Biodegradation and utilization of quaternary alkylammonium compounds by specialized methylotrophs

by O. Ghisalba and M. Küenzi

Central Research Laboratories and Pharmaceuticals Division, Ciba-Geigy Ltd, CH-4002 Basel (Switzerland)

Dealkylations in organic syntheses are often carried out with trimethylamine (TMA) as the alkylacceptor. In such reactions an ethyl group is transferred from an alkyldonor (e.g. a substituted diethyl phosphate or diethyl thiophosphate) to TMA and a trimethylethylammonium salt (TMEA) is formed in stoichiometric quantities. The production of large-scale chemicals such as agrochemicals or dyestuffs involving deethylations (dealkylations) with trimethylamine thus yields large volumes of mother liquors containing trimethylethylammonium salt (or trimethylalkylammonium salt). The regeneration of TMA from TMEA is unfortunately rather difficult to achieve. Alkaline hydrolysis of TMEA at elevated temperature yields TMA as the main product but also other methylated amines and by-products (stoichiometry rather unclear). The purification of TMA by distillation of such a hydrolysate is very expensive (much energy is needed for cooling). One of the methods best suited for its disposal is the incineration of mother liquors containing TMEA.

Since biodegradation of trimethylethylammonium salts would be an alternative to physical or chemical methods of disposal we searched for TMEA degrading microorganisms.

Trimethylethylammonium salts are (at least partially) C_1-compounds. We therefore decided to investigate and isolate methylotrophs (C_1-utilizing microorganisms).

The various C_1-compounds utilized according to the literature by specialized methylotrophs as the sole source of carbon and energy have been mentioned in the preceding publication[4].

For the degradation of tetramethylammonium chloride, a homologue of TMEA, only two microorganisms have been described so far: *Bacterium* 5H2[5] and *Bacillus* PM6[2]. However, many of the methylotrophs reported to utilize methylamine, dimethylamine and/or trimethylamine have never been tested for their ability to grow on tetramethylammonium salts. Mackrell and Walker[6] were able to enrich cultures of microorganisms growing on 10 mM tetramethylammonium chloride as the sole carbon source. These microorganisms could be adapted to grow on 10 mM trimethylethylammonium chloride. While no attempts were made to isolate and characterize the TMEA-degrading organisms from these enrichment cultures, it was suggested that pseudomonads might be involved. Concentrations higher than 10 mM tetramethylammonium chloride or TMEA were not tested (10 mM TMEA-chloride ≙ 1.23 g/l).

Evaluation of methylotrophs from culture collections

More than 30 methylotrophic bacteria obtained from culture collections and listed in the accompanying paper[4] were tested for TMEA utilization under the conditions described for testing growth on monomethyl sulfate[4]. The test concentrations were 1.0, 5.0 and 10.0 g/l of trimethylethylammonium chloride.

None of these strains was able to utilize TMEA at significant rates! TMEA utilization is thus not a common property of methylotrophs. All the strains were also tested for the utilization of tetramethylammonium chloride (5 g/l) and the results were always negative. The two methylotrophs *Bacterium* 5H2[5] and *Bacillus* PM6[2] reported as tetramethylammonium chloride utilizers were unfortunately not available from any of the strain collections ATCC, NCIB, NRRL or DSM.

Enrichment and isolation of trimethylethylammonium chloride utilizing microorganisms

TMEA-utilizing microorganisms were obtained from sewage sludge of an industrial sewage treatment plant by the enrichment procedure described[4]. Pure cultures of TMEA degraders were isolated by repeated plating of the enrichment cultures on MV7-agar containing 5 g/l of TMEA chloride. Nine of these pure cultures, designated TMEA 14, TMEA 81, TMEA 83, TMEA 84, TMEA 86, TMEA 87, TMEA 89, TMEA 199 and TMEA 211, were then further investigated and taxonomically characterized.

Taxonomical characterization of the isolated TMEA utilizing microorganisms

Two commercially available taxonomy kits were utilized for the taxonomical characterization of the 9 TMEA degrading pure cultures: Oxi/Ferm Tube (Roche) and API 20 E (API System Inc.). For details see the preceding paper[4].

The biochemical data for the 9 TMEA-utilizing strains (all of them are gram-negative) obtained with the two taxonomy systems are listed in table 1.

All the tests were performed in duplicate, once with inoculum from nutrient agar and once with inoculum from TMEA-containing mineral agar. They were carried out according to the instructions in the test kit, but incubation was at 28 °C instead of 37 °C.

The biochemical data indicate that the 9 strains are not identical but can be divided into 5 distinct groups. The strains TMEA 14 and TMEA 84 are related not only with respect to their biochemical reactions but also with respect to their tendency to aggregate in

Table 1. Biochemical reactions of trimethylethylammonium chloride utilizers

Strain	ONPG: Hydrolysis by β-galactosidase[A]	ADH: Arginine dihydrolase[A,O]	LDC: Lysine decarboxylase[A]	ODC: Ornithine decarboxylase[A]	CIT: Citrate utilization[A,O]	H_2S: H_2S from thiosulfate[A,O]	URE: Urease[A,O]	TDA: Tryptophan desaminase[A]	IND: Tryptophan degradation→formation of indole[A,O]	VP: Acetoin test[A]	GEL: Gelatine liquefaction[A]	GLU: Glucose utilization[A]	MAN: Mannitol utilization[A]	INO: Inositol utilization[A]	SOR: Sorbitol utilization[A]	RHA: Rhamnose utilization[A]	SAC: Saccharose utilization[A]	MEL: Melibiose utilization[A]	AMY: Amygdaline utilization[A]	ARA: L-Arabinose utilization[A]	AND: Dextrose, anaerobic degradation[O]	AD: Dextrose, aerobic degradation[O]	XYL: Xylose utilization[O]	OX: Oxidase[A]	CAT: Catalase[A]	NO_2/N_2: Nitrate reduction[A,O]
Pseudomonas TMEA 14	±	−	−	−	−	−	±	−	−	±	−	−	−	−	−	±	±	−	±	±	−	−	−	+	+	±
Pseudomonas TMEA 81	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	±	+	+	+
Pseudomonas TMEA 83	+	−	−	−	+	−	±	−	−	−	±	−	−	−	−	−	−	−	−	−	−	±	±	+	+	+
Pseudomonas TMEA 84	+	−	−	−	±	−	−	−	−	±	−	±	−	−	−	−	+	−	+	−	−	−	−	+	+	±
Pseudomonas TMEA 86	−	±	−	−	±	−	−	−	−	±	−	±	−	−	−	−	−	+	−	−	−	+	+	+	+	±
Pseudomonas TMEA 87	+	−	+	+	+	−	−	−	−	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas TMEA 89	+	−	±	−	+	−	+	−	−	±	−	−	−	−	−	−	±	−	±	−	−	±	±	+	+	+
Pseudomonas TMEA 199	±	−	−	−	+	−	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
Pseudomonas TMEA 211	−	−	−	−	+	−	+	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+

A, API 20 E. O, Oxy/Ferm Tube. A, O, reaction in both systems.
+, 3 or 4 of the parallel tests were positive[A,O], or both of 2 parallel tests were positive[A] or [O].
−, all parallel tests were negative, or only one of 4 tests was positive.
±, 2 of 4 or one of 2 parallel tests were positive.

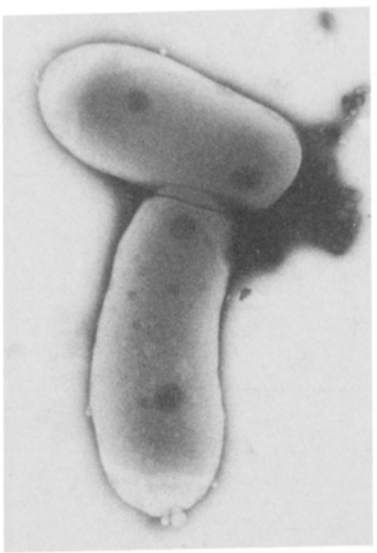
liquid cultures (flocculation). In strains TMEA 81 and TMEA 83 are closely related and show similarity to strain TMEA 89. The strains TMEA 199 and TMEA 211 are very similar with respect to their biochemical reactions. Strain TMEA 87 is clearly different from all the other strains with respect to carbohydrate utilization and ornithine decarboxylase. Strain TMEA 86 also seems to be different from the other strains. According to the numerical code of the Oxi/Ferm Tube test the 9 TMEA-utilizing strains would be tentatively classified as *Pseudomonas*-like, *Pseudomonas* sp., *Achromobacter* sp. or *Alcaligenes faecalis*. However, the classification of these strains as *Achromobacter* sp. or *Alcaligenes faecalis* would be inconsistent with the morphology of the strains (see below).

Here are the morphology and growth parameters of the strains TMEA 14, 81, 83, 84, 86, 87, 89, 199 and 211 (parameters similar for all strains):

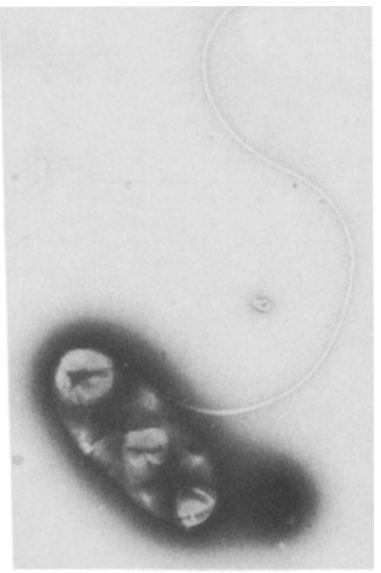
- Optimum temperature: 28–30 °C; practically no or only very slow growth is observed at 37 °C.
- Doubling time (28 °C, with trimethylethylammonium chloride as the sole carbon source, medium MV7 in shake flasks at 250 rpm): 20–30 h.
- Tolerance of electrolytes: osmotolerant, halotolerant, acid tolerant.
- Relation to oxygen: aerobic.
- Colonies on nutrient agar or TMEA containing MV7-agar: small (1–2 mm diameter), circular, convex, smooth, opaque, cream or yellowish in color.
- TMEA tolerance (maximum TMEA concentrations tolerable to start an inoculated culture): strains TMEA 14 and TMEA 84 (≥ 60 g/l), strains TMEA 81, TMEA 83, TMEA 86, TMEA 87, TMEA 89 and TMEA 211 (≥ 20 g/l), TMEA 199 (≥ 40 g/l).
- TMEA (and certainly all the other methyl/ethylamines listed below) can serve as both carbon *and* nitrogen source for all the TMEA strains.
- Light microscopy (identical for all the strains): cells 1×2 µm, rod-shaped; occurring singly, in pairs or in aggregates; single cells are motile.
- Electron microscopy (negative staining with sodium phosphotungstate): the strains are not identical; at least 3 groups of morphologically different cells can be distinguished: TMEA 14, TMEA 84 and TMEA 86 (rods sometimes slightly curved, significantly bigger than those from the other strains), TMEA 81, TMEA 83, TMEA 87 and TMEA 89 (cells shrink due to the preparation, not observed with the other strains, only one polar or subpolar flagellum, rods shorter than in the first group),

TMEA 199 and TMEA 211 (rods smaller than those in the other two groups, one or two polar or subpolar flagella). Flagella are easily lost (depending on the age of cultures) and in all the preparation identical cells without flagella, as well as isolated flagella are visible. The strains of the first group seem to loose their flagella very easily as only a small number of flagellated cells was visible. This is perhaps due to the high tendency of aggregation observed with strains TMEA 14 and TMEA 84 and to a lesser extent also with TMEA 86.

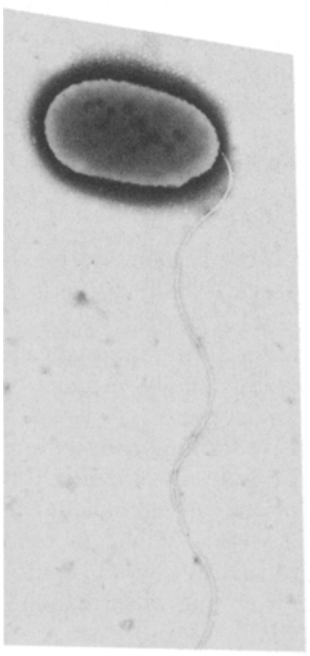
The morphology of the trimethylethylammonium chloride-utilizing microorganisms excludes their classification as *Alcaligenes faecalis* (cocci or coccal rods, usually occurring singly, motile with 1–8 peritrichous flagella) or *Achromobacter sp.* (similar description)[1]. According to Bergey's Manual of Determinative Bacteriology[1] the TMEA-utilizing strains are best classified as *Pseudomonas* sp. and therefore attributed to this genus.



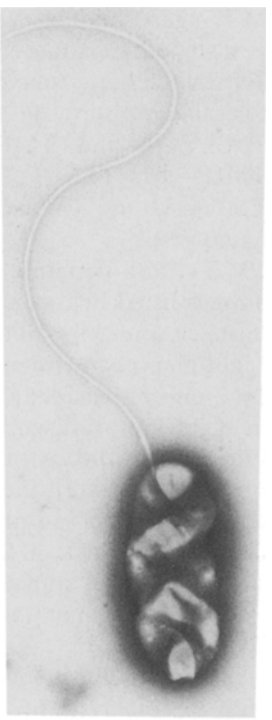
TMEA 14, ×22,750.



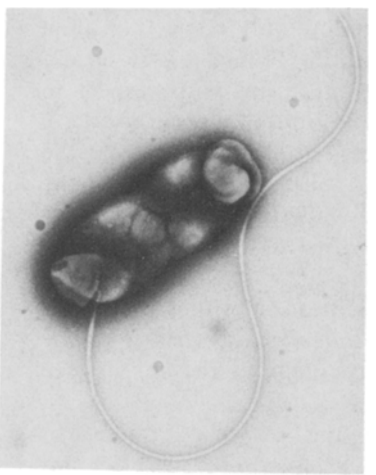
TMEA 81, ×22,750.



TMEA 211, ×15,500.



TMEA 89, ×22,750.



TMEA 87, ×22,750.

Figure 1. Electron micrographs of trimethylethylammonium chloride utilizing *Pseudomonas* sp. (3 morphological groups, cells grown on TMEA).

Substrate spectra of the trimethylethylammonium chloride utilizing Pseudomonas strains

The substrates utilized by the 9 strains of *Pseudomonas* described in the preceding section are listed in table 2. The list also comprises the substrates that are not utilized by the 9 strains. All the substrates were tested as the sole source of carbon and energy under the conditions described starting from precultures grown on trimethylethylammonium chloride. TMEA is utilized as both carbon and nitrogen source by all the 9 strains (tested in medium MV7 without NH_4NO_3). The 9 *Pseudomonas* TMEA strains show some differences with respect to their substrate spectra, but also with respect to the cell densities (OD_{650}) and growth rates observed on the individual substrates. These differences are also listed in table 2.
The substrate spectra show clearly that the TMEA-utilizing *Pseudomonas* strains are specialized facultative methylotrophs.

Table 2. Substrate spectra of the trimethylethylammonium chloride utilizing *Pseudomonas* strains TMEA 14, TMEA 81, TMEA 83, TMEA 84, TMEA 86, TMEA 87, TMEA 89, TMEA 199 and TMEA 211

Substrates utilized as the sole source of carbon and energy by all of the nine strains: (test concentrations in brackets)

Trimethylethylammonium chloride (5–60 g/l)
Ethanol (5 g/l), not utilized by TMEA 86
Acetate (2 g/l)
Glucose (5 g/l)
Nutrient broth
Methylamine (5 g/l)*
Dimethylamine (5 g/l)*
Trimethylamine (5 g/l)*
Methylethylamine (5 g/l)*
Dimethylethylamine (5 g/l)*
Trimethylamine-N-oxide (5 g/l)
Tetramethylammonium chloride (5 g/l)

Substrates utilized only by part of the strains:

Methanol (5 g/l), only TMEA 14, 199 and 211 (211 moderately)
Formate (2 g/l), moderate growth only with TMEA 199
Ethylamine (5 g/l)*, moderate growth onyl with TMEA 211
Diethylamine (5 g/l)*, only with TMEA 14 and 86 (86 moderately)
Triethylamine (5 g/l)*, good growth with TMEA 14 and 86
moderate growth with TMEA 81, 83, 84, 87, 199 and 211, no growth with TMEA 89
Dimethyldiethylammonium chloride (5 g/l), moderate growth only with TMEA 14
Tetraethylammonium chloride (5 g/l), moderate growth only with TMEA 199

Substrates not utilized by the 9 *Pseudomonas* strains:

Methyltriethylammonium chloride(5 g/l)
Sodium monomethyl sulfate (5 g/l)
Formamide (2 g/l)
N,N-Dimethylformamide (2 g/l)
Urea (2 g/l)
Dichloromethane (1 g/l in sealed bottles)
1,2-Dichloroethane (1 g/l in sealed bottles)
Methane – air (1:1 in sealed bottles)
Methylamine anaerobic (5 g/l)*

* Amines were added as hydrochlorides.
Conditions: medium MV7, buffered pH 7, incubation at 28 °C, 250 rpm for 6–10 days. The chemicals were bought from FLUKA chemicals or synthesized in the laboratories of Ciba-Geigy (Dr F. Rigamonti).

The following observations are of special interest:
- Growth on methylamine, dimethylamine, trimethylamine, methylethylamine or dimethylethylamine, is faster than growth on trimethylethylammonium chloride or tetramethylammonium chloride. This seems to indicate that the degradation of the quaternary alkylammonium salt to the tertiary alkylamine might be the rate-limiting step in the biodegradation of TMEA or tetramethylammonium chloride.
- Tetraethylammonium chloride, methyltriethylammonium chloride and dimethyldiethylammonium chloride are practically not utilized. This is in good agreement with the work of Mackrell and Walker[6]. They also found that their enrichment culture grown on tetramethylammonium chloride and adapted to TMEA was not able to grow on the higher homologue tetraethylammonium bromide. They speculate that the close-packed tetrahedral structure protects the central N atom against biodegradative attacks.
  However, this might not be the only explanation if one takes into consideration that also ethylamine, diethylamine and triethylamine are not readily utilized (with the exception of TMEA 14 and 86 and triethylamine). Possibly some of the degradative enzymes are relatively specific for methyl groups. Surprisingly, triethylamine seems to be a somewhat better carbon source than mono- or diethylamine.
- In conclusion, only one methyl group in tetramethylammonium salts can be replaced by an ethyl group to allow significant growth of our TMEA strains with these salts as the sole carbon source. However, a cooxidation of higher substituted homologues in the presence of either TMEA or tetramethylammonium chloride can not be excluded.
- Surprisingly, formate is not utilized and methanol is a good growth substrate for only 2 of the 9 TMEA utilizers.

Degradation pathway for trimethylethylammonium chloride

The pathways proposed for the biodegradation and utilization of trimethylethylammonium chloride by *Pseudomonas* TMEA strains are depicted in figure 2. It is not easy to predict whether the cleavage of the C_2-unit takes place in the first, second or third degradation step. If this cleavage takes place in the first degradation step we have the situation depicted on the left side of figure 2. The products would then be trimethylamine (trimethylammonium chloride) and a C_2-unit (acetaldehyde if we assume a mechanism as for the cleavage of methyl groups). The further degradation of trimethylammonium chloride would then follow the same route as shown for tetramethylammo-

nium chloride[5] after the degradation of the first methyl group.

This mechanism would be in agreement with the substrate spectra of the TMEA-utilizing strains as all of the postulated intermediates trimethylamine, dimethylamine and methylamine are utilized by these strains as the sole carbon source.

The mechanism depicted on the right side of figure 2, however, would also be in agreement with the substrate spectra of the TMEA utilizers because dimethylethylamine and methylethylamine are good carbon sources too. Again one cannot predict whether the cleavage of the C_2-unit is more likely in the second (not shown) or in the third degradation step. However, based on the data from the substrate spectra one restriction can be made: the cleavage of the C_2-unit in the fourth degradation step is unlikely as ethylamine is not accepted as a carbon source by the TMEA utilizing strains. Clarification could be achieved by testing the substrate specificity of tetramethyl (trimethylethyl) mono-oxygenase, trimethylamine mono-oxygenase, trimethylamine-N-oxide demethylase (not shown in fig. 2), dimethylamine mono-oxygenase and methylamine dehydrogenase. Probably some of these enzymes do not discriminate between ethyl and methyl groups.

Some of the medium chain trimethylalkylammonium compounds such as trimethyldecylammonium- and trimethylhexadecylammonium bromide are reported to be utilized as the sole carbon source by specialized strains of *Pseudomonas* and *Xanthomonas*[3,6]. Some evidence was provided that the degradation of the decyl or hexadecyl group was initiated by ω-oxidation of the terminal carbon followed by β-oxidation to release acetyl units. This mechanism would then lead to our well-known TMEA in the last β-oxidation step.

Stirred tank fermentations and mass balances with trimethylethylammonium chloride

In shake flask experiments with unbuffered medium MV7 and 5 g/l of trimethylethylammonium chloride as the carbon source a decrease of pH (from 7 to 3.5) due to liberation of hydrochloric acid was observed.

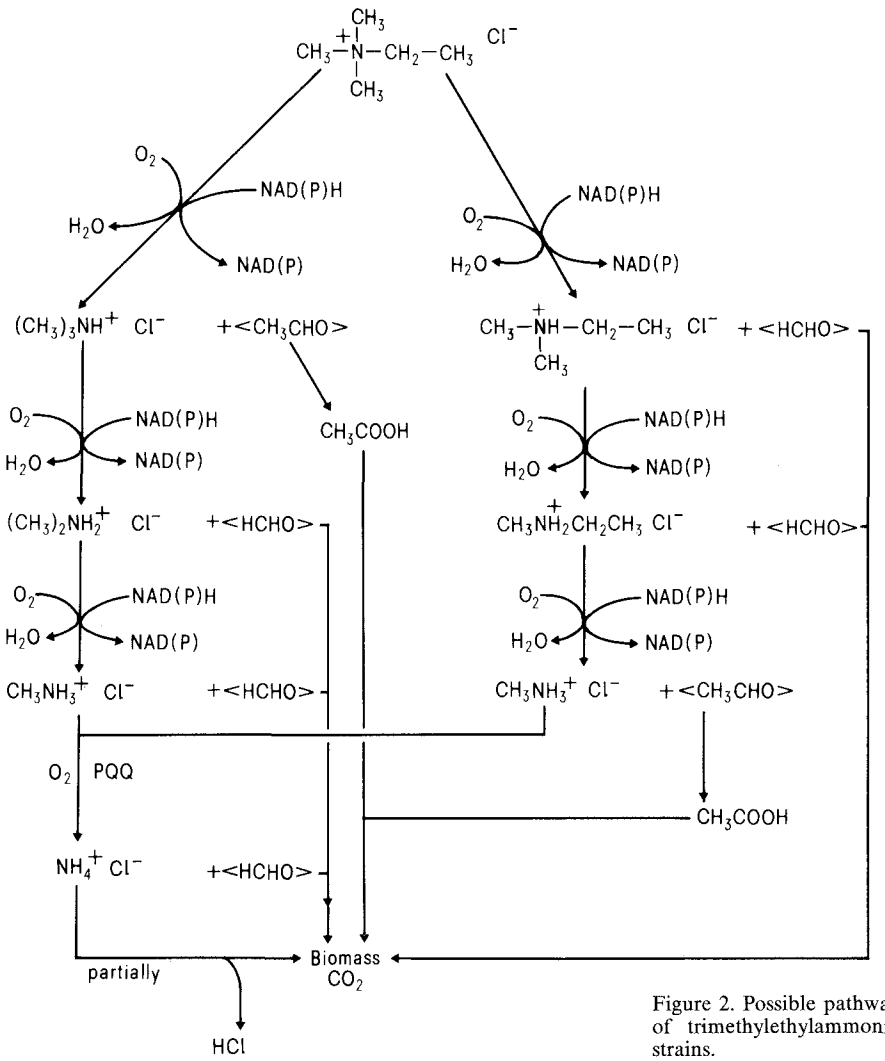


Figure 2. Possible pathways for the biodegradation and utilization of trimethylethylammonium chloride by *Pseudomonas* TMEA strains.

In buffered medium MV7[4] cell densities of OD_{650} 1.8–2.1 ≙ 1.0–1.25 g/l dry weight can be obtained, corresponding to a total consumption of the substrate. The TMEA titer at the end of the shake flask experiments was determined by potentiometric gravimetry. The following degradation efficiencies were found: *Pseudomonas* TMEA 14, TMEA 84, TMEA 86, TMEA 87, TMEA 89, TMEA 199 and TMEA 211: 99% degradation; TMEA 81 and TMEA 83: 80–90% degradtion. The tests were carried out once with TMEA as the sole carbon source (in medium MV7) and once with TMEA as the sole carbon and nitrogen source (in medium MV7 without NH_4NO_3). No significant differences in the degradation efficiencies were observed between the parallel fermentations with the two media.

Fermentation studies were carried out in a 14-l Chemap-fermenter with *Pseudomonas* TMEA 199 under the following conditions (typical examples):

- Preculture in 500-ml shake flasks containing 100 ml of medium MV7 (without NH_4NO_3) and 5 g/l of pure trimethylethylammonium chloride (72 h, 28 °C, 250 rpm).
- 500 ml of this preculture were then inoculated into the fermenter containing 10 l of sterilized medium MV7 (without NH_4NO_3) and 10 g/l TMEA chloride (sterilized by filtration of a 10% solution) → OD_{650} 0.1 ≙ 0.05 g/l dry weight.
  Operation: pH, 5.5 (regulated with 4N NaOH and 1 N HCl); temperature, 28 °C; aeration, 0.26 l air/l culture/min; stirring velocity, 400–700 rpm (agitator with 2 six-bladed turbines), regulated to keep the dissolved oxygen tension above 40% saturation.
  Controlled parameters: growth (OD_{650} and dry weight), dissolved oxygen (pO_2-electrode), O_2-consumption and CO_2-production (gas analyzer), NaOH used for neutralization, concentrations of TMEA, NH_4^+ etc. After 186 h of fermentation 1.75 g/l biomass (dry weight) were formed and about 60% of the substrate degraded and utilized. For technical reasons the fermentation was stopped at this point.

Samples taken from this first fermentation were analyzed with the following methods:

- The concentration of TMEA was determined by potentiometric gravimetry and by ^{1}H-NMR spectroscopy with lyophilized culture filtrate in D_2O with tert-butanol as an internal standard.
- Amines such as methylamine, ethylamine, NH_3, dimethylamine and methylethylamine were detected by high-resolution gas chromatography. The samples were derivatized with benzoyl chloride and the resulting benzamides analyzed with N,N-diethylbenzamide as an internal standard → sensitivity ≤ 10^{-4} M.
  In addition, NH_4^+ was determined with an ion-sensitive electrode.
- Trimethylamine and dimethylethylamine were detected by ^{1}H-NMR spectroscopy in D_2O with TMEA as an internal standard.

The following results were obtained with the samples from the first fermentation:

- No significant accumulation of the postulated degradation intermediates (fig. 2) trimethylamine, dimethylethylamine, dimethylamine, methylethylamine, methylamine and ethylamine was found in the culture filtrate (limit of detection ~ 1.10^{-4} M). The only N-compound accumulated in amounts increasing with the reaction time was NH_3/NH_4^+. This was correlated with the decrease of TMEA concentration.
- For each of 3 test times (98, 165 and 186 h) the total nitrogen content (determined by Kjeldahl's method) of the culture filtrate was found to be the sum of N in free ammonia, N in the remaining TMEA and N incorporated into the biomass of *Pseudomonas* TMEA 199 (determined by elemental analysis of lyophilized cells). The recovery of nitrogen varied between 95% and 99%.
- The determination of the total organic carbon (TOC) present in the culture filtrate (1.94 g C/l at 186 h) indicated that besides the unreacted TMEA (1.86 g C/l at 186 h) no significant amounts of other organic compounds such as alkylamines or acetate were present.

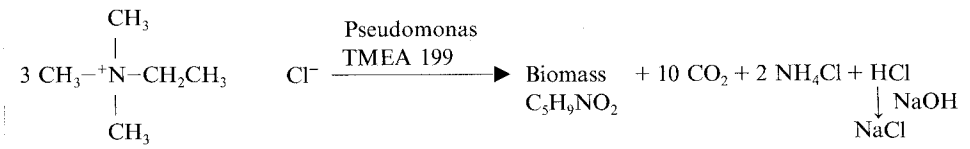
All these data are in good agreement with the biodegradation pathways for TMEA proposed in figure 2. TMEA is biodegraded and utilized without an intermediary accumulation of degradation products and transformed directly into biomass and CO_2 with release of NH_4Cl and HCl.

A second fermentation test was carried out, using a TMEA-containing mother liquor from an industrial production process as the sole source of carbon and nitrogen for *Pseudomonas* TMA 199:

- The preculture was prepared as described for the first fermentation.
- 500 ml of preculture were inoculated into the fermenter containing 10 l of sterilized medium MV7 (without NH_4NO_3) and 22.2 g/l of a mother liquor (composition: 45.2% TMEA chloride, 6.4% HCl, ≤ 1% organic by-products and 47.5% water, neutralized with NaOH) corresponding to 10 g/l TMEA chloride. OD_{650} 0.1 ≙ 0.05 g/l dry weight.
  The operating conditions were as described for the first fermentation. Exponential growth was observed and growth stopped after 190 h of fermentation at OD_{650} 9.5 ≙ 3.1 g/l dry weight.
  In a sample taken after 193 h no trace of undegraded TMEA could be detected (TMEA concentration < 10 mg/l).
- 6 h after substrate depletion (i.e. after 196 h in total) another batch of 22.2 g/l of TMEA containing mother liquor corresponding to 10 g/l TMEA

chloride was added and exponential grwoth was restored immediately (growth rate identical with that in the first phase).
- After 215 h (in total) 55.5 g/l of TMEA mother liquor corresponding to 25 g/l TMEA chloride were fed over a period of 58 h. The fermentation was stopped after 330 h at OD_{650} 30.0 ≙ 9.5 g/l dry weight.

The growth parameters calculated for the first and the second phase with TMEA-containing mother liquor are:
Maximum specific growth rate: $\mu_{max} = 0.023\text{–}0.024\ h^{-1}$,
Medium generation time: $\bar{g} = 29\text{–}30$ h,
Growth yield: $Y_{X/S} = 0.34$ (all values for TMEA chloride as the sole carbon and nitrogen source for *Pseudomonas* TMEA 199).
The following mass balances were established for the utilization of TMEA chloride by *Pseudomonas* TMEA 199 (corrected for sampling):
- After the first phase (see above) TMEA was utilized quantitatively. After 330 h (end of phase 3) 11 g/l of TMEA were left from a total of 45 g/l added consecutively in the 3 phases of the experiment. Thus 75% of the total amount of TMEA added to the fermentation were utilized.
  After about 215 h of fermentation (start of phase 3) the growth rate slowly decreased. As the concentration of dissolved oxygen was never below 40% saturation it is assumed that one of the components in the mineral medium MV7 was depleted and therefore growth limitation occurred.
- Carbon balance: 1/3 of the total amount of carbon from TMEA is incorporated into the biomass of *Pseudomonas* TMEA 199 and 2/3 are lost as CO_2 (needed for energy metabolism).
- Nitrogen balance: 1/3 of the total amount of nitrogen from TMEA is incorporated into the biomass of *Pseudomonas* TMEA 199 and 2/3 are liberated as NH_4Cl. For the quantity of nitrogen incorporated into the biomass a stoichiometric amount of HCl is liberated and has to be neutralized with NaOH (pH-stat). The NaOH consumption is therefore growth-related and exponential (linearity in a semi-logarithmic plot was observed).
- As already described for the first pilot test with pure TMEA, in this second pilot test with TMEA-containing mother liquor no significant amounts of degradation intermediates (methylated amines, methylethylamines, acetate etc.) were detectable.
- The following stoichiometry was established for the biodegradation of TMEA chloride (empirical approximation):



Disposal of trimethylethylammonium chloride wastes under practical conditions – an outlook

Our fermentation studies have demonstrated that trimethylethylammonium chloride, or bromide and tetramethylammonium chloride or bromide, can be degraded in pilot scale by *Pseudomonas* TMEA 199, or other TMEA strains, either as the pure substance or as a constituent of process effluents or mother liquors from dealkylation processes.
For the practical application of such specialized strains different biological waste treatment processes could be envisaged:
- Biodegradation of TMEA containing mother liquors in a classical sewage treatment plant after inoculation with TMEA utilizers or after acclimatization. However, as TMEA is not one of the substrates most easily degraded by such organisms (see substrate spectrum) the overall degradation efficiency for TMEA would probably not be very good and the organisms would prefer utilizing substrates that are easier to degrade. An unstabilized plant with changing substrate composition would probably require a continuous inoculation with TMEA degraders.
- As TMEA-containing mother liquors are produced in large quantities by big chemical companies, a separate treatment of such defined process effluents (without mixing with different effluents from other processes) in small- or medium-sized bioreactors using pure or enriched cultures of TMEA utilizers could be established. Such a process could operate at cell densities even higher than in classical treatment systems.
- The mineralization of TMEA yields NH_4Cl. As large quantities of ammonia can not be tolerated in the effluents from sewage treatment plants (fish toxicity) a treatment in a classical sewage treatment device would require an additional nitrification/denitrification step. On the other hand, the mineralization of TMEA by a separate treatment in a bioreactor could easily be combined with recycling of ammonia (stripping) from the culture broth.
  Another possibility would be to use the culture filtrate from TMEA degradation as a nitrogen source for related biodegradation processes with nitrogen free substrates.

In ecologically integrated systems the sewage sludge (or single cell protein in the case of pure cultures) produced in the specific biodegradation facilities proposed above should be considered as a potential source for the production of chemicals, enzymes or

energy. The following possibilities could be envisaged:

- Use of the biomass as single cell protein, fertilizer or compost (after processing). These applications would be restricted to biomass from waste degradation processes without toxic organic or metallic impurities.
- Production of biogas (methane) by anaerobic digestion of sewage sludge. Such biogas plants are often combined with municipal sewage treatment plants (for instance in Zürich-Werdhölzli). 1 kg of biomass (dry weight) yields 500 l of biogas (~ 70% CH_4, 29% CO_2 and 1% H_2; heat of combustion: 5500–6500 kcal/m^3). The biogasification of industrial sewage sludge would also result in a significant decrease of the remaining sludge volumes to be deposited in landfills or incinerated.
- Extraction of interesting cell components from pure cultures: polyhydroxy butyrate, enzymes, nucleic acids etc.
- In the future, the production of interesting chemicals by genetically modified microorganisms grown on waste substrates instead of the usual C- and N-sources could be of interest.
- A number of ideas and methods for the processing and application of biomass or modified biomass have recently been patented by scientists of Bayer Ltd[7].

Acknowledgment. The authors thank H. Elmiger, F. Heinzer, B. Rudolph, J. Suter and L. Winkler for technical assistance, F. Rigamonti for the synthesis of chemicals (trimethylethylammonium chloride, monomethyl sulfate etc.) and J. Nüesch and J.A.L. Auden for reviewing the manuscript.

1 Bergey's Manual of Determinative Bacteriology, 8th edn. Ed. R.E. Buchanan and N.E. Gibbons. The Williams & Wilkins Company, Baltimore 1974.
2 Colby, J., and Zatman, L.J., Tricarboxylic acid-cycle and related enzymes in restricted facultative methylotrophs. Biochem. J. *148* (1975) 505–511.
3 Dean-Raymond, D., and Alexander, M., Bacterial metabolism of quaternary ammonium compounds. Appl. environ. Microbiol. *33* (1977) 1037–1041.
4 Ghisalba, O., and Küenzi, M., Biodegradation of monomethyl sulfate by specialized methylotrophs. Experientia *39* (1983) 1257–1263.
5 Hampton, D., and Zatman, L.J., The metabolism of tetramethylammonium chloride by bacterium 5H2. Biochem. Soc. Trans. *1* (1973) 667–668.
6 Mackrell, J.A., and Walker, J.R.L.: The biodegradation of quaternary ammonium compounds. Int. Biodeterior. Bull. *14* (1978) 77–83.
7 Wagner, K., Reischl, A., Findelsen, K., Mann, T., Mack, K., and Hamburger, B., Verfahren zur Aufarbeitung von Biomassen; modifizierte Biomassen und deren Verwendung. Europäische Patentschrift 10243 (1981).

0014-4754/83/111264-08$1.50 + 0.20/0

Bacterial growth on 1,2-dichloroethane[1*]

G. Stucki, U. Krebser and T. Leisinger[2]

Mikrobiologisches Institut ETH, Weinbergstrasse 38, ETH-Zentrum, CH-8092 Zürich (Switzerland), May 19, 1983

Summary. 1,2-Dichloroethane (5 mM) served as the only carbon and energy source for bacterium DE2, a gram-negative, oxidase-positive, motile rod. The specific growth rate μ of strain DE2 on 1,2-dichloroethane was 0.08 h^{-1}. A NAD-dependent 2-chloroacetaldehyde dehydrogenase activity and a 2-chloroacetate halidohydrolase activity were detected in extracts of cells grown on 1,2-dichloroethane.

1,2-Dichloroethane or ethylene dichloride is one of the highest volume chemicals produced in the world with an estimated annual production of 13 million tons[3]. About 10% of the compound, which is mainly used as an intermediate in the production of vinyl chloride and other chemicals, is released into the environment[3]. Oxidation of the compound in the atmosphere is believed to result in the formation of the mutagens 2-chloroacetaldehyde and formylchloride and of 2-chloroacetic acid[4–6]. The same degradation products were detected in mice and rats fed with 1,2-dichloroethane[7,8]. Despite its importance as an industrial and environmental chemical, the microbial degradation of 1,2-dichloroethane has not been studied thus far. Here we present evidence for the quantitative degradation of 1,2-dichloroethane by the bac-