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The anaerobic biodegradation of diethanolamine by a nitrate reducing bacterium

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Abstract

The ability of bacterial cultures to degrade diethanolamine under anoxic conditions with nitrate as an electron acceptor was investigated. A mixed culture capable of anaerobic degradation of diethanolamine was obtained from river sediments by enrichment culture. From this a single bacterial strain was isolated which could use diethanolamine, monoethanolamine, triethanolamine and N-methyl diethanolamine as its sole carbon and energy sources either aerobically or anaerobically. Growth on diethanolamine was faster in the absence of oxygen. The accumulation of possible metabolites in the culture medium was determined as was the ability to grow on certain putative intermediates in the degradation of diethanolamine. A possible pathway for the degradation of ethanolamines by this organism is suggested.

Introduction

Although the aerobic biodegradation of a wide variety of aliphatic amines and alkanolamines has been investigated in considerable detail (see Emtiazi & Knapp 1994; Jones & Turner 1973; Large 1971; Rothkopf & Bartha 1984), there have however been few reports of the anaerobic degradation of such compounds. The fermentative breakdown of ethanolamine and choline by a *Clostridium* sp. has been reported (Bradbeer 1965 a,b) and Frings et al. (1994) have described the fermentation of triethanolamine by an anaerobe ascribed to the genus *Acetobacterium*. Other reports on anaerobic degradation of amines relate to the breakdown of the chelating agent nitrilotriacetic acid (NTA) under anoxic conditions which has been described by several authors (e.g. Enfors & Molin 1973 a,b; Egli & Weilenmann 1986). An organism has now been isolated in pure culture which can degrade NTA under denitrifying conditions (Wanner et al. 1990).

Diethanolamine is an industrially important alkanolamine (an estimated 91,000 tons being produced in the USA alone in 1990) used for a wide range of purposes including as an ingredient in cosmetics, soaps, and shampoos and on a larger scale in metal cutting fluids (Anon 1983; Edens & Lochary 1991). There are few reports on the biodegradation of diethanolamine (Emtiazi & Knapp 1994; Gannon et al. 1978; Rothkopf & Bartha 1984; Williams & Callely 1982) and all refer to degradation under aerobic conditions.

We report here for the first time the isolation of a pure bacterial culture which is able to grow on diethanolamine as a sole source of carbon and energy under anaerobic conditions while using nitrate as an electron acceptor.

Materials and methods

Materials

Unless otherwise stated all chemicals were obtained from Merck Ltd. (Poole, Dorset, UK) and were of Analar or the next purest grade available. Diethanolamine and N -methyl diethanolamine were from Aldrich Ltd. (Gillingham, Dorset, UK). Yeast extract and all agar-based microbial growth media were obtained from Oxoid (Basingstoke, Hants., UK).

Anaerobic riverine sediments were obtained from the River Aire in central Leeds.

Analysis

Ammonia was measured directly by Nessler's method using Nessler's reagent (Merck Ltd.). Nitrite was measured by the Griess-Ilosvay method. Nitrate was measured by the brucine sulphate method of Jenkins and Medsker (1964).

Gas chromatography

All the ethanolamines were assayed by gas chromatography using a Pye Unicam 204 chromatograph (Unicam, Cambridge, UK) with a flame ionisation detector. A Tenax (80/100 mesh) column (Jones Chromatography, Hengoed, Mid-Glamorgan, UK) was used with direct injection of aqueous samples; the carrier gas was nitrogen. For analysis of amines the initial temperature was 150 °C, this was held for 2 min. after which it was increased at a rate of 8 °C/min. up to 250 °C. Injector and detector temperatures were 200 and 250 °C, respectively. For detection of neutral volatile compounds and glycolic and glyoxylic acids the initial temperature was 50 °C, this was held for 5 min. after which it was increased at a rate of 8 °C/min. up to 150 °C. Injector and detector temperatures were 100 and 125 °C, respectively.

Growth media

For enrichment cultures in the chemostat a medium was used containing per litre of distilled water : diethanolamine - 5mmol; glucose - 1mmol; KNO_3 - 6mmol; K_2HPO_4 - 4g; KH_2PO_4 - 4g; Yeast extract - 50mg; $MgSO_4.7H_2O$ - 0.04g; $FeSO_4.6H_2O$ - 0.004g. The pH was adjusted to 7.0.

For the isolation of diethanolamine degrading bacteria and their subsequent growth in liquid batch cultures the following medium was used which contained per litre of distilled water : diethanolamine - 6mmol; KNO_3 - 18mmol; K_2HPO_4 - 4g; KH_2PO_4 - 4g; $MgSO_4.7H_2O$ - 0.04g; $FeSO_4.6H_2O$ - 0.004g. The pH was adjusted to 7.0. Where needed this medium was solidified by the addition of Noble agar (Difco, East Molesy, Surrey, UK) at 12 g/l. The ability of the bacterial culture to grow on other substrates was tested using similar media in which diethanolamine was substituted with ethanolamine, triethanolamine or N-methyl diethanolamine at 5mmol/l or formaldehyde, acetaldehyde, glycolaldehyde, glyoxal, glyoxylic acid or glycolic acid at 10mmol/l. In amine-free media $(NH_4)_2SO_4$was added at 1 g/l as a nitrogen source. All media were sterilised by autoclaving at 121 °C for 15 minutes, except for volatile compounds listed above which were sterilised by membrane filtration. Ferrous and magnesium salts and glucose solutions were autoclaved separately as concentrates and added to the rest of the media after sterilisation.

Enrichment and cultivation of microorganisms

For enrichment studies a continuous fermenter was used. The culture vessel was a 5 l stirred tank reactor with an operating volume of 3 l and it was made anaerobic by sparging continuously with oxygen-free nitrogen at a rate of 270 ml per min.. The vessel was inoculated with 500 ml of anaerobic river sediment and then fed continuously with medium at a rate of 71 ml/hour giving a dilution rate of 0.023 hr^{-1}(retention time *ca.* 42 hours.), the incubation temperature was controlled at 27 °C. Samples were removed at regular intervals for analysis. The composition of the microbial population was determined by cultivation of the organisms on nutrient agar or solidified diethanolamine mineral salts medium under anaerobic conditions in an anaerobic jar using triplemix gas (10% H_2, 10%CO_2, 80%N_2). The handling of the cultures was not done under anaerobic conditions and so the isolation of any strict anaerobes was unlikely. Anaerobic cultivation of selected organisms was done either in 100 ml medical flat bottles in an anaerobic jar or in a small anaerobic culture vessel in which 200 ml volumes of culture were continuously sparged with oxygen-free nitrogen (20 ml/min). Aerobic cultivation was carried out in 250 ml Erlenmeyer flasks with 100ml of medium which were shaken on an orbital incubator at 100 r.p.m.. All incubations were at 27 °C.

Results

Initial enrichment studies

About 5 days after inoculation the concentration of diethanolamine (initially 5mmol/l) in the culture began to fall and thereafter decreased steadily over the next 40 days to reach a concentration of about 1.5 mmol/l. For the next 100 days the diethanolamine concentration was maintained in the range 1.5 to 2.5 mmol/l. Monoethanolamine accumulated in the culture at a

concentration of *ca.* 0.5 mmol/l and ammonia was present at *ca.* 1 to 1.75 mmol/l. The nitrate concentration was reduced by *ca.* 95% and nitrite accumulated to *ca.* 1.8 to 2 mmol/l (30 to 33% of the added nitrate). Glucose was always completely removed. pH remained roughly constant at about 7.2. The incomplete utilisation of diethanolamine suggests that the culture is nutrient limited, probably by the concentration of electron acceptor.

Isolation of a diethanolamine degrading bacterium

After 100 days operation the microflora of this anaerobic reactor was investigated. Four strains of bacteria were isolated on nutrient agar all were facultative anaerobes. Of these isolates only one, nominated as strain DEA 4, was able to grow anaerobically using diethanolamine as its sole source of carbon and energy. The other organisms were presumably utilising glucose or yeast extract or diethanolamine-degradation products as their substrates. DEA 4 is a Gram-negative, rod-shaped (0.7 by 1.5 μm), motile bacterium . It is a facultative anaerobe, oxidase and catalase positive and is oxidative in Hugh and Leifson's O/F test. It can reduce nitrate to nitrite and beyond. It is positive for arginine dihydrolase but cannot grow at 42 °C and produces no pigments on King's media A or B. It could not be typed using the API 20NE system.

Growth of strain DEA4 on diethanolamine

Strain DEA4 was able to grow on diethanolamine as its sole source of carbon and energy either aerobically or anaerobically with nitrate as an electron acceptor. It did not grow anaerobically on diethanolamine in the absence of nitrate. Figure 1 (a to d) shows the growth, diethanolamine degradation, nitrate removal and accumulation of metabolites when DEA4 was grown in the same medium either aerobically or anaerobically. Growth occurred at a similar rate under either anaerobic or aerobic conditions with mean generation times of *ca.* 3.8 and 5.5 hours respectively. Growth was exponential for the first 4 hours for anaerobic cultures and the first 7 hours for aerobic cultures. Diethanolamine was degraded to a similar extent (*ca.* 70%) under both conditions. During growth monoethanolamine accumulated transiently in the culture fluid to a concentration of about 0.5 to 0.7 mmol/l (10 to 14% of the added diethanolamine). Degradation of diethanolamine also resulted in the accumulation of ammonia to a concentration of *ca.* 1 to 1.5 mmol/l in the culture fluids. Nitrate was reduced under both anaerobic and aerobic conditions but while in the former case it was completely removed in the later only *ca.* 22% was removed. There was some accumulation of nitrite in both cultures - with maximum concentrations of 1.5 and 0.75 mmol/l in anaerobic and aerobic cultures respectively.

Growth of DEA4 on other amines

Strain DEA4 was also able to grow anaerobically on monoethanolamine, triethanolamine and N-methyl diethanolamine. Growth occurred most rapidly on mono and diethanolamines (mean generation time 3.8 hours) while growth was a little slower on the other amines tested (mean generation time 4.5 hours) and growth on them only proceeded after a short (*ca.* 2 hour) lag period.

Accumulation of possible intermediates during anaerobic incubation of DEA4 on ethanolamines

During growth of DEA4 on N- methyl diethanolamine and triethanolamine transitory accumulation of small amounts of diethanolamine (never more than 0.5mmol/l) was noted. At no time was N-methyl ethanolamine observed in cultures growing on N-methyl diethanolamine.

Low molecular weight volatile intermediates were detected by GC, it was not possible to optimise conditions for all the analytes and the analysis was unfortunately only semi-quantitative, demonstrating the presence, or absence, of a compound and the approximate range of concentration. The results presented in Table 1 clearly show that acetaldehyde accumulated transiently in cultures with all ethanolamines, normally accumulating to a concentration of over 100mg/l (>2.2mmol/l) before decreasing to trace concentrations. Ethanol and acetic acid were also present at low concentrations for all ethanolamines. Formaldehyde, formic acid and methanol were found only during growth on N-methyl diethanolamine. Glycolaldehyde was found in cultures with diethanolamine, N-methyl diethanolamine and triethanolamine, but not monoethanolamine, peak concentrations in excess of 100mg/l being observed. Glycolic acid appeared at some stage for all substrates but glyoxylic acid was not observed.

Anaerobic growth of DEA4 on possible intermediates

The ability of DEA4 to grow on possible intermediates was assessed in static cultures in the anaerobic

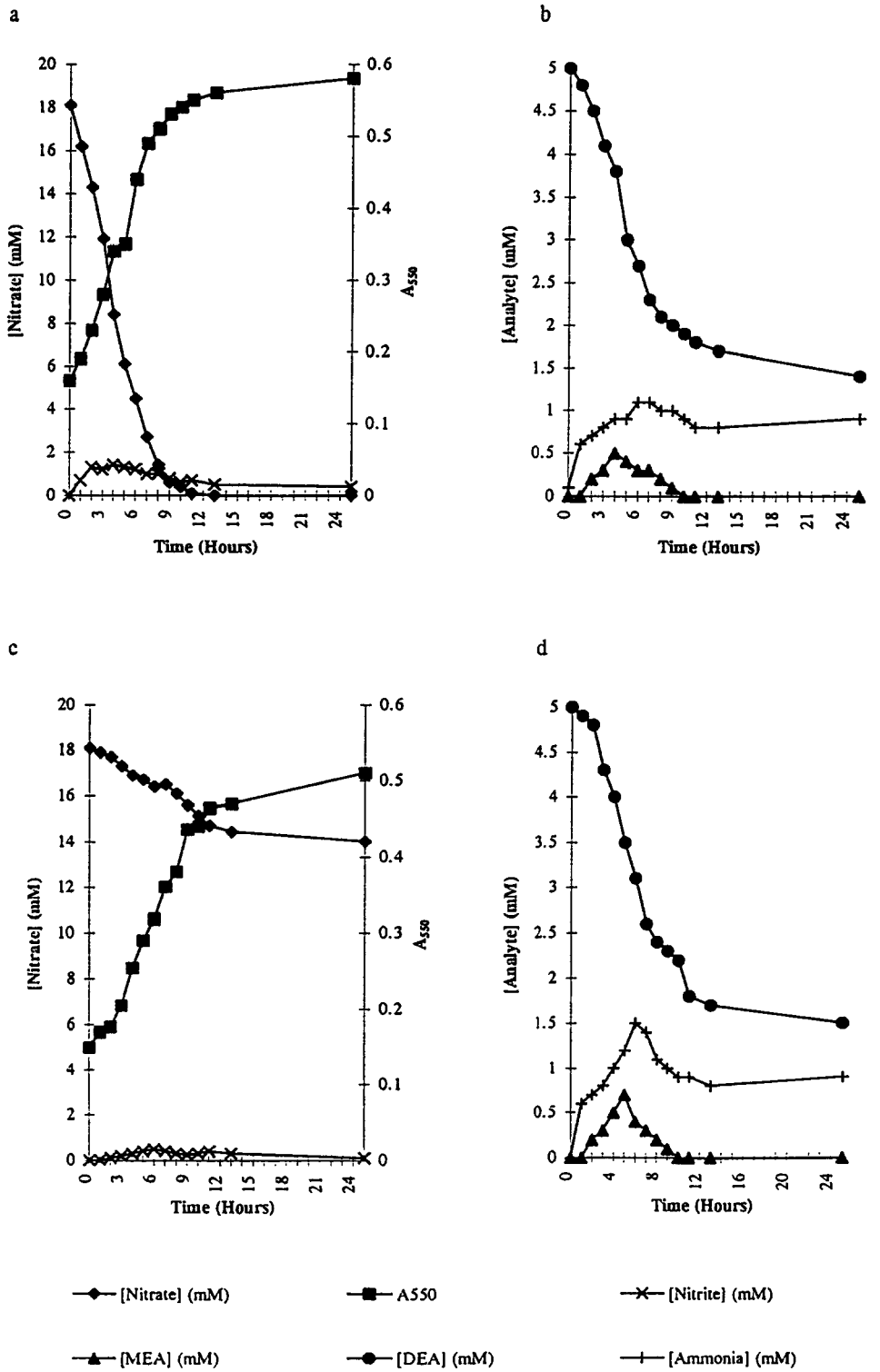

Figure 1. (*a-d*) Growth of DEA4 on diethanolamine mineral salts medium with nitrate, either anaerobically (*a,b*) or aerobically (*c,d*). Graphs a and c show growth and changes in nitrate and nitrate concentration, while b and d show changes in concentrations of diethanolamine, monoethanolamine and ammonia.

Table 1. Production of putative intermediates during the anaerobic growth of strain DEA4 on ethanolamines in the presence of nitrate.

Amine	MEA			DEA			MDEA			TEA		
Time - days	3	7	14	3	7	14	3	7	14	3	7	14
Putative intermediate	Concentration of intermediate - mg/l											
Formaldehyde	-	-	-	-	-	-	10–50	50–100	10–50	-	-	-
Methanol	-	-	-	-	-	-	1–10	1– 10	1–10	-	-	-
Formic acid	-	-	-	-	-	-	1–10	10– 50	1–10	-	-	-
Acetaldehyde	>100	1–10	1–10	10–50	50 -100	1–10	50 -100	>100	1–10	10–50	>100	1–10
Ethanol	1–10		1–10	1–10	1– 10	1–10	1– 10	1– 10	10–50	1–10	1–10	1–10
Acetic acid	-	1–10	1–10	1–10	1– 10	10–50	-	1– 10	10–50	-	1–10	1–10
Glycolaldehyde	-	-	-	1–10	50 -100	10–50	10– 50	>100	1–10	50 -100	>100	1–10
Glyoxal	-	-	1–10	-	-	1–10	-	-	-	-	-	-
Glycolic acid	1–10	-	-	1–10	10– 50	-	-	1– 10	1–10	1–10	-	-
Glyoxylic acid	-	-	-	-	-	-	-	-	-	-	-	-

DEA was present in the medium at 5 mmol/l.
The presence of the putative intermediates was determined by Gas chromatography, the estimation of concentrations was only semi-quantitative, concentrations are therefore given as ranges.
- means not detectable.

jar. Acetaldehyde, glycolaldehyde, glyoxal, glyoxylic acid and glycolic acid all served as sole sources of carbon and energy for anaerobic growth in the presence of nitrate. Growth did not however occur on formaldehyde. This failure is unlikely to be due to toxicity of formaldehyde as it was shown that at 10mmol/l, formaldehyde only caused a moderate (40%) decrease in the rate of growth of DEA4 on glucose mineral salts medium. Under the conditions pertaining, growth was most rapid on acetaldehyde and glycolaldehyde (data not shown).

Discussion

This study has shown for the first time that diethanolamine can be degraded without the involvement of molecular oxygen and confirms the finding of Wanner et al. (1990) and others that amines can be degraded anaerobically under nitrate reducing conditions. The observation that growth of DEA4 is more rapid under anaerobic conditions than aerobic is unexpected, but might be due to inhibition by oxygen or oxygen metabolites in the aerobic cultures. The limited nitrate reduction observed under aerobic conditions is not however surprising in view of recent observations (Lloyd et al. 1987; Patureau et al. 1994; Robertson & Kuenen 1984) that dissimilatory nitrate reduction by bacteria does always not require totally anoxic conditions.

From the data presented it is clear that ethanolamine is an intermediate in diethanolamine degradation and diethanolamine is an intermediate in degradation of the tertiary amines. The accumulation of acetaldehyde and glycolaldehyde by DEA4 and the ability of the organism to utilise them suggests that acetaldehyde is an intermediate for all ethanolamines, and glycolaldehyde an intermediate for the secondary and tertiary ethanolamines. For N-methyl diethanolamine it appears that the methyl group is converted to formaldehyde and this is oxidised to formic acid, presumably allowing some energy generation by electron transport. DEA4 does not appear able to grow on formaldehyde anaerobically. The fact that N-methyl ethanolamine was not observed suggests (but does not proove) that the first step in N-methyl diethanolamine degradation is demethylation to yield diethanolamine rather than removal of a hydroxyethyl group. Small amounts of ethanol and acetic acid were found in all cultures and methanol in the N-methyl diethanolamine culture. It is likely that the methanol and ethanol are side-products derived from reduction of the corresponding aldehydes and it is not clear whether they are later oxidised by the bacterium. Acetaldehyde is probably oxidised to acetic acid which will then enter the tricarboxylic acid cycle.

Acetaldehyde has been demonstrated to be an intermediate in the degradation of monoethanolamine by a range of bacteria (e.g. Bradbeer 1965, a,b; Jones & Turner 1973; Scarlett & Turner 1976) and also of

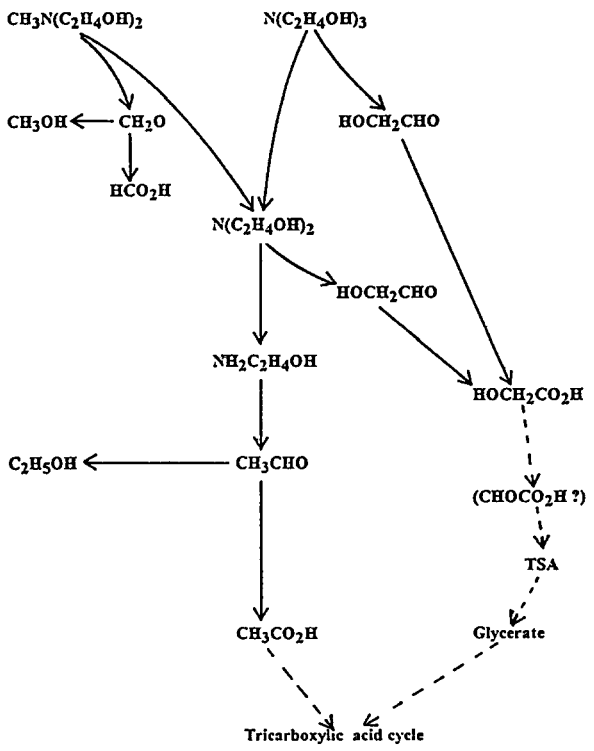

Figure 2. Proposed pathway for the anaerobic metabolism of ethanolamines by strain DEA4 grown in the presence of nitrate. TSA is tartronic semialdehyde. Solid lines represent likely transformations. Dashed lines represent possible transformations for which no evidence exists.

diethanolamine (Williams & Callely 1982). The mechanism employed for the deamination of ethanolamine in DEA4 is not clear - two mechanisms have been previously reported. One proceeds via conversion to ethanolamine -O -phosphate which is then deaminated by a phospho-lyase (e.g. Jones & Turner 1971; Jones & Turner 1973; Swain et al. 1991; Williams & Callely 1982), while the other involves a direct deamination by a cobamide- or coenzyme B_{12}-dependant ethanolamine-ammonia lyase (e.g. Bradbeer 1965b; Chang & Chang 1975; Scarlett & Turner 1976).

While glycolaldehyde is certainly an intermediate, we cannot yet be sure how it is further metabolised. The presence of small concentrations of glycolic acid suggest that it may be involved. Glyoxal was also found but the evidence for its direct involvement is less strong, although it too can act as a growth substrate. DEA4 can grow on glyoxylate but this compound was not detected, it is however a probable intermediate in the metabolism of glycolic acid. Williams and Callely (1982) propose that glycolaldehyde, derived from diethanolamine, is degraded via glycolic acid and glyoxylic acids and thence via tartronic semialdehyde and glycerate to yield pyruvate (the 'glycerate pathway' - Kornberg 1966). A similar pathway is proposed for glycolate degradation in a *Mycobacterium* by Swain et al. (1991). A possible pathway for the degradation of ethanolamines by DEA4 is given in Figure 2.

Overall it appears that anaerobic diethanolamine degradation by DEA4 may occur by a similar route to that reported for an aerobic culture by Williams and Callely (1982). It is not clear however if the enzymes employed will be the same. In particular it would be of interest to determine the nature of the enzymes cleaving the alkyl-N bonds. In aerobic bacteria such reactions are often catalysed by mono-oxygenases (e.g. Cripps & Noble 1973; Uetz et al. 1992), however these enzymes utilise molecular oxygen and therefore will not be involved in this anaerobic process. It is likely that the alkyl-N bonds are cleaved by a dehydrogenase enzyme of the type reported by Jenal-Wanner and Egli (1993) to be involved in the anaerobic degradation of nitrilotriacetate by a denitrifying bacterium. Incidentally the pathway proposed here is analogous to those proposed for the aerobic bacterial degradation of NTA (Cripps & Noble 1973). Interestingly in the only description of anaerobic bacterial degradation of triethanolamine a different pathway has been reported. Frings et al. (1994) demonstrated that an *Acetobacterium* sp. utilised a triethanolamine-degrading enzyme which yielded acetaldehyde rather than glycolaldehyde from tri- and diethanolamine breakdown. This enzyme is co-enzyme B_{12}-dependent and the reaction is thought to be analogous to a diol dehydratase reaction. *Acetobacterium* sp. is of course fermentative and a strict anaerobe and generates its energy by substrate level phosphorylation, disposing of surplus electrons via CO_2 reduction to acetate. Such a pathway is unlikely in a facultative bacterium such as DEA4 which can dispose of electrons, and generate energy, via nitrate reduction. It would be of interest to determine whether DEA4 uses the same enzymes for both the aerobic and anaerobic degradation of diethanolamine.

The confirmation of the anoxic utilisation of aliphatic amines suggests that their degradation will occur in more environmental niches than might previously have been thought possible. The involvement of nitrate reducing bacteria in the degradation of secondary amines is of interest in view of the reports (e.g. Calmels et al. 1987; Leach et al. 1987; Smith et al. 1992) that many nitrate reducing bacteria can catalyse

the N-nitrosation of secondary amine at around neutral pH. It is thus possible that amine-degrading, denitrifying organisms may be involved in the environmental generation of potent carcinogens and this possibility merits further investigation.

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