Purification and Properties of Trimethylamine *N*-Oxide Reductase from *Shewanella* sp. NCMB 400

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Two major trimethylamine-*N*-oxide reductases were detected in the periplasmic fraction of the marine bacterium *Shewanella* sp. NCMB 400 grown in the presence of trimethylamine *N*-oxide (TMAO). The high- M_r enzyme was purified to homogeneity and consisted of a single polypeptide of M_r 86 000 as judged by SDS-PAGE. The second enzyme had an M_r of 47 000. On isoelectric focusing, multiple forms of the purified enzyme were revealed with isoelectric points of 5.1 and 5.2. The K_m values for the *N*-oxides of trimethylamine, pyridine and γ -picoline were 0.02, 2.41 and 6.95 mM, respectively. The purified TMAO reductase is a molybdoenzyme containing $1.32 \text{ mol Mo (mol enzyme)}^{-1}$.

INTRODUCTION

In the absence of oxygen, many facultatively and obligately anaerobic bacteria can respire by using a variety of inorganic or organic terminal electron acceptors, including nitrate, nitrite, sulphate, sulphite and fumarate (Konings & Michels, 1980). This process of anaerobic respiration is usually accompanied by changes in the components of the electron transport chain, particularly with respect to the appropriate terminal reductases (for reviews see Haddock & Jones, 1977; Ingledew & Poole, 1984).

Anaerobic respiration with trimethylamine *N*-oxide (TMAO) as terminal electron acceptor has been demonstrated in a number of bacteria, including members of the Enterobacteriaceae (Kim & Chang, 1974; Yamamoto & Ishimoto, 1977), *Rhodopseudomonas* spp. (Shultz & Weaver, 1982; McEwan *et al.*, 1983) and *Shewanella* spp. (Easter *et al.*, 1983; Ringo *et al.*, 1984). The components functioning in electron transport with TMAO in *Escherichia coli* and *Salmonella typhimurium* have been partially characterized and include specific dehydrogenases (Sakaguchi & Kawai, 1977; Ishimoto & Shimokawa, 1978), quinones (Cox & Knight, 1981; Bragg & Hackett, 1983), cytochromes *b* and *c* (Sakaguchi & Kawai, 1978; Kwan & Barrett, 1983*a*) and multiple TMAO reductase enzymes (Shimokawa & Ishimoto, 1979; Kwan & Barrett, 1983*b*). The latter are reported to be membrane bound and have been purified from *E. coli* (Shimokawa & Ishimoto, 1979) and *Salmonella typhimurium* (Kwan & Barrett, 1983*b*). TMAO reduction in the enterobacterial species appears unusual in that TMAO is not present in the natural habitat of these bacteria, TMAO being a low- M_r component of marine fish, comprising up to 7% of their dry weight (Groninger, 1959). *Shewanella* spp. are marine, pseudomonad-like bacteria that are associated with spoiling fish where they may account for up to 30% of the total bacterial population (Lee *et al.*, 1977). The TMAO reductase in these bacteria appears to be either periplasmic (Easter *et al.*, 1983) or loosely bound to the outer part of the cytoplasmic membrane

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Abbreviations: BV, benzyl viologen; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); FAD, flavin adenine dinucleotide (oxidized form); FMN, flavin mononucleotide (oxidized form); LDAO, *N,N*-dimethyldodecylamine *N*-oxide; MV, methyl viologen; PMS, *N*-methylphenazonium methosulphate; PCMB, *p*-chloromercuribenzoate; TMAO, trimethylamine *N*-oxide.

(Stenberg *et al.*, 1984), depending upon the method of cell disruption. As part of an investigation on electron transport in alteromonads, we here report the purification of a periplasmic TMAO reductase from *Shewanella* sp. NCMB 400 and a comparison of its properties with those of other bacteria.

METHODS

Organism and growth. *Shewanella* sp. NCMB 400 (Lee *et al.*, 1977) was cultured in the medium of Wood & Baird (1943), containing (g l⁻¹): NaCl, 20; K₂HPO₄, 1; MgSO₄·7H₂O, 1; peptone (Oxoid), 5; yeast extract (Oxoid), 2; (pH 7.2). Aerobic cultures were obtained by inoculation of 500 ml medium in a 2.5 l flask with 10 ml of an overnight starter culture and incubated for 4–6 h at 20 °C in an orbital incubator (120 r.p.m.). Microaerobic conditions were obtained by diluting the aerobic culture into 2 l fresh medium containing TMAO (10 mM) in a 2.5 l flask without stirring for 14–18 h at 20 °C. The alternative electron acceptors KNO₃ (10 mM) and sodium fumarate (10 mM) replaced TMAO in the growth medium when required.

Preparation of periplasm. Spheroplasts were prepared by the method of Birdsall & Cota-Robles (1967) as modified by Easter *et al.* (1983). MgSO₄ was omitted to prevent activity of proteases requiring divalent cations. The periplasm was separated from the spheroplast suspension by centrifugation at 35000 *g* for 30 min at 4 °C.

TMAO reductase assay. TMAO reductase activity was assayed by the method of Jones & Garland (1977) as used by Easter *et al.* (1983) following the TMAO-dependent oxidation of reduced methyl viologen (MV^{•+}) at 600 nm. Specific activity was expressed as nmol MV^{•+} oxidized min⁻¹ (mg protein)⁻¹ (U). Samples were assayed in TMAO reductase buffer composed of 0.1 M-KH₂PO₄, pH 6.5, containing 0.1 M-NaCl and 1 mM-MgSO₄·7H₂O. For inhibition studies with Na₂MoO₄·2H₂O, MacLeod buffer B (Martin & MacLeod, 1971), containing 50 mM-Tris/HCl (pH 7.0), 300 mM-NaCl, 50 mM-MgSO₄ and 10 mM-KCl, was used to prevent autooxidation of TMAO reductase buffer.

Purification of TMAO reductase. All procedures were done at 4 °C. The periplasm obtained from a 7.5 l culture grown microaerobically in the presence of TMAO was fractionated by precipitation for 2 h with a saturated solution of (NH₄)₂SO₄, pH 7.2, followed by centrifugation at 30000 *g* for 30 min. The precipitate obtained between 60–80% saturation was resuspended in ion-exchange buffer, containing 50 mM-Tris/HCl, pH 7.0 (about 36 ml), and dialysed against the same buffer for 16 h. The dialysed fraction was applied to a column of DEAE-Sephacrose CL 6B (2.5 × 10 cm, Pharmacia) equilibrated with ion-exchange buffer. The column was washed with two volumes of the same buffer and bound protein was eluted with a 300 ml linear gradient of 0–0.3 M-NaCl in the same buffer (flow rate, 12 ml h⁻¹; fraction volume, 7.5 ml). The major peak of activity eluting at 0.1 M-NaCl was pooled (about 36 ml) and dialysed for 16 h against 10 mM-K₂HPO₄, pH 7.2. The pooled fraction was applied to an hydroxyapatite column (2.6 × 15 cm) and washed with two column volumes of the same buffer. Bound protein was eluted with a 300 ml linear gradient of 10–500 mM-K₂HPO₄, pH 7.2 (flow rate, 12 ml h⁻¹; fraction volume, 7.5 ml). Active fractions were pooled and concentrated by ultrafiltration (Amicon 8010 stirred cell and PM 10 membrane filter). The pooled and concentrated material (< 10 ml) was applied to a column of Sephacryl S-300 (1.6 × 100 cm, Pharmacia) and eluted with 10 mM-Tris/HCl, pH 7.2, containing 0.2 M-NaCl (flow rate, 8 ml h⁻¹; fraction volume, 3 ml). The active fractions were pooled, concentrated by ultrafiltration and stored at -20 °C.

PAGE. This was done according to Laemmli (1970), using gel slabs (1 mm thickness; 12%, w/v, acrylamide) prepared in the presence or absence of SDS (1%, w/v). For *M_r* determinations, samples were prepared in the presence of SDS (2%, w/v); reducing conditions were provided by incubation with 5 mM-DTT at 100 °C for 5 min. Standard proteins were prepared in a similar manner and included β-galactosidase (*M_r* 114000), phosphorylase *b* (92000), ovalbumin (43000), lysozyme (14000) and horse-heart cytochrome *c* (12500). Gels were stained for protein either with Coomassie blue R250 or the silver-stain method of Merrill *et al.* (1981). A TMAO reductase activity stain was done on samples electrophoresed in the presence or absence of SDS but without DTT incubation at 100 °C. Gels were stained according to Shimokawa & Ishimoto (1979) in 50 mM-Tris/HCl, pH 7.4, containing 40 mM-TMAO, 7.5 mM-KHCO₃, 2 mM-MV^{•+}, and 5 mM-sodium dithionite added just prior to use. Gels were incubated for 5–10 min at room temperature until zones of decolourization associated with enzyme activity were observed against the blue background of the gel. The stained gel was either photographed directly or fixed using 2,3,5-triphenyltetrazolium chloride (2.5%, w/v). In some experiments the method was modified by using 100 mM-Tris/HCl 100 mM-KH₂PO₄, pH 7.8, instead of 50 mM-Tris/HCl, pH 7.4, and adding sodium dithionite as a concentrated solution made up in 100 mM-Tris/HCl adjusted to pH 8.0.

Analytical gel filtration. The *M_r* of TMAO reductase was estimated by gel filtration on Sephacryl S-300 (1.5 × 100 cm) equilibrated in 10 mM-Tris/HCl, pH 7.2, containing 0.2 M-NaCl. Calibration of the column was achieved using the standard proteins thyroglobulin (669 000), BSA (67000), chymotrypsinogen A (25000) and horse-heart cytochrome *c* (12500).

Determination of the isoelectric point. Flat-bed isoelectric focusing was done on an LKB Ultraphor with prepared isoelectric focusing polyacrylamide gels of pH gradient 3.5–9.5 (LKB). Samples of enzyme were dialysed against 10 mM-Tris/HCl, pH 7.2, prior to electrophoresis to remove NaCl. Following electrophoresis, the gel was

sectioned; the position of TMAO reductase was determined by the enzyme activity stain described for PAGE and the pH gradient was determined by sectioning part of the electrofocusing gel in 0.5 mm increments, each piece being placed into a separate tube containing 1 ml degassed NaCl (0.5%, w/v), with the pH of each being recorded after 16 h.

Other assays. Protease activity in samples of periplasm was determined by the neutral protease assay of Levy *et al.* (1976) and by the clearing of opacity in 1% (w/v) casein/1% (w/v) agar plates (Oxoid) after incubation for 1–7 d at 4 °C. Non-haem iron was determined by the method of Doeg & Ziegler (1962) as described by Brumby & Massey (1967) using water-soluble bathophenanthroline sulphonate. Molybdenum was determined by atomic absorption spectroscopy using a Varian AA 1475 model with GTA 95 graphite tube atomizer. Absorption spectrophotometry was done using a Pye Unicam SP 1800 recording spectrophotometer with samples containing approximately 1 mg protein ml⁻¹ and a path length of 1 cm. Samples were oxidized in the presence of air and reduced with sodium dithionite. Protein was estimated by the method of Peterson (1977).

Chemicals. All chemicals were obtained from Sigma or BDH unless otherwise stated. *N,N*-Dimethyl-dodecylamine *N*-oxide (LDAO) was obtained from Ceca.

RESULTS

Multiple TMAO reductase enzymes

Periplasm (1.56 mg ml⁻¹) from cells grown microaerobically in the presence of TMAO was analysed by PAGE and SDS-PAGE to determine the multiplicity of TMAO reductase enzymes. Activity staining by the normal method revealed two enzymes after both PAGE and SDS-PAGE, designated high-*M_r* and low-*M_r* TMAO reductase by their relative mobility. When the high-*M_r* activity was excised after PAGE and re-electrophoresed in the presence of SDS, the activity migrated as a single band in the high-*M_r* position indicating that there is no common monomer for the two enzymes. No protease activity was detected in the periplasm.

Using the modified procedure at normal loadings the same two bands were seen on SDS-PAGE gels (Fig. 1, track 1). At low loadings (track 2) only the low-*M_r* band was seen due to partial inactivation of the high-*M_r* enzyme in the presence of SDS. This was confirmed by running native PAGE gels at the same loading; the high-*M_r* band was then much more prominent (tracks 3 and 6). At higher loadings a further minor band was observed beyond the low-*M_r* band (track 3).

Induction of TMAO reductase

The periplasmic fractions from cells cultured under various growth conditions were analysed by PAGE and activity staining to determine the relative induction pattern of the two TMAO reductases. PAGE and SDS-PAGE gels were run using periplasm from cells grown either in the presence or the absence of TMAO, DMSO, nitrate or fumarate as electron acceptors. Periplasm isolated from cells grown with TMAO or DMSO showed a single high-*M_r* band on PAGE gels (track 6) but corresponding tracks from cells grown with nitrate, fumarate or without any added electron acceptor gave either faint bands or none at all using the normal staining method (track 7). Similar results were obtained with SDS gels. However, using the modified staining method the difference between results obtained for cells grown in the presence of TMAO or DMSO and those grown with nitrate, fumarate or without added electron acceptor was more apparent. The higher contrast obtained with this modified staining method enabled better resolution and the visualization of further faint bands on SDS-PAGE gels. The faint staining in the region corresponding to the high-*M_r* TMAO reductase band was due to multiple bands (tracks 4 and 5). In addition a number of bands of very high mobility corresponding to proteins with *M_r* values of about 12000–20000 were observed.

The low-*M_r* band was observed in gels of the periplasmic fraction of all cells irrespective of the exogenous electron acceptor (Fig. 1). With the modified procedure further minor bands were observed on SDS-PAGE gels for cells grown on nitrate, fumarate or without added electron acceptor, located close to the main low-*M_r* band. Only one of these was observed with periplasm from cells grown in the presence of TMAO or DMSO. On the corresponding native gels the main low-*M_r* enzyme stained less well; it was detected usually as a single band but occasionally as a doublet with extracts from cells grown with fumarate, nitrate or without added electron acceptor.

Fig. 1. Zones of TMAO reductase activity on PAGE and SDS-PAGE gels. The figure is composed from three gels using the same batch of periplasms from cells grown with various electron acceptors (10 mM). Gel type, electron acceptor in growth medium and loading of protein (μ g) and TMAO reductase activity (U) were as follows for each track: (1) SDS gel 1, TMAO, 0.18, 4; (2) SDS gel 1, TMAO, 0.01, 0.26; (3) SDS gel 2, TMAO, 0.7, 17; (4) SDS gel 2, nitrate, 25, 13; (5) SDS gel, none, 71, 25; (6) native gel, TMAO, 0.7, 17; (7) native gel, fumarate, 9, 13.

Purification of TMAO reductase

The high- M_r TMAO reductase was purified from the periplasm of cells grown microaerobically in the presence of TMAO as detailed under Methods. A summary of the purification scheme is given in Table 1. Separation of the high- M_r enzyme from the low- M_r enzyme occurred during $(\text{NH}_4)_2\text{SO}_4$ precipitation. The purified enzyme was judged to be homogeneous by the presence of a single band of protein coincident with a single band of enzyme activity.

The M_r and isoelectric point of TMAO reductase

The native M_r of the purified enzyme was estimated by analytical gel filtration on Sephacryl S-300 to be 84000. This was confirmed by SDS-PAGE under reducing conditions, which indicated an M_r of 86000; there was no detectable dissociation of the protein into subunits. The low- M_r TMAO reductase was estimated to have an M_r of 47000 by SDS-PAGE in the absence of reducing conditions by activating staining with a gel run under the same conditions.

Electrofocusing of the purified enzyme gave two zones of activity corresponding to pH 5.1 and 5.2 whereas unfractionated periplasm gave bands of activity at pH 4.4, 4.9 and 5.2.

Table 1. Purification scheme for TMAO reductase

Sample	Protein (mg ml ⁻¹)	Specific activity (U)	Purification (-fold)	Total protein (mg)	Total activity (U)	Yield* (%)
Periplasm	1.91	3846	1.0	343.38	1320639	100
(NH ₄) ₂ SO ₄ (60–80%)	1.36	10671	3.7	48.96	522452	30.5
Ion exchange	0.13	72525	16.8	4.68	339417	18.0
Hydroxyapatite	0.13	121591	34.5	0.92	112257	7.9
Gel filtration	0.44	211530	55.0	0.44	92447	6.9

* Total units of activity recovered after each step.

Electron donors to TMAO reductase

Periplasmic and purified TMAO reductase were analysed for their ability to oxidize a variety of potential electron donors. Only reduced benzyl viologen (BV^{•+}) was effective, giving approximately 50% and 60% of the activity measured with MV^{•+} in periplasmic and purified enzyme fractions, respectively. No activity was measured with FADH₂, NADH, NADPH and the reduced forms of methylene blue and *N*-methylphenazonium methosulphate (PMS).

Electron acceptors for TMAO reductase

Compounds possessing oxide and/or multiple alkyl moieties together with known electron acceptors from other respiratory systems were analysed for their ability to act as alternative electron acceptors to TMAO with periplasmic and purified TMAO reductase. Specific activities were expressed as a percentage of that obtained using TMAO as acceptor. LDAO gave the highest activity with the periplasmic and purified enzyme sources (63% and 41%, respectively). Adenosine *N*-oxide gave 40% and 35%, hydroxylamine *N*-oxide gave 19% and 3%, and γ -picoline *N*-oxide gave 11% and 12% with the two respective enzyme sources. Little or no activity was detected with KNO₃, sodium fumarate, DMSO, NaClO₃, betaine, carnitine, pyridine *N*-oxide or nicotinamide *N*-oxide.

The *K_m* values for TMAO, LDAO and γ -picoline *N*-oxide were determined by Lineweaver-Burk plots at 0.02, 2.41 and 6.92 mM, respectively.

Inhibitors of TMAO reductase

The effects of various metal ions and potential inhibitors were examined by preincubation of samples of periplasm or purified enzyme with each compound for 5 min (Table 2). Molybdate was assayed in MacLeod buffer B. The effect of Cu²⁺, Fe²⁺ and Fe³⁺ could not be measured because these ions oxidized MV^{•+} and BV^{•+} in both TMAO reductase buffer and MacLeod buffer B. The reduction of TMAO was inhibited by high concentrations of molybdate and tungstate. Both 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzoate (PCMB) inhibited activity; inhibition by PCMB was relieved by incubation with DTT, suggesting the involvement of thiol group(s) in catalysis. Iodoacetate, sodium chlorate and KCN gave only partial inhibition. Little inhibition was obtained with the metal-chelating compounds 1,10-phenanthroline or 2,2'-dipyridyl.

Other properties

Activity was not stimulated in the presence of FAD or FMN. Molybdenum was present in the purified enzyme at 1.3 mol (mol enzyme)⁻¹, but iron was not detected.

DISCUSSION

The reduction of TMAO in *Shewanella* spp. has been shown to be a respiration-linked process on the basis of growth-yield studies (Easter *et al.*, 1982) and by the demonstration of a collapsible membrane potential generated in the presence of TMAO (Stenberg *et al.*, 1984). Two major

Table 2. *Inhibitors of TMAO reductase*

Metal ions were added as their chloride salts at the concentration indicated.

Inhibitor	Concn (mM)	Specific activity* (%)	
		Periplasm	Pure enzyme
None	—	100	100
Ca ²⁺	1	97	78
Mn ²⁺	1	100	78
Na ₂ WO ₄	1	93	36
Na ₂ MoO ₄	1	39	28
	0.1	63	59
NaN ₃	5.0	67	91
KCN	5.0	62	69
	1.0	88	98
KSCN	5.0	101	91
1,10-Phenanthroline	1.0	97	84
2,2-Dipyridyl	2.5	96	85
NaClO ₃	5.0	67	91
Iodoacetate	5.0	53	76
	1.0	90	100
DTNB	1.0	15	33
DTNB + 1 mM-DTT	1.0	26	35
PCMB	0.3	12	10
PCMB + 1 mM-DTT	0.3	87	93
DTT	1.0	103	100

* Specific activities of TMAO reductase for the periplasm and purified enzyme without inhibitor (i.e. 100% values) were 8328 and 113730 U (mg protein)⁻¹, respectively.

TMAO reductase activities were detected in periplasm from cells of *Shewanella putrefaciens* grown in the presence of TMAO. The periplasmic nature of these enzymes was consistent with previous data on their cellular location and function (Easter *et al.*, 1983). The multiplicity of enzymes appears to be a common feature of TMAO reduction in that cultures of both *E. coli* and *Salmonella typhimurium*, induced for TMAO reductase synthesis, contain four TMAO-reducing enzymes (Shimokawa & Ishimoto, 1979; Kwan & Barrett, 1983*a, b*). The two major enzymes from *Shewanella* sp. NCMB 400 could be partially resolved on the basis of their regulation. The high-*M_r* enzyme was induced by TMAO and DMSO and was absent or present at only low levels in cells grown on fumarate or nitrate. This was shown by zymogram staining and is supported by the high specific activities measured. By contrast the low-*M_r* enzyme appeared to be constitutively synthesized under microaerobic conditions, the activity being independent of the electron acceptor present. The high- and low-*M_r* enzymes differed in their sensitivity to inactivation by SDS, the low-*M_r* enzyme being more resistant. A similar distribution of enzymes was observed in *E. coli* with three inducible forms and one constitutive form of the reductase (Shimokawa & Ishimoto, 1979), and in *Salmonella typhimurium* with two inducible forms and one constitutive form (Kwan & Barrett, 1983*a*). The origin of the multiple faint bands observed in gels using periplasm from cells grown with nitrate, fumarate or without added electron acceptor is not known. They may be either precursors or degradation products of the high- and low-*M_r* TMAO reductases. The absence of periplasmic protease activity is evidence against them being degradation products and the simpler pattern of staining observed with extracts from TMAO- and DMSO-grown cells is consistent with them being precursors. However there are alternative plausible explanations. It should be emphasized that using equivalent protein loadings rather than equal activity loadings (as shown here) these minor bands cannot be seen.

The inducible high-*M_r* enzyme was purified to homogeneity with an approximate 56-fold increase in purity and a 7% recovery. The 60% loss in activity following (NH₄)₂SO₄ fractionation was most probably due to the resolution between the two TMAO reductase enzymes that occurred during this step, the low-*M_r* TMAO reductase precipitating between 40–

60% saturation (not shown). The loss in yield after hydroxyapatite was surprising as most of the eluted protein was recovered, suggestive of some denaturation during this stage.

The estimated M_r of 86000 differs from the values obtained for the major inducible amine oxide reductases of *E. coli* (M_r 220000) and *Salmonella typhimurium* (M_r 332000) (Shimokawa & Ishimoto, 1979; Kwan & Barrett, 1983b), with no evidence of aggregation into oligomeric activities (Kwan & Barrett, 1983b). In addition, the purified enzyme from *Shewanella* sp. NCMB400 was not dissociated into subunits unlike the enzymes of *E. coli* and *Salmonella typhimurium*. The purified enzyme gave rise to two activities after isoelectric focusing, possibly indicating two ionic species of the same M_r protein.

The purified enzyme reduced a restricted range of alternative electron acceptors, particularly in comparison with the range of electron acceptors reduced by the *E. coli* enzyme, also termed a tertiary amine *N*-oxide, reductase EC 1.7.99 (Shimokawa & Ishimoto, 1979). In this respect, the enzyme from *Shewanella* sp. NCMB 400 is best described as a TMAO reductase. Although the purified enzyme was unable to reduce DMSO, this compound was shown to be an effective inducer of the enzyme in culture, indicating a gratuitous type of induction. Molybdenum was shown to be present in the purified enzyme, in agreement with molybdenum cofactor complementation studies indicating the presence of the molybdenum cofactor (Nasser & Ward, 1983). The molybdenum may be assumed to be tightly associated with the enzyme due to the lack of both inactivation by SDS during SDS-PAGE and by molybdenum-chelating agents such as KSCN (Lam & Nicholas, 1969).

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REFERENCES

- BIRDELL, D. C. & COTA-ROBLES, E. H. (1967). Production and ultrastructure of lysozyme and EDTA-lysozyme spheroplasts of *Escherichia coli*. *Journal of Bacteriology* **93**, 427-437.
- BRAGG, P. D. & HACKETT, N. R. (1983). Cytochromes of the trimethylamine *N*-oxide anaerobic respiratory pathway of *Escherichia coli*. *Biochimica et biophysica acta* **725**, 168-177.
- BRUMBY, P. E. & MASSEY, V. (1967). Determination of nonhaem iron, total iron and copper. *Methods in Enzymology* **10**, 463-474.
- COX, J. C. & KNIGHT, R. (1981). Trimethylamine *N*-oxide (TMAO) reductase activity in chlorate-resistant or respiration-deficient mutants of *Escherichia coli*. *FEMS Microbiology Letters* **12**, 249-252.
- DOEG, K. A. & ZIEGLER, D. M. (1962). Simplified method for the estimation of iron in mitochondria and submitochondrial fractions. *Archives of Biochemistry and Biophysics* **97**, 37-40.
- EASTER, M. C., GIBSON, D. M. & WARD, F. B. (1982). A conductance method for the assay and study of bacterial trimethylamine oxide reduction. *Journal of Applied Bacteriology* **52**, 357-365.
- EASTER, M. C., GIBSON, D. M. & WARD, F. B. (1983). The induction and location of trimethylamine *N*-oxide reductase in *Shewanella* sp. NCMB 400. *Journal of General Microbiology* **129**, 3689-3696.
- GRONINGER, H. S. (1959). Occurrence and significance of trimethylamine oxide in marine animals. *Special Scientific Report Fisheries*, no. 333, pp. 1-22. Washington, DC: US Fish and Wildlife Service.
- HADDOCK, B. A. & JONES, C. W. (1977). Bacterial respiration. *Bacteriological Reviews* **41**, 47-99.
- INGLEDEW, W. J. & POOLE, R. K. (1984). The respiratory chains of *Escherichia coli*. *Microbiological Reviews* **48**, 222-271.
- ISHIMOTO, M. & SHIMOKAWA, O. (1978). Reduction of trimethylamine *N*-oxide by *Escherichia coli* as anaerobic respiration. *Zeitschrift für allgemeine Mikrobiologie* **18**, 173-181.
- JONES, R. W. & GARLAND, P. B. (1977). Sites and specificity of the reaction of bipyridylum compounds with anaerobic respiratory enzymes of *Escherichia coli*. Effects of permeability barriers imposed by the cytoplasmic membrane. *Biochemical Journal* **164**, 199-211.
- KIM, K. E. & CHANG, G. W. (1974). Trimethylamine oxide reduction by *Salmonella*. *Canadian Journal of Microbiology* **20**, 1745-1748.
- KONINGS, W. N. & MICHELS, P. A. M. (1980). Electron-transfer-driven solute translocation across bacterial membranes. In *Diversity of Bacterial Respiratory Systems*, vol. 1, pp. 33-86. Edited by C. J. Knowles. Boca Raton, Fla: CRC Press.
- KWAN, H. S. & BARRETT, E. L. (1983a). Roles for menaquinone and the two trimethylamine oxide (TMAO) reductases in TMAO respiration in *Salmonella typhimurium*: Mud (Ap^+ lac) insertion mutations in *men* and *tor*. *Journal of Bacteriology* **155**, 1147-1155.
- KWAN, H. S. & BARRETT, E. L. (1983b). Purification and properties of trimethylamide oxide reductase from *Salmonella typhimurium*. *Journal of Bacteriology* **155**, 1455-1458.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680-685.
- LAM, Y. & NICHOLAS, D. J. D. (1969). A nitrate reductase from *Micrococcus denitrificans*. *Biochimica et biophysica acta* **178**, 225-234.
- LEE, J. V., GIBSON, D. M. & SHEWAN, J. M. (1977). A numerical taxonomic study of some pseudomonas-

- like marine bacteria. *Journal of General Microbiology* **98**, 439–451.
- LEVY, M. R., SISKIN, E. E. & MCCONKEY, C. L. (1976). A protease that increases during a period of enzymic and metabolic adjustment in *Tetrahymena*. *Archives of Biochemistry and Biophysics* **172**, 634–647.
- MARTIN, E. L. & MACLEOD, R. A. (1971). Isolation and chemical composition of the cytoplasmic membrane of a Gram-negative bacterium. *Journal of Bacteriology* **105**, 1160–1167.
- MCEWAN, A. G., FERGUSON, S. J. & JACKSON, J. B. (1983). Electron flow to dimethylsulphoxide or trimethylamine-*N*-oxide generates a membrane potential in *Rhodopseudomonas capsulata*. *Archives of Microbiology* **136**, 300–305.
- MERRILL, C. R., GOLDMAN, D., SEDMAN, S. A. & EBERT, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437–1438.
- NASSER, Z. M. & WARD, F. B. (1983). Assay and location of a molybdenum cofactor in strains of *Alteromonas putrefaciens*. *Society for General Microbiology Quarterly* **10**, M21.
- PETERSON, G. L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry* **83**, 346–356.
- RINGØ, E., STENBERG, E. & STRØM, A. R. (1984). Amino acid and lactate catabolism in trimethylamine oxide respiration of *Shewanella putrefaciens* NCMB 1735. *Applied and Environmental Microbiology* **47**, 1084–1089.
- SAKAGUCHI, M. & KAWAI, A. (1977). Electron donors and carriers for the reduction of trimethylamine *N*-oxide in *Escherichia coli*. *Bulletin of the Japanese Society of Scientific Fisheries* **43**, 437–442.
- SAKAGUCHI, M. & KAWAI, A. (1978). Presence of *b*- and *c*-type cytochromes in the membrane of *Escherichia coli* induced by trimethylamine *N*-oxide. *Bulletin of the Japanese Society of Scientific Fisheries* **44**, 999–1002.
- SHIMOKAWA, O. & ISHIMOTO, M. (1979). Purification and some properties of inducible tertiary amine-*N*-oxide reductase from *Escherichia coli*. *Journal of Biochemistry* **86**, 1709–1717.
- SHULTZ, J. E. & WEAVER, P. F. (1982). Fermentation and anaerobic respiration by *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*. *Journal of Bacteriology* **149**, 181–190.
- STENBERG, E., RINGØ, E. & STRØM, A. R. (1984). Trimethylamine oxide respiration of *Shewanella putrefaciens* NCMB 1735: Na⁺-stimulated anaerobic transport in cells and membrane vesicles. *Applied and Environmental Microbiology* **47**, 1090–1095.
- WOOD, A. J. & BAIRD, E. A. (1943). Reduction of trimethylamine oxide by bacteria. I. The Enterobacteriaceae. *Journal of the Fisheries Research Board of Canada* **6**, 195–207.
- YAMAMOTO, I. & ISHIMOTO, M. (1977). Anaerobic growth of *Escherichia coli* on formate by reduction of nitrate, fumarate and trimethylamine *N*-oxide. *Zeitschrift für allgemeine Mikrobiologie* **17**, 235–242.