Nitrate Dissimilation by *Vibrio* spp. Isolated From Estuarine Sediments

By G. T. MACFARLANE AND R. A. HERBERT*

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

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The generic distribution of nitrate-respiring bacteria in tidal mudflats of six Scottish east-coast river estuaries has been determined. The predominant bacteria involved were identified as *Aeromonas/Vibrio* spp., together with lower numbers of enterobacteria. Pseudomonads accounted for only a small proportion of the nitrate-respiring populations. Samples of sediment from all the estuaries produced nitrite and ammonia after 7 d incubation at 15 °C with added nitrate. Studies with the isolate *Vibrio* V48 and strains of *Vibrio parahaemolyticus* (NCMB 1903 and NCMB 2047) demonstrated that when these bacteria were grown anaerobically under conditions of glucose limitation, nitrite was the primary product of nitrate respiration, whereas ammonia was excreted into the medium when nitrogen was limiting. Nitrate reduction was accompanied by the synthesis of a particulate nitrate reductase and under N-limitation a nitrite reductase was also induced.

INTRODUCTION

Many bacteria can use nitrate as a terminal electron acceptor in the absence of molecular O₂ (Payne, 1973; Herbert *et al.*, 1980). The end-products of nitrate respiration depend upon the bacterium and the growth conditions. In bacteria such as *Paracoccus denitrificans*, *Thiobacillus denitrificans* and *Pseudomonas aeruginosa* the products are gaseous (N₂O and N₂) and the process is more correctly termed denitrification. Alternatively, nitrate may be reduced via nitrite to ammonia. This process has been demonstrated in a number of fermentative bacteria such as *Klebsiella* spp. (Hadjipetrou & Stouthamer, 1965; Dunn *et al.*, 1978), *Escherichia coli* (Cole, 1978) and *Achromobacter fischeri* (Prakash & Sadana, 1973). The respiratory reduction of nitrate is catalysed in most bacteria by a membrane-bound nitrate reductase (Thauer *et al.*, 1977) and a soluble nitrite reductase (Kemp & Atkinson, 1966; Dunn *et al.*, 1978). Energetically, nitrate respiration is superior to fermentative metabolism and a number of studies have demonstrated that this respiratory process is coupled to phosphorylation (Stouthamer, 1977; Thauer *et al.*, 1977).

Dunn *et al.* (1979) showed that in *K. aerogenes* K312 nitrate reduction occurred at dissolved O₂ tensions of 15 mmHg (2 kPa) and below. These conditions prevail immediately below the oxidized surface layer of marine and estuarine sediments. Studies with [¹⁵N]nitrate have shown that nitrate reduction to ammonia occurred in these sediments (Koike & Hattori, 1978; Sorensen, 1978). Dunn *et al.* (1980) showed that *Aeromonas/Vibrio* spp. were the predominant nitrate-reducing bacteria present in sediments in the Tay estuary. Preliminary physiological studies with one isolate (*Vibrio* V25) indicated that this bacterium was unable to reduce nitrate beyond nitrite even under N-limiting conditions. In this paper we describe the role of nitrate as an alternative electron acceptor in another isolate, *Vibrio* V48, and in two strains of *Vibrio parahaemolyticus* when grown under anaerobic conditions, and consider how this correlates with the synthesis of a membrane-bound nitrate reductase and a soluble nitrite reductase.

METHODS

Organisms. Nitrate-respiring bacteria were isolated from sediment samples obtained from the estuaries of the Don, North Esk, South Esk, Tay and Leven according to the scheme of Dunn *et al.* (1980). Samples (10 ml) of

sediment were diluted in sterile Ringer's solution and 0.1 ml volumes of suitable dilutions were plated on to nitrate agar. Plates were incubated anaerobically in an atmosphere of H_2 and CO_2 for 7 d at 15 °C. For each estuary, 50 colonies were picked at random from the plates and replated on the same medium to check purity. Each isolate was then tested for its ability to grow in nitrate broth culture and further characterized by the Gram-stain, catalase, oxidase and oxidation-fermentation test (glucose as substrate) using methods described by Cowan (1974).

Vibrio V48 was isolated by direct plating of sediment samples from Kingoodie Bay, Tay estuary, Scotland, on nutrient agar plates plus 0.1% (w/v) KNO_3 . Type strains of *Vibrio parahaemolyticus* (NCMB 1903 and NCMB 2047) were obtained from the National Collection of Marine Bacteria, Torry Research Station, Aberdeen.

Growth in continuous culture. The bacteria were grown in a single-stage 1 litre chemostat as described by Baker (1968). The defined medium of Evans *et al.* (1970) was used, with the concentration of all nutrients lowered by 50%. The concentrations of the carbon source (glucose) and nitrogen source (KNO_3) were varied as required. A dilution rate (D) of $0.05\ h^{-1}$ was used throughout. The growth temperature was maintained at 25 °C by the use of a Churchill thermocirculator (Churchill Instruments, Perivale, London). The culture was aerated by sparging with sterile compressed air ($2.5\ l\ min^{-1}$), whilst for anaerobic conditions the air supply was replaced by high-purity N_2 (Air Products).

Preparation of cell extracts. Extracts were prepared from bacteria grown in the chemostat, as described by Dunn *et al.* (1979).

Enzyme assays. Nitrate reductase activity was determined in soluble and particulate fractions using the method of Lowe & Evans (1964), in which benzylviologen reduced with dithionite served as electron donor. This method was not suitable for the two strains of *V. parahaemolyticus*, and nitrate reductase activity in these bacteria was determined by following the nitrate-dependent oxidation of NADH in Thunberg cuvettes. The method used was a modification of the nitrite reductase assay (Cole *et al.*, 1974) in which $20\ \mu mol\ NaNO_3$ was substituted for NO_2^- . Nitrite reductase was determined in soluble fractions, incubated anaerobically in Thunberg cuvettes, according to the method of Cole *et al.* (1974). Nitrite reductase was only detected in the soluble fractions.

Determination of bacterial populations. Population densities were determined as described by Dunn *et al.* (1979).

Nitrate reduction by sediment slurries. This was determined according to the method of Dunn *et al.* (1980).

Analysis of spent media. Spent media were analysed for nitrite and ammonia according to the methods described by Dunn *et al.* (1978).

Protein estimation. The Lowry method was used with bovine serum albumin as standard.

Chemicals. Benzylviologen was obtained from Sigma. All other chemicals were of Analar grade and obtained from B.D.H.

RESULTS

Nitrate reduction in sediment slurries

Preliminary experiments were performed to determine whether sediment samples obtained from five different Scottish east-coast river estuaries reduced nitrate to nitrite and ammonia. All the samples tested produced both nitrite and ammonia following the addition of nitrate (Table 1). Sediment samples from the Tay and Don produced nitrite as the principal product, whereas with those from the North Esk, South Esk and Leven ammonia was the major product. The proportion of added nitrate dissimilated varied considerably from estuary to estuary (13% for the Don to 41% for the Leven).

Characterization of nitrate-reducing bacteria

Serial dilutions of sediment from each river estuary were plated on to nitrate agar and incubated under anaerobic conditions. After incubation for 7 d at 15 °C, 50 colonies were selected at random and classified to genus level (Table 2). On the basis of a limited number of morphological and biochemical tests, *Aeromonas/Vibrio* spp. were predominant, together with lower numbers of enterobacteria. Pseudomonads only occurred in appreciable numbers in the coarser sandy sediments from the North Esk. Sediment samples were taken in autumn, spring and summer from each river estuary and, whilst the exact proportion of each genus present showed some variation, the predominance of fermentative bacteria identified as *Aeromonas/Vibrio* spp. was confirmed. None of the *Aeromonas/Vibrio* spp. or enterobacteria produced gas when inoculated into nitrate broth. One isolate, *Vibrio* V48, was selected for further study together with two strains of *V. parahaemolyticus* (NCMB 1903 and 2047).

Table 1. Nitrite and ammonia production from sediment samples obtained from Scottish east-coast rivers

Sediment samples were incubated at 15 °C for 7 d and then the concentrations of nitrite and ammonia measured.

Estuary	Addition	Nitrite produced [µg N (ml sediment) ⁻¹]	Ammonia produced [µg N (ml sediment) ⁻¹]
Don	None	0	0.57
	2 mM-KNO ₃	2.49	0.52
	10 mM-KNO ₃	6.97	1.37
North Esk	None	0	0.42
	2 mM-KNO ₃	1.14	2.01
	10 mM-KNO ₃	1.85	1.76
South Esk	None	0.51	0.84
	2 mM-KNO ₃	2.87	5.19
	10 mM-KNO ₃	2.26	3.13
Tay	None	2.56	0.18
	2 mM-KNO ₃	6.63	2.22
	10 mM-KNO ₃	8.35	2.46
Leven	None	0	0.37
	2 mM-KNO ₃	0.03	8.04
	10 mM-KNO ₃	2.13	5.87

Table 2. Identification to genus level of 250 randomly selected nitrate-respiring bacteria isolated from Scottish east-coast estuarine sediments

Estuary	Number of isolates					Unidentified
	<i>Aeromonas/Vibrio</i>	<i>Alcaligenes</i>	<i>Enterobacteriaceae</i>	<i>Pseudomonas</i>	Gram-positive bacteria	
Don	35	5	7	1	2	0
North Esk	27	1	5	14	1	2
South Esk	29	3	7	5	4	2
Tay	24	0	12	9	5	0
Leven	25	1	14	2	5	3

Physiology of nitrate reduction

When *Vibrio* V48 was grown anaerobically on nitrate under C-limitation, the cell population density decreased to 42% of that recorded under aerobic conditions and there was a significant increase in the particulate (membrane-bound) nitrate reductase (Table 3). No nitrite reductase activity was observed under these growth conditions; this is reflected by the accumulation of nitrite in the spent medium (Table 3). The excreted nitrite under anaerobic conditions accounted for 42% of the nitrate input. On the other hand, under N-limitation the cell population density of *Vibrio* V48 under anaerobic conditions was 70% of that observed aerobically and high activities of both a particulate nitrate and a soluble nitrite reductase were recorded. Spent media analyses showed the almost complete absence of nitrite and the accumulation of ammonia (Table 3). In addition to the data presented on *Vibrio* V48, we have obtained similar results for a number of other *Vibrio* spp. (*Vibrio* V27, *Vibrio* V29, *Vibrio* V39 and *Vibrio* V44) isolated from Tay estuary sediments (R. A. Herbert, unpublished results).

To confirm that nitrate reduction to nitrite and ammonia was not confined to these *Vibrio* isolates, the experiments were repeated with *V. parahaemolyticus* strains NCMB 1903 and 2047. When *V. parahaemolyticus* NCMB 2047 was grown anaerobically under C-limitation, a cell population density was 29% of that observed under aerobic conditions, and this was associated with the synthesis of a membrane-bound nitrate reductase (Table 3). Unlike the dissimilatory enzyme from *Vibrio* V48, however, nitrate reductase activity in both strains of *V. parahaemolyticus* was linked only to NADH and no activity was observed with reduced benzylviologen. Spent

Table 3. *Influence of nitrogen and carbon limitation on the aerobic and anaerobic reduction of nitrate by Vibrio V48 and V. parahaemolyticus NCMB 2047*

Under C-limitation the medium input contained 5 g glucose l⁻¹ and 20 mM-nitrate, whereas under N-limitation the glucose concentration was increased to 15 g l⁻¹ and nitrate concentration decreased to 5 mM. *D* = 0.05 h⁻¹, 25 °C.

Bacterium	Growth conditions	Population density (ml culture) ⁻¹	Enzyme activity* [nmol min ⁻¹ (mg protein) ⁻¹]			Concn in medium (mM)	
			Nitrate reductase		Nitrite reductase Soluble	NO ₂ ⁻	NH ₄ ⁺
			Soluble	Particulate			
<i>Vibrio</i> V48†	N-limitation: aerobic	1.1 × 10 ⁹	53	16	0	0	0
	N-limitation: anaerobic	7.8 × 10 ⁸	80	2000	373	0.1	1.6
	C-limitation: aerobic	1.4 × 10 ⁹	55	20	0	0.2	0
	C-limitation: anaerobic	6.2 × 10 ⁸	30	625	0	8.6	0.04
<i>V. parahaemolyticus</i> (NCMB 2047)	N-limitation: aerobic	5.4 × 10 ⁸	6	207	0	0	0
	N-limitation: anaerobic	2.1 × 10 ⁸	18	708	150	0.1	2.2
	C-limitation: aerobic	6.6 × 10 ⁸	23	98	0	0	0
	C-limitation: anaerobic	1.9 × 10 ⁸	37	597	0	6.4	0.08

* Mean values of four replicate determinations on separate occasions.

† *Vibrio* V48 nitrate reductase activities are nmol NO₂⁻ produced min⁻¹ (mg protein)⁻¹; all other enzyme activities are nmol NADH oxidized min⁻¹ (mg protein)⁻¹.

media analyses showed that nitrite was the principal product of nitrate reduction under C-limitation (Table 3) and accounted for 32% of the nitrate input. Under N-limitation the anaerobic population density was 39% of that obtained with aerobically grown cultures, and high activities of nitrate reductase and nitrite reductase were recorded in extracts. Ammonia was the principal end-product and accounted for 44% of the nitrate input. Similar results were obtained with *Vibrio parahaemolyticus* NCMB 1903. These results unequivocally demonstrate that *Vibrio* spp. can use nitrate as electron acceptor under anaerobic conditions with the concomitant excretion of nitrite under C-limitation, and ammonia under N-limitation.

DISCUSSION

Sediment samples from a number of Scottish east-coast river estuaries produced nitrite and ammonia when supplemented with nitrate (Table 1). These data support the conclusions of Sorensen (1978) and Koike & Hattori (1978) that dissimilatory nitrate reduction may account for a significant proportion of the ammonia present in estuarine and marine sediments. The rate of ammonia production observed in the Scottish east-coast estuarine sediments investigated ranged from a maximum of 1.14 µg N ml⁻¹ d⁻¹ (Leven estuary) to a minimum of 0.4 µg N ml⁻¹ d⁻¹ (Don estuary). These rates are approximately 10–30% of those reported by Sorensen (1978) for the Limfjorden, Denmark. In experiments using [¹⁵N]nitrate, Koike & Hattori (1978) showed that in sediments from Tokyo bay between 7 and 52% of the added nitrate was dissimilated to ammonia.

The predominant nitrate-reducing bacteria isolated from the estuaries studied belong to the genera *Aeromonas/Vibrio*. These data confirm and extend the initial studies by Dunn *et al.* (1980) that *Aeromonas/Vibrio* spp. were the principal nitrate-dissimilating bacteria in estuarine and marine environments. Preliminary physiological studies with one isolate, *Vibrio* V25, indicated that this bacterium had a restricted ability to dissimilate nitrate and that it would not compete effectively *in situ* with those bacteria e.g. *Klebsiella* spp. which reduced nitrite to ammonia when nitrate is limiting (Herbert *et al.*, 1980). It would appear paradoxical that the dominant nitrate-respiring bacteria in these estuarine sediments should have such a restricted role.

Re-examination of nitrate dissimilation by vibrios, as reported here, showed that individual *Vibrio* spp. were able to dissimilate nitrate under anaerobic conditions and that this was

associated with a membrane-bound nitrate reductase and a soluble nitrite reductase (Table 3). Since our classification scheme for the *Vibrio* spp. was based on a limited number of characters, we used known strains of marine vibrios (*V. parahaemolyticus* NCMB 1903 and NCMB 2047) to confirm that members of this genus could dissimilate nitrate to ammonia (Table 3). Nitrate reduction has also been reported previously in *Achromobacter fischeri* (Prakash & Sadana, 1973), which has now been reclassified as *V. fischeri* (Hendry *et al.*, 1970).

A major feature of the nitrate reductase from the type strains of *V. parahaemolyticus* and a number of the *Vibrio* spp. studied (*Vibrio* V27, *Vibrio* V29, *Vibrio* V39 and *Vibrio* V44), but not of *Vibrio* V48, was the requirement *in vitro* for NADH rather than reduced benzylviologen as electron donor. *Vibrio* V48 nitrate reductase was able to use both benzylviologen and NADH as electron donor. These data are in agreement with a number of authors who have demonstrated that NADH may serve as an electron donor for nitrate reduction in extracts (Cole & Wimpenny, 1968; Lester & De Moss, 1971). The levels of NADH-linked nitrate reductase activity observed in the anaerobically grown *Vibrio* cultures were similar to those reported for the benzylviologen-linked enzyme in *Klebsiella* K312 (Herbert *et al.*, 1980) and *Klebsiella aerogenes* NCIB 418 (Cole & Brown, 1980).

In conclusion, these results show that fermentative bacteria, and particularly isolates identified as *Aeromonas/Vibrio* spp., are numerically dominant and potentially the most active nitrate-reducing bacteria in a number of Scottish east-coast estuarine sediments. Nitrate dissimilation in these bacteria was associated with the synthesis of a membrane-bound nitrate reductase and, under nitrate-limiting conditions, a soluble nitrite reductase. Neither enzyme was repressed when cultures were pulsed with ammonia (10 mM-NH₄Cl), except when nitrate was omitted from the growth medium. However, the synthesis of both enzymes was repressed by oxygen, thereby confirming their dissimilatory role in nitrate reduction. Whilst these results establish that nitrate reduction to ammonia can occur in estuarine sediments, the relative significance of dissimilatory nitrate reduction to ammonia and ammonification from organic nitrogen detritus in estuarine sediments still needs to be assessed.

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