salt concentrations

Aharon Oren,[1] Peter Gurevich,[1] Malkit Azachi[2] & Yigal Henis[2]
[1] *Division of Microbial and Molecular Ecology, The Alexander Silberman Institute of Life Sciences and The Moshe Shilo Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel;* [2] *Department of Plant Pathology and Microbiology, and the Otto Warburg Center for Biotechnology in Agriculture, The Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel*

Key words: halophilic, archaeobacteria, eubacteria, pollutants, hydrocarbons, formaldehyde, nitroaromatic compounds

Abstract

Though our knowledge on microbial degradation of organic pollutants at high salt concentrations is still limited, the list of toxic compounds shown to be degraded or transformed in media of high salinity is growing. Compounds transformed aerobically include saturated and aromatic hydrocarbons (by certain archaeobacteria), certain aromatic compounds, organophosphorus compounds, and formaldehyde (by halotolerant eubacteria). Anaerobic microbial transformations of toxic compounds occurring at high salt concentrations include reduction of nitroaromatic compounds, and possibly transformation of chlorinated aromatic compounds.

1. Introduction

The association of hypersaline lakes with unpleasant and harmful chemical compounds has been known for thousands of years, in any case as long as written documentation on these lakes exists:

> 'Then the Lord rained on Sodom and Gomor'rah brimstone and fire from the Lord out of heaven.'
> (Genesis 19:24)

Solutions to pollution problems in hypersaline water bodies can be achieved in different ways. As we will discuss below, not all biological degradation processes known to function in freshwater environments have been shown to be operative also in the presence of high salt concentrations. Therefore, from a microbiological point of view the easiest way to stimulate the degradation of pollutants is dilution to a sufficiently low salinity. In the case of the Dead Sea it was already suggested more than two thousand years ago that when the biology of hypersaline lakes does not perform as desired, dilution with fresh water can work wonders:

> 'And he said to me, 'This water flows toward the eastern region and goes down into the Arabah; and when it enters the stagnant waters of the sea, the water will become fresh. And wherever the river goes every living creature which swarms will live, and there will be very many fish, for this water goes there, that the waters of the sea may become fresh; so everything will live where the river goes.'
> (Ezekiel 47:8–9)

However, dilution with sufficient quantities of fresh water, if these are available at all, cannot always be expected to be feasible from the point of view of the engineer or the economist.

Another approach, and one that poses much greater challenges to the microbiologist, is to make microorganisms do the job in the presence of the high salt concentrations found in the polluted envi-

ronment, without prior dilution to lower the salinity.

To our knowledge no systematic survey has ever been made on the potential of bacteria to transform and/or degrade polluting chemicals at high salt concentrations. One possible reason is that preliminary attempts to demonstrate the degradation of otherwise relatively easily biodegradable pollutants in hypersaline lakes often yielded disappointing results. For example, no significant breakdown of long-chain straight hydrocarbons such as hexadecane could be demonstrated in Great Salt Lake brines at salinities exceeding 20% (Ward & Brock 1978). The ability of halophilic anaerobic microorganisms to degrade different organic substrates has been reviewed (Oren 1988; Oren 1990a), the main conclusion being that only a few easily degradable substrates such as simple sugars and amino acids can be fermented.

The ability of halophilic or halotolerant bacteria to degrade or transform different organic pollutants in the presence of high salt concentrations – when salt stress is superimposed on pollution stress, is of both basic and applied importance. Applied aspects include answers to the questions: How well can naturally occurring bacteria degrade pollutants in hypersaline lakes (the Great Salt Lake, Utah, or the Dead Sea, to give two major examples)? Can specialized halophilic or halotolerant bacteria be introduced to deal with the degradation of specific pollution events in these lakes? Can halophilic or halotolerant bacteria be used in the biological treatment of highly saline industrial waste effluents that contain toxic materials?

The first section of this chapter reviews the presently available information on microbial degradation of pollutants at high salt concentrations, under aerobic as well as under anaerobic conditions, and the nature of the bacteria involved in these processes. The discussion is limited to naturally occurring bacteria. Table 1 summarizes the range of toxic compounds that have been shown to be degraded by halophilic or halotolerant microorganisms. Another possible approach, not discussed here, would be the introduction of genes derived from non-halophilic microorganisms coding for the ability to transform selected pollutants into halophilic or halotolerant bacteria, using techniques of genetic engineering. To our knowledge this approach has not yet been used.

In the second part of the chapter two processes, currently studied in our laboratories will be discussed: aerobic transformation of formaldehyde at high salt concentrations by halotolerant eubacteria, and anaerobic transformation of nitroaromatic compounds by obligately anaerobic halophilic eubacteria.

Table 1. Toxic compounds shown to be degraded or transformed at high salt concentrations.

Compound degraded or transformed	Halophilic organisms involved	Salt concentrations	Reference
Saturated hydrocarbons Pristane Aromatic hydrocarbons	*Halobacterium* sp.	15–31%	Bertrand et al. 1990
Hexadecane	Unidentified bacteria in the Great Salt Lake	< 20%	Ward & Brock 1978
Benzoate and other aromatic compounds (?)	*Pseudomonas halodurans*	1.8–15.5%	Rosenberg 1983
Organophosphorus compounds	Unidentified moderately halophilic bacterium	2–24% (?)	DeFrank & Cheng 1991
Pretreated lignitic material	Unidentified anaerobes and methanogens	8–12%	Oren 1990e
Chlorophenols, chlorophenoxyphenols	Unidentified	9–12% or higher (?)	Boone et al. 1989
Formaldehyde	Unidentified moderately halophilic bacterium	1–20%	This work
Nitroaromatic compounds	*Haloanaerobium, Sporohalobacter*	13–16%, possibly higher	Oren et al. 1991, This work

2. Halophilic bacteria – an overview

The ability to grow at high salt concentrations – even up to saturating concentrations of NaCl, is widespread in the prokaryotic world. Halophilic or halotolerant bacteria are found both in the kingdom of the archaeobacteria (Archaea), and in the eubacteria. Halophilic bacteria can be found in many of the major subgroups within the eubacteria: the Proteobacteria, the Gram-positive branch, the cyanobacteria, and others.

Two major physiological groups of aerobic heterotrophic bacteria are generally found in hypersaline environments:

1. Halophilic archaeobacteria – generally red-pigmented cells, most of which require salt concentrations of at least 15–20% for growth, and often grow at salt concentrations of up to saturation. This group is represented by the genera *Halobacterium, Haloferax, Haloarcula,* and *Halococcus*. Exposure to lower salt concentrations causes lysis of the cells (with the exception of *Halococcus* cells, which possess a rigid cell wall). The halophilic archaeobacteria are heterotrophs, using amino acids, and to a lesser extent carbohydrates, as carbon and energy source. Certain strains can obtain additional energy from light, using the bacteriorhodopsin proton pump. The halophilic archaeobacteria maintain an osmotic balance of their cytoplasm with the hypersaline environment by accumulating high concentrations of salts (mainly K^+ and Cl^-, to a lesser extent Na^+). This mechanism of osmoregulation requires special adaptations of the intracellular enzymatic machinery, which has to be operative in the presence of high salt concentrations, which in some species may exceed 5 M.

2. Halophilic or halotolerant eubacteria – belonging to a great variety of taxonomic and physiological groups within the eubacterial kingdom. Metabolic diversity in the salt-tolerant eubacteria is much greater than in the archaeobacteria. In contrast to the halophilic archaeobacteria, which maintain high intracellular salt concentrations, their intracellular salt concentration is low. Halophilic aerobic eubacteria maintain an osmotic balance of their cytoplasm with the external medium by accumulating high concentrations of variety of organic osmotic solutes (Trüper et al. 1991).

Though many known halotolerant eubacteria are able to grow in the laboratory at salt concentrations comparable to those preferred or tolerated by the halophilic archaeobacteria, and can be isolated from sites dominated by halophilic archaeobacteria, experiments have shown that hypersaline water bodies with salt concentrations exceeding 25% are dominated by archaeobacteria. On the basis of experiments with specific inhibitors (bile salts, causing lysis of non-coccoid halophilic archaeobacteria, and antibiotics selectively inhibiting protein synthesis in either group) it was concluded that in saltern ponds with salinities below 250 g dissolved salts per liter all heterotrophic activity can be attributed to eubacteria, while at higher salinities the archaeobacteria take over (Oren 1990b; Oren 1990c; Oren 1991a). Similarly, in the Dead Sea (total dissolved salts about 340 g per liter) specific inhibitors of archaeobacterial activity abolished all heterotrophic activity (Oren 1990c; Oren 1991a).

Relatively little is known about anaerobic degradation processes at high salt concentrations (Oren 1988; Oren 1990a). Only during the past ten years have investigators isolated fermentative bacteria which are both obligately anaerobic and halophilic from environments such as the bottom sediments of the Dead Sea and the Great Salt Lake. A new family (the Haloanaerobiaceae) was created to accomodate the three new genera described (*Haloanaerobium, Halobacteroides,* and *Sporohalobacter*) (Oren 1991b). *Haloanaerobium praevalens,* an organism present in high numbers in sediments of the Great Salt Lake, is able to grow at salt concentrations from 3 to as high as 25%, with an optimum at 13%; *Halobacteroides* and *Sporohalobacter* species described to date grow in a more restricted range of salt concentrations of 5 to 22% (Oren 1991b). All of these species ferment simple substrates (sugars, certain amino acids) to products including acetate, butyrate, hydrogen and carbon dioxide. To what extent representatives of the Haloanaerobiaceae are widespread and contribute to anaerobic degradation processes at high salt concentrations is unknown.

The anaerobic halophilic eubacteria of the fam-

ily Haloanaerobiaceae present an exception to the general finding that halophilic or halotolerant eubacteria have low intracellular salt concentrations, and balance their intracellular environment by accumulating high concentrations of organic osmotic solutes. In the Haloanaerobiaceae no organic osmotic solutes could be detected, and intracellular salt concentrations were reported to be high (Oren 1986; Rengpipat et al. 1988). This means that intracellular enzymes should be adapted to the presence of high salt concentrations, and this may, as discussed above, restrict the metabolic potential of such organisms.

Further metabolism of the primary fermentation products (acetate, hydrogen, and others) at high salt concentrations is also limited. The upper limit of salinities at which dissimilatory sulfate reduction has been established to occur is about 20%. Methanogenesis does occur at higher salinities, but is based on the transformation of a few specialized substrates only (notably methylated amines) (Oren 1990d). No halophilic or halotolerant methanogenic bacteria are known that derive their energy from the acetate or from hydrogen + carbon dioxide, the main substrates for methanogenesis in freshwater environments.

3. Microbial degradation or organic pollutants at high salt concentrations – a literature survey

3.1. Degradation under aerobic conditions

3.1.1. Processes performed by archaeobacteria

Little is known to what extent the assumption of the apparently limited metabolic potential of the halophilic archaeobacteria is due to the restricted range of compounds that have been tested as potential substrates for members of the *Halobacterium – Haloferax – Haloarcula* group. Only recently some indications were obtained that the halophilic archaeobacteria may have a greater potential in the degradation of pollutants than previously assumed. Using enrichment cultures with the saturated C-20 hydrocarbon eicosane as single carbon and energy source, a number of red extremely halophilic archaeobacterial strains able to degrade eicosane were isolated from a salt marsh in the south of France with a salinity of 310 g per liter (Bertrand et al. 1990). One of these isolates (designated strain EH4) was found to be able to degrade a range of different hydrocarbons, both even and odd carbon number saturated hydrocarbons (tetradecane, hexadecane, eicosane, heneicosane), saturated isoprenoid alkanes (pristane) and aromatic hydrocarbons (acenaphtene, phenantrene, anthracene and 9-methyl anthracene). After 30 days of incubation at 32 °C with 0.5 g per liter of hydrocarbon, and at a salinity of 310 g per liter, between 48 and 88% of the added amount of straight-chain hydrocarbons was degraded. Aromatic hydrocarbons were biodegraded to a lesser extent, but nevertheless significantly (between 19 and 24%). Palmitic acid was also degraded by the strain. NaCl concentrations of at least 2.5–3 M were required for growth and eicosane degradation. No information is available as yet on the geographic distribution of these or similar strains in hypersaline environments. The only field study known to us on the degradation of hydrocarbons at high salt concentrations was performed in the Great Salt Lake, Utah (Ward & Brock 1978). Enrichment cultures with mineral oil as substrate yielded growth at salinities of up to 17.2%, while no growth was obtained above 20% salt. No measurable amounts of $^{14}CO_2$ were evolved during 5 days incubation of brine samples of salinities of 20.4% and higher supplemented with [1-^{14}C]hexadecane – though upon longer incubation some signs of degradation were observed. The published information does not identify the bacteria involved. However, the relatively low salinities at which the process did occur suggest that eubacteria rather than archaeobacteria were primarily involved. Marine eubacteria degrading petroleum components show an optimum salinity of 0.4 M (close to that of seawater), but significant degradation was observed at 2 M NaCl; the inhibitory effect of salinity was larger for the biodegradation of aromatic and polar fractions than of the saturated fraction (Mille et al. 1991).

In view of the finding of the ability of hydrocarbon degradation in certain halophilic archaeobacteria (Bertrand et al. 1990) a reexamination of the metabolic potential of existing and newly isolated

strains of the *Halobacterium – Haloferax – Haloarcula* group may be worthwile, as the group may yield additional surprises.

3.1.2. Processes performed by eubacteria

The halophilic or halotolerant aerobic eubacteria are more promising than the halophilic archaeobacteria as a source of organisms degrading organic pollutants. First, eubacteria in general have a much greater metabolic versatility than the archaeobacteria. Eubacterial genera such as *Pseudomonas* are known for their wide range of substances they degrade, including toxic organic compounds. Second, halophilic eubacteria may be more useful than halophilic archaeobacteria in the degradation of unusual and toxic compounds because of their mode of haloadaptation. In contrast to the halophilic archaeobacteria, which maintain high intracellular salt concentrations, their intracellular salt concentration is low. Halophilic aerobic eubacteria maintain an osmotic balace of their cytoplasm with the external medium by accumulating high concentrations of a variety of organic osmotic solutes (Trüper et al. 1991). Thus, the intracellular enzymes responsible for the degradation processes may be 'conventional' enzymes of the types encountered in non-halophilic bacteria, and there is no need for special salt-tolerant (or even salt-requiring) enzymes such as found in the archaeobacterial halophiles.

In view of the above it is surprising that no systematic study of the potential of halophilic or halotolerant eubacteria to degrade unusual compounds has ever been done. Studies in which halophilic eubacteria were characterized, especially by means of numerical taxonomy, which involves the examination of a large number of properties, hardly ever mention the ability or lack of ability to degrade aromatic compounds or other unusual and/or toxic chemicals. The only substrate that was regularly tested in the course of studies published on the characterization of halophilic or halotolerant eubacteria by numerical taxonomy is benzoate. Only a small percentage of the isolates examined were found to grow on benzoate (Del Moral et al. 1988; Garcia et al. 1987; Ventosa et al. 1982). In addition, out of the 58 strains examined, one single strain of *Vibrio costicola,* growing at salt concentrations between 0.5 and 20%, was reported to degrade quinate (1,3,4,5-tetrahydrocyclohexanecarboxylic acid) (Garcia et al. 1987). Degradation of benzoate was investigated in somewhat more detail in *Pseudomonas halodurans,* an isolate growing at salt concentrations between 1.8 and 15.5% (or maybe higher – it was stated that 22% NaCl, and 'NaCl concentrations greater than 10-fold that of seawater' are tolerated). The stain was found to cleave aromatic rings by *ortho* cleavage (Rosenberg 1983).

The range of substrates that can be transformed and/or degraded at high salt concentrations by aerobic halophilic or halotolerant eubacteria may be much greater than commonly assumed, and may include certain pollutants, even those considered to be among the more recalcitrant compounds. This was illustrated by the recent isolation of a halotolerant eubacterium degrading organophosphorus compounds (DeFrank & Cheng 1991). The isolate – designated JD6.5 – is a moderately halophilic eubacterium, and was isolated from Grantsville Warm Springs, just south of the Great Salt Lake, Utah, a spring with a salt content of 24%, a temperature of 24° C, and a pH of 6.0. No data were published on the range of salt concentrations tolerated by strain JD6.5, but it was described as being obligatory halophilic, requiring at least 2% NaCl for growth. It was routinely grown in a medium containing 5% NaCl and 1% $MgSO_4 \cdot 7H_2O$ as major salts. The strain is a Gram-negative short rod, and was tentatively identified as an *Alteromonas* sp. The organism was found to possess high levels of enzymatic activity against several highly toxic organophosphorus pesticides and chemical warfare agents, acting as cholinesterase inhibitors. Compounds degraded by strain JD6.5 include DFP (diisopropylfluorophosphate), NPMPP (*p*-nitrophenylmethyl-(phenyl)phosphinate), MPEPP (*p*-nitrophenylethyl(phenyl)phosphinate), and Paraoxon (diethyl *p*-nitrophenyl phosphate). The predominant enzyme responsible for the degrading activity (designated organophosphorus acid anhydrase 2) was purified to homogeneity and characterized. In assays using diisopropylfluorophosphate as substrate optimal activity was obtained at

a pH of 8.5 at 50 °C. Assays were performed in the presence of 0.5 M NaCl, but no information was given on the salt requirement or tolerance of the enzyme. The natural substrate and function of organophosphorus acid anhydrases is not known, and at present it is impossible to speculate what role it plays in the normal metabolism of strain JD6.5.

3.2. Degradation under anaerobic conditions

To a certain extent it can be expected that those compounds that can be degraded aerobically by halophilic aerobic archaeobacteria can also be oxidized anaerobically using alternative electron acceptors. Many halophilic archaeobacteria are denitrifiers (Mancinelli & Hochstein 1986), and some can also use other terminal electron acceptors, such as dimethylsulfoxide or trimethylamine N-oxide (Oren & Trüper 1990). Possible exceptions are those cases in which molecular oxygen is required in the biochemical reactions involved in the transformation process, as can for example be expected to be the case in the degradation of hydrocarbons by certain representatives of the extremely halophilic archaeobacteria (Bertrand et al. 1990). As discussed above, the range of compounds known to be degraded by this group is very limited anyhow, and therefore its potential use in degradation of pollutants at high salt concentrations under anaerobic conditions is doubtful.

Few attempts have been made to exploit the properties of anaerobic halophilic bacteria in the biotransformation of unusual chemicals. One preliminary study dealt with the possibility of anaerobic biotransformation of chemically pretreated lignitic material with the production of methane (Oren 1990e). This study was initiated in view of the development of biological gasification processes for the utilization of Texas lignite after chemical pretreatment (alkaline hydrolysis, leading to breakdown to simple water-soluble aromatic compounds). One of the methods proposed for the biogasification of the pretreated lignite makes use of underground salt caverns in Texas as cheaply available bioreactors. In the course of time the salt concentration in these reactors is expected to increase as a result of dissolution of salt from the underground salt domes. Therefore a study was made on the feasibility of methanogenic biodegradation of the complex mixture of chemicals obtained at high salt concentrations (Oren 1990e; Wise 1987). It was shown that chemically pretreated lignite, inoculated with anaerobic hypersaline sediment samples as a source of bacteria, can give rise to a limited amount of methane and hydrogen evolution in an anaerobic fermentation at high salt concentrations. The optimal salt concentration for the process was found to be between 8 and 12%, and above 16% salt the reaction rates were negligible. The optimal temperature was between 35 and 42 °C. Methane yields were low – between 0.006 and 0.01 ml of methane per ml of lignite preparation containing 2.4 g total solids per liter. No methane could be shown to be formed from hydrogen + carbon dioxide or acetate at these salt concentrations. It was postulated that the methane evolved was derived from $-O\text{-}CH_3$ groups that are abundant in the alkaline hydrolysis products formed during the lignite pretreatment. The nature of the microorganisms responsible for the formation of the methanogenic precursor (probably methanol) was not clarified.

The possible occurrence of reductive dechlorination of chlorophenols and chlorophenoxyphenols under anaerobic hypersaline conditions was reported in hypersaline groundwaters near Alkali Lake, a hypersaline alkaline lake in the desert of south central Oregon, U.S.A. (Boone et al. 1989). No clear data were given with regards to the actual salinity of the groundwater; it was reported that the lake has a salinity of 90 to 120 gram per liter total dissolved salts, but this value seems much too low in view of the statement that 'The lakes and groundwater contain large amounts of dissolved cations sodium (5.1 M) and potassium (0.23 M), with carbonate and bicarbonate (1.6 M, total concentration), chloride (1.3 M), and sulphate (0.48 M) being the major anions'. Between 1969 and 1971 more than 25,000 55-gallon drums containing chlorophenols, chlorophenoxyphenols, and other herbicide manufacturing wastes were deposited in the valley near the lake. In 1976, the drums were placed in shallow trenches, crushed, and covered with gravel. The

contents contaminated the groundwater. In a study performed in 1982 chlorophenols (2,4-dichlorophenol and 2,4,6-trichlorophenol) and chlorophenoxyphenols were readily detectable in the upper, oxic groundwater at a level concentration about 20 mg/kg of soil, but in the anoxic zone the concentration of the compounds dropped more rapidly than their diffusion and dispersion would predict. All data obtained led to the conclusion that the chlorinated compounds are probably biologically degraded in the anoxic subsurface. The partial pressure of hydrogen in samples from this anoxic zone was found to be high, and this was suggested to be related to the rapid reductive transformation of chlorinated phenolic compounds (Boone et al. 1989). No information was obtained on the nature of the microorganism(s) involved in the process.

4. Aerobic transformation of formaldehyde at high salt concentrations by a halotolerant eubacterium

Formaldehyde is a carbonyl compound with a high polarity and a very high reactivity. As a nucleophilic agent it reacts with amines (it readily combines with amino groups in proteins), amides, sulfides, purines, and a variety of other compounds, with the formation of C- or N-methyloyl groups and methylene bridges. Formaldehyde is widely used in medicine, agriculture, and industry. Among its applications we may mention its use as a high effective disinfectant for the killing of bacteria, fungi and viruses, as a fixing fluid and preservative in histological technique, and its use in the transformation of toxins to toxoids. Formaldehyde is known to have mutagenic activity to both eukaryotic and prokaryotic organisms.

In spite of its toxicity at higher concentrations, formaldehyde is a common intermediate in metabolic pathways of bacteria. Table 2 gives a – not necessarily exhaustive – survey of some biochemical reactions by which formaldehyde may enter the carbon and/or energy metabolism of bacteria. Certain reactions are limited to specialized groups of bacteria. For example, formaldehyde can be used as sole carbon and energy source in methylotrophic bacteria – a small group of organisms that can grow on methane, methanol and other one-carbon compounds. In these organisms formaldehyde is an intermediate in the oxidation of methane or methanol to carbon dioxide (Attwood & Quayle 1984). To our knowledge no halophilic or halotolerant representatives of this group have been described.

Many bacteria which do not use formaldehyde as sole carbon and energy source do incorporate and/or transform formaldehyde. Possible mechanisms may either be based on reaction with the coenzyme tetrahydrofolate to yield methylene-tetrahydrofolate, or may involve oxidation to formate by the enzyme formaldehyde dehydrogenase (Attwood & Quayle 1984). The latter reaction may serve as a detoxification mechanism, enabling bacteria to tol-

Table 2. Some pathways for the microbial utilization of formaldehyde.

Pathway	Organisms performing the reaction
A. Assimilatory pathways	
Ribulose monophosphate pathway	Methylotrophic bacteria
Reaction with xylulose 5-phosphate	Methylotrophic bacteria
Serine pathway	Methylotrophic bacteria
Formation of methylene-tetrahydrofolate	Probably widespread
B. Dissimilatory pathways	
Oxidation to CO_2	Methylotrophic bacteria
Oxidation to formate by formaldehyde dehydrogenase	Possibly widespread
Reduction or disproportionation, via methylene-tetrahydromethanopterin	Methanogenic bacteria

Information given in this table was derived from Attwood & Quayle (1984), Escalante-Semerena et al. (1984), and Kaulfers & Marquardt (1991).

erate high formaldehyde concentrations. This phenomenon has been investigated in some depth in clinical isolates of formaldehyde-resistant members of the Enterobacteriaceae (Kaulfers & Marquardt 1991). Resistant strains of *Escherichia coli* and *Serratia marcescens* can be grown in media containing 200 ppm formaldehyde and higher, one strain even up to 700 ppm (Kaulfers & Brandt 1987; Kaulfers & Laufs 1985). In these strains formaldehyde resistance appeared to be plasmid-bound, as shown by conjugation, transformation, and plasmid-curing experiments, and the property can be transferred from *Serratia* to *E. coli*. A 62 MDa conjugative plasmid determining formaldehyde resistance was isolated from formaldehyde-resistant *E. coli* strains. Resistant strains could be cured of the plasmid by treatment with acridine orange, and as a result they became formaldehyde-sensitive (Kaulfers & Brandt 1987). Clinical formaldehyde-resistant isolates of different Enterobacteriaceae were shown to possess a NAD- and glutathione-dependent formaldehyde dehydrogenase activity, which was absent in formaldehyde-sensitive strains.

In the course of a search for formaldehyde-transforming halophilic or halotolerant bacteria soil was collected at a storage site for formaldehyde near a chemical plant that uses formaldehyde in the production of glue (S. Sarig, Bar-Ilan University, Ramat-Gan, pers. comm.). Soil samples were used to inoculate enrichment cultures containing a mineral medium with 10% NaCl, other salts, and supplemented with 100 ppm glycine betaine and 20 or 50 ppm formaldehyde. After incubation for 4 weeks at 30° C in a shaker bacterial growth was obtained. Upon repeated transfers in the same medium, in all cases accompanied by disappearance of the added 20 or 50 ppm formaldehyde within 3 days, a consortium of bacteria was obtained that could be grown in media containing 100 ppm glycine betaine and 100 ppm formaldehyde. This consortium was found to consist of at least four different bacteria, which were obtained in pure cultures. The isolate (designated MA-C) that proved to be the most formaldehyde-resistant (growing well in media supplemented with 100 ppm formaldehyde), and the most active in the transformation of formaldehyde, was selected for further study.

Isolate MA-C is an as yet unidentified rod-shaped Gram-negative, motile eubacterium. It is highly halotolerant, and grows well at NaCl concentrations from 0 to 20% and possibly higher. Mineral media with single carbon sources (several sugars, acetate, succinate, citrate, glutamate) support excellent growth. Under anaerobic conditions acid is produced from a variety of sugars. A complete account on the strain and its formaldehyde metabolism will be published elsewhere; the relevant results are summarized below.

We performed assays for formaldehyde dehydrogenase in cell-free extracts of strain MA-C, using the procedure described by Kaulfers & Marquardt (1991). A high NAD- and glutathione-dependent activity was measured. NAD could not be replaced by NADP, and no activity could be measured in the absence of glutathione. The enzyme proved constitutive, as was the case in the Enterobacteriaceae (Kaulfers & Marquardt 1991), but activities measured were somewhat higher in cells previously exposed to 20 or 50 ppm formaldehyde, compared with cells that had not been in contact with formaldehyde for many subsequent transfers. Activities measured were between 650 and 850 nmol NAD reduced/min·mg protein at room temperature (in cells grown in the presence of 20 ppm formaldehyde, either in medium containing 0.5% yeast extract, 0.5% tryptone, or in medium containing 0.5% Na-succinate and 0.05% yeast extract, in addition to 10% NaCl, other salts). In cell extracts prepared from cells grown in similar media, but in the absence of formaldehyde activities were between 280 and 480 nmol/min·mg protein. In formaldehyde-resistant Enterobacteriaceae, rates between 14 and 25 units/mg protein were reported (Kaulfers & Marquardt 1991); as no definition of the units used was given, comparison with our results is not possible. The formaldehyde dehydrogenase activity of cell-free extracts of strain MA-C is sensitive to salt: when 2.5, 5.0 or 7.5% NaCl were present in the assay mixture, the rates obtained were respectively 63, 41, and 31% of the control without salt. Being a eubacterium,

strain MA-C probably has a low intracellular salt concentration, and it can be expected that the intracellular formaldehyde dehydrogenase is not exposed to high salt concentrations such as found in the outside medium. It remains to be determined whether in strain MA-C the formaldehyde dehydrogenase activity is plasmid-coded, or even possibly due to a plasmid identical or similar to those responsible for formaldehyde resistance in *E. coli* or *Serratia* (Kaulfers & Brandt 1987; Kaulfers & Laufs 1985).

To examine the fate of the formaldehyde metabolized by strain MA-C, we used radioactively labeled formaldehyde as a tracer, and tested whether label from formaldehyde is incorporated into the cells, oxidized to CO_2, or to non-volatile dissolved products. ^{14}C-labeled formaldehyde was added to cultures in the presence of 25 ppm unlabeled formaldehyde, and the amount of label incorporated into the cells, and that remaining in the culture supernatant before and after acidification, was determined. No significant incorporation of radioactivity into the cells was observed, and the radioactivity of the culture supernatant slowly declined (within days), probably as formaldehyde is transformed to volatile products.

To assay for the accumulation of formate in the supernatant of formaldehyde-metabolizing culturres of strain MA-C we used an enzymatic assay for formate, based on the reduction of NAD by formate dehydrogenase from *Pseudomonas oxalaticus*. No significant amounts of formate could be demonstrated in the supernatant fluid of cultures fed with formaldehyde. Formate, added to cultures at concentrations of 3.7 or 7.4 mM (250 or 500 mg/l) sodium formate) disappeared from the medium within one day, while no significant change in formate concentration was observed in sterile control experiments, showing that strain MA-C is able to further metabolize formate. Experiments with ^{14}C-labeled formate showed that only a minor part of the added formate (less than 5%) was incorporated by the cells. A NAD-dependent formate dehydrogenase activity was detected in cell extracts of cells grown in the presence of 7.4 mM formate (activity between 14 and 34 nmol NAD reduced/mg protein·min at 45 °C). In extracts of cells not exposed to formate, the activity was only 10–25% of that of formate-grown cells. NAD could not be replaced by NADP. The optimum temperature of the formate dehydrogenase was 45 °C, and the activity of the enzyme was greatly inhibited by NaCl.

5. Anaerobic transformation of nitroaromatic compounds by obligately anaerobic halophilic bacteria

Nitrosubstituted aromatic compounds such as nitrobenzene and nitrophenols are widely used in the manufacture of azo dyes, explosives, pharmaceuticals, and especially pesticides: insecticides (e.g. parathion), herbicides, and fungicides. During chemical or biological breakdown of these pesticides in soil and water nitroaromatic compounds are released into the environment.

Though nitrophenols and other nitrosubstituted aromatic compounds are generally considered to be highly resistant to microbial degradation, a number of aerobic bacteria have been shown to degrade nitrophenols. In this process the nitro group is released as nitrite, followed by oxidation of the aromatic ring. Also under anaerobic conditions degradation of nitroaromatic compounds occurs, and in this case the initial step in the process is probably a reduction of the nitro group to an amino group. A number of bacteria have been shown to reduce nitrosubstituted aromatic compounds to their corresponding amino derivatives. These include obligately anaerobic *Clostridium* and *Eubacterium* species isolated from the human intestinal tract (Rafii et al. 1991), and unidentified bacteria present in the rumen of sheep (Gurevich et al. 1993). A few reports have been published on anaerobic bacterial consortia degrading nitrophenols to methane (Berry et al. 1987; Boyd et al. 1983). During methanogenic fermentation of nitrophenols in sewage sludge, the nitro group was again presumed to undergo reduction to form a substituent amino group. The resulting intermediate aminophenol subsequently underwent further mineralization (Boyd et al. 1983).

In the course of our studies on the metabolic abilities of obligately anaerobic halophilic eubacteria belonging to the family Haloanaerobiaceae (Oren 1991b) it was found that two species tested, *Haloanaerobium praevalens* (isolated from the Great Salt Lake), and *Sporohalobacter marismortui* (isolated from sediments on the shore of the Dead Sea) rapidly reduced *p*-nitrophenol to *p*-aminophenol (Oren et al. 1991). The experiments were performed in the presence of 13–14% NaCl (13.5–15.8 g/liter total dissolved salts). Other nitrosubstituted aromatic compounds transformed by both strains included nitrobenzene, *o*-nitrophenol, *m*-nitrophenol, *o*-nitroaniline, *m*-nitroaniline, *p*-nitroaniline, 2,4-dinitrophenol, and 2,4-dinitroaniline. Most of these compounds, when added to growing cultures at concentrations of 50 to 100 ppm, were completely transformed within 24 h, but at the highest concentrations growth rates were somewhat lowered.

The transformation of *p*-nitrophenol by *H. praevalens* was investigated in more detail. Concentrations of up to 125 ppm were tolerated, but at concentrations above 25 ppm growth was slowed down significantly. However, at all concentrations tested, the *p*-nitrophenol added had disappeared completely within 24 h, and was quantitatively converted to *p*-aminophenol. No degradation of aromatic amines could be demonstrated by the organisms tested, and it was concluded that the only transformation that took place was a reduction of the nitro groups to amino groups. Nitrite was not detected in *p*-nitrophenol-transforming cultures. Incubation of growing cultures of *H. praevalens* in the presence of ^{14}C-labeled *p*-nitrophenol showed that the compound was neither incorporated by the cells, nor degraded to acid-volatile compounds.

The obligately anaerobic bacteria of the family Haloanaerobiaceae were not previously known for their metabolic versatility, and the range of substrates used was reported to be restricted to simple sugars and amino acids (Oren 1988; Oren 1990a; Oren 1991b). It is not clear what enzymatic activity is involved in the reduction of nitroaromatic compounds in these organisms. In fact, the nature of similar activities in non-halophilic anaerobic microorganisms is still to be determined. It is highly improbable that the halophilic strains examined have ever been exposed to significant concentrations of nitrophenols and other nitroaromatic compounds in their natural habitat (the Great Salt Lake, the Dead Sea), and therefore the function of the activity remains enigmatic. It may be imagined that the reaction serves for the detoxification of toxic nitro compounds. Another possibility is that the nitro groups are used as an electron sink during fermentation processes, allowing the formation of more oxidized end products and a higher energy yield. No microorganisms are yet known able to further transform aminophenols under either aerobic or anaerobic conditions at high salt concentrations. In this context it may be stated that our knowledge on microbial transformations of aminophenols in freshwater environments is likewise extremely limited.

The discovery of anaerobic reduction of nitroaromatic compounds by members of the Haloanaerobiaceae indicates that the variety of organic compounds that can be transformed and/or degraded in hypersaline environments may be much greater than previously assumed. The results also suggest that anaerobic halophilic bacteria show a certain promise in biotechnological processes, and practical use may be found for their newly discovered properties.

6. Conclusions

The above survey shows that our knowledge on the degradation of pollutants at high salt concentrations is still extremely limited. This may simply be due to the fact that the potential of halophilic bacteria for biodegradation of toxic compounds has never been systematically investigated. Our lack of knowledge by no means implies that halophilic bacteria do not have a potential to degrade toxic compounds. The opposite may be true – a world of unusual microorganisms is waiting to be tested for useful properties. The finding of features such as hydrocarbon degradation in certain halophilic archaeobacteria (Bertrand et al. 1990), degradation of organophosphorus compounds (DeFrank & Cheng 1991), transformation of formaldehyde in

halotolerant eubacteria (described here), and reductive transformation of nitroaromatic compounds (Oren et al. 1991) and possibly chlorophenols and chlorophenoxyphenols (Boone et al. 1989) by obligately anaerobic halophilic eubacteria suggests that a reexamination of the metabolic potential of extant and newly isolated strains of halophilic or halotolerant bacteria may yield additional surprises.

Acknowledgements

We thank Shlomo Sarig (Bar-Ilan University, Ramat-Gan, Israel), for the use of unpublished data on the formaldehyde-transforming strain MA-C, and Carol A. Baker (Monsanto Co.) for useful suggestions and thorough reading of the manuscript. Our studies on the transformation of formaldehyde and nitroaromatic compounds are supported by a grant from Monsanto Co., St. Louis, MO.

Note added in proof

The use of hydrocarbons by members of the Halobacteriaceae (Bertrand et al. 1991) was discovered independently by a Russian group (Kulichevskaya IS, Milekhina EI, Borzenkov IA, Zvyagintseva IS & Belyaev SS (1991) Oxidation of petroleum hydrocarbons by extremely halophilic archaebacteria. Mikrobiologiya (English translation) 60: 596–601).

References

Attwood MM & Quayle JR (1984) Formaldehyde as a central intermediary metabolite of methylotrophic metabolism. In: Crawford RL & Hanson RS (Eds) Microbial Growth on C_1 Compounds (pp 315–323). American Society for Microbiology, Washington, DC

Berry DF, Francis AJ & Bollag J-M (1987) Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol. Rev. 51: 43–59

Bertrand JC, Almallah M, Acquaviva M & Mille G (1990) Biodegradation of hydrocarbons by an extremely halophilic archaebacterium. Lett. Appl. Microbiol. 11: 260–263

Boone DR, Johnson RL, Chen DC, Mathrani IM & Mah RA (1989) Methanogenesis and reductive dechlorinations in an alkaline, hypersaline sediments and groundwater. In: Da Costa MS, Duarte JC & Williams RAD (Eds) Microbiology of Extreme Environments and its Potential for Biotechnology (pp 205–215). Elsevier Applied Science, London

Boyd SA, Shelton DR, Berry D & Tiedje JM (1983) Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46: 50–54

DeFrank J & Cheng T-C (1991) Purification and properties of an organophosphorus acid anhydrase from a halophilic bacterium. J. Bacteriol. 173: 1938–1943

Del Moral A, Prado B, Quesada E, Garcia T, Ferrer R & Ramos-Cormenzana A (1988) Numerical taxonomy of moderately halophilic Gram-negative rods from an inland saltern. J. Gen. Microbiol. 134: 733–741

Escalante-Semerena JC, Leigh JA, Rinehart KL, Jr. & Wolfe RS (1984) Formaldehyde activation factor, tetrahydromethanopterin, a coenzyme of methanogenesis. Proc. Natl. Acad. Sci. USA 81: 1976–1980

Garcia MT, Ventosa A, Ruiz-Berraquero F & Kocur M (1987) Taxonomic study and amended description of *Vibrio costicola*. Int. J. Syst. Bacteriol. 37: 352–360.

Gurevich P, Oren A, Sarig S & Henis Y (1993) Reduction of aromatic nitro compounds in anaerobic ecosystems. Water Sci. Technol. (in press)

Kaulfers P-M & Brandt D (1987) Isolation of a conjugative plasmid in *Escherichia coli* determining formaldehyde resistance. FEMS Microbiol. Lett. 43: 161–163

Kaulfers P-M & Laufs R (1985) Übertragbare Formaldehydresistenz bei *Serratia marcescens*. Zbl. Bakt. Hyg., I. Abt. Orig. B 181: 309–319

Kaulfers P-M & Marquardt A (1991) Demonstration of formaldehyde dehydrogenase activity in formaldehyde-resistant Enterobacteriaceae. FEMS Microbiol. Lett. 79: 335–338

Mancinelli S & Hochstein LI (1986) The occurrence of denitrification in extremely halophilic bacteria. FEMS Microbiol. Lett. 35: 55–58

Mille G, Almallah M, Bianchi M, van Wambeke F & Bertrand JC (1991) Effect of salinity on petroleum degradation. Fresenius J. Anal. Chem. 339: 788–791

Oren A (1986) Intracellular salt concentrations of the anaerobic halophilic eubacteria *Haloanaerobium praevalens* and *Halobacteroides halobius*. Can. J. Microbiol. 32: 4–9

— (1988) Anaerobic degradation of organic compounds at high salt concentrations. A. v. Leeuwenhoek 54: 267–277

— (1990a) Anaerobic degradation of organic compounds in hypersaline environments: possibilities and limitations. In: Wise DL (Ed) Bioprocessing and Biotreatment of Coal (pp 155–175). Marcel Dekker, New York

— (1990b) Estimation of the contribution of halobacteria to the bacterial biomass and activity in solar salterns by the use of bile salts. FEMS Microbiol. Ecol. 73: 41–48

— (1990c) The use of protein synthesis inhibitors in the estimation of the contribution of halophilic archaebacteria to bacterial activity in hypersaline environments. FEMS Microbiol. Ecol. 73: 187–192

— (1990d) Formation and breakdown of glycine betaine and

trimethylamine in hypersaline environments. A. v. Leeuwenhoek 58: 291–298

— (1990e) Microbial formation of methane from pretreated lignite at high salt concentrations. In: Wise DL (ed) Bioprocessing and Biotreatment of Coal (pp 449–463). Marcel Dekker, New York

— (1991a) Estimation of the contribution of archaebacteria and eubacteria to the bacterial biomass and activity in hypersaline ecosystems: novel approaches. In: Rodriguez-Valera F (Ed) General and Applied Aspects of Halophilic Microorganisms (pp 25–31). Plenum Press, New York

— (1991b) The genera *Haloanaerobium, Halobacteroides,* and *Sporohalobacter.* In: Balows A, Trüper HG, Dworkin M, Harder W & Schleifer K-H (Eds) The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications 2nd ed, Vol II (pp 1893–1900). Springer-Verlag, New York

Oren A & Trüper HG (1990) Anaerobic growth of halophilic archaeobacteria by reduction of dimethylsulfoxide and trimethylamine *N*-oxide. FEMS Microbiol. Lett. 70: 33–36

Oren A, Gurevich P & Henis Y (1991) Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui.* Appl. Environ. Microbiol. 57: 3367–3370

Rafii F, Franklin W, Heflich RH & Cerniglia CE (1991) Reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. Appl. Environ. Microbiol. 57: 962–968

Rengpipat S, Lowe SE & Zeikus JG (1988) Effect of extreme salt concentrations on the physiology and biochemistry of *Halobacteroides acetoethylicus.* J. Bacteriol. 170: 3065–3071

Rosenberg A (1983) *Pseudomonas halodurans* sp. nov., a halotolerant bacterium. Arch. Microbiol. 136: 117–123

Tindall BJ (1991) The family Halobacteriaceae. In: Balows A, Trüper HG, Dworkin M, Harder W & Schleifer K-H (Eds) The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, 2nd ed, Vol I (pp 768–808). Springer-Verlag, New York

Trüper HG, Severin J, Wohlfahrt A, Müller A & Galinski EA (1991) Halophily, taxonomy, phylogeny and nomenclature. In: Rodriguez-Valera F (Ed) General and Applied Aspects of Halophilic Microorganisms (pp 3–7). Plenum Press, New York

Ventosa A, Quesada E, Rodriguez-Valera F, Ruiz-Berraquero F & Ramos-Cormenzana A (1982) Numerical taxonomy of moderately halophilic gram-negative rods. J. Gen. Microbiol. 128: 1959–1968

Ward DM & Brock TD (1978) Hydrocarbon degradation in hypersaline environments. Appl. Environ. Microbiol. 35: 353–359

Wise DL (1987) Meeting report – first international workshop on biogasification and biorefining of Texas lignite. Res. Conserv. 15: 229–247