Indian Journal of Marine Sciences
Vol. 18, December 1989, pp. 238-241

Diazotrophic activity and ferric phosphate mineralizing ability in estuarine sulfate reducing bacteria*

S Banik† & A Ninawe†
Central Marine Fisheries Research Institute, P.O. Box 2704, Cochin 682 031, India
Received 17 April 1989; revised 19 September 1989

Twenty strains of sulfate reducing bacteria were isolated from water and sediments of Cochin backwaters, India. Five of them were identified as *Desulfovibrio desulfuricans* and fifteen as *Desulfotomaculum orientis*. All the *Desulfovibrio* were isolated from sediments whereas *Desulfotomaculum* was present in both water and sediment. All the bacteria grew at *p*H of slightly alkaline reaction and lowered the E_h from −105 to −270 mV of the growth medium. Complete reduction of sulfate to sulfide by the bacteria was evidenced by blackening of Postgate culture media. All isolates were able to fix dinitrogen in anaerobic N-free modified Postgate broth. In addition to nitrogen fixing ability, 5 of the *Desulfotomaculum* and 3 of the *Desulfovibrio* were able to produce soluble phosphorus in growth medium from insoluble $FePO_4$. A scheme for $FePO_4$ mineralization in marine and estuarine sediments has been proposed.

Heterotrophic sulfate reducing activity in marine and estuarine sediments is predominantly of microbial origin[1,2]. Species of *Desulfovibrio* and *Desulfotomaculum* are the major genera active in sulfate reducing activity anaerobically in these habitats[3]. Members of the genus *Desulfovibrio* fix dinitrogen in marine sediments[4,5]. In marine sediments large quantities of phosphorus are chemically bound to iron when the latter is in the oxidised state and the soluble form of phosphorus is thought to be released when ferric is reduced to ferrous sulfide[6]. In submerged terrestrial soils, hydrogen sulfide from anaerobic biological reduction of sulfate has been found to bring soluble form of phosphorus to solution from insoluble ferric phosphate[7]. Similarly, H_2S produced by anaerobic sediment bacteria as a consequence of reduction of sulfate in marine and estuarine habitat may play a crucial role in mineralization of phosphorus from insoluble ferric phosphate. Consequently the sulfate reducing bacteria in marine and estuarine habitat may be ecologically important in controlling primary producers in that habitat. In the present study an attempt has been made to verify the diazotrophic as well as ferric phosphate mineralization ability of sulfate reducing bacteria isolated from an estuary. A scheme for the probable pathway of $FePO_4$ mineralization in marine or estuarine sediments has also been proposed.

Materials and Methods

Sediment samples were collected from 4 stations in Cochin backwaters (Fig. 1) during April–May 1985, using Peterson's grab and stored aseptically in sterilised glass bottles. Water samples were collected from the surface in 125 ml presterilized glass bottles for bacteriological analysis and in 500 ml narrow mouthed plastic bottles for determining

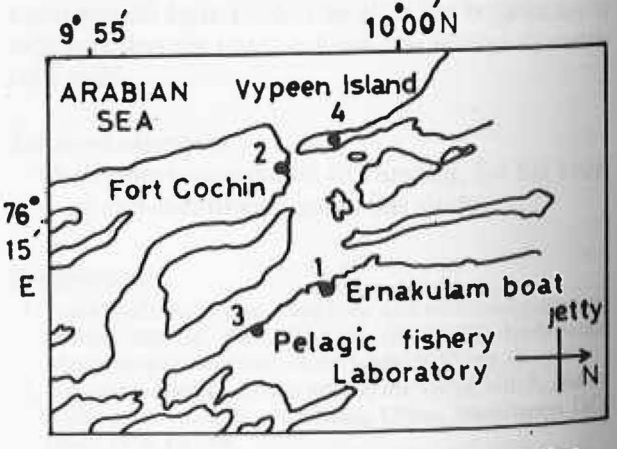

Fig. 1—Station locations [1, Ernakulam boat jetty; 2, Fort Cochin; 3, Pelagic fishery laboratory; and 4, Vypeen island]

*Paper presented in 73rd Indian Science Congress, 3–8 January 1986, New Delhi, India

Present address:
†Jute Technological Research Laboratories, 12 Regent Park, Calcutta 700 040, India
‡Department of Science and Technology, New Mehrauli Road, New Delhi 110 016, India

other parameters. Analyses were made on the same day of collection. Temperature, *p*H and E_h of the collected samples were determined immediately[8]. Salinity of water sample was estimated by Mohr–Knudson argentometry method[9] and dissolved oxygen (DO) content by modified Winkler's method[9]. Organic carbon in water and sediment was estimated[10]. Sulfate reducing bacterial population was enumerated using Postgate medium[12] modified by the use of 50% (w/v) aged seawater as dilutant and 2% (w/v) agar, following usual pour plate method, and *p*H was adjusted to 7.4. Petridishes were incubated at 30°±1°C in anaerobic jar filled with nitrogen and carbon dioxide for 7 d and the number of sulfate reducing bacteria was determined by counting only black coloured colonies.

Twenty sulfate reducing bacteria were isolated from well grown colonies. The isolated bacteria were maintained in the same liquid medium[12] in screw capped bottles covered with sterilized liquid paraffin. The isolated bacteria were purified by repeated subculture and subsequent microscopic examination. Sulfate reducing ability of the isolated bacteria was confirmed by the release of H_2S when inoculated in Postgate broth[13]. Identification of the pure cultures of the bacterial isolates was carried out with the help of taxonomic scheme[14]. Pure cultures of the isolated bacteria were tested in modified Postgate broth for determining N_2–fixing ability and $FePO_4$ mineralization power. N_2–fixation was tested by inoculating isolated bacteria in 100 ml Postgate broth medium in 250 ml Erlenmayer flasks, in duplicate, by replacing soluble phosphate source with $FePO_4$ (6 $g.l^{-1}$) and using 1% lactate as sole source of carbon. The *p*H of the media was adjusted to 7.4 and 6 respectively in both the tests. Uninoculated controls were kept for comparison. After inoculation of bacterial cultures the mouth of the flask was sealed with sterilized paraffin wax in case of N_2–fixing experiments and covered with sterile liquid paraffin in case of $FePO_4$ mineralization experiment and incubated at 30°±1°C for 15 d. After 15 d E_h and *p*H of the broth were recorded. Broth (10 ml) from each of $FePO_4$ added sets was centrifuged at 10000 rpm for 10 min. A suitable volume of supernatant was then used for determining soluble phosphate content in broth colorimetrically[9] after adjusting control to 'zero value. After 15 d incubation, 10 ml sample each from N–free sets were digested with conc. H_2SO_4 and N was determined by micro-Kjeldhal technique[15].

Results

Variations in temperature, salinity and dissolved oxygen content in water samples were negligible (Table 1). Similarly, *p*H, E_h, organic carbon contents and soluble sulfate contents in both water and sediment did not vary much. E_h in both was in oxidised range. Sulfate reducing bacterial population was higher in sediments than in water.

Study of morphological and biochemical characteristics of the isolates revealed that all the isolates were Gram negative and 15 of them were spore formers. All isolates formed black circular colonies in anaerobic Postgate agar and were straight or curved rods. Their size varied from 0.5 to 1 μm in width and 3 to 5 μm in length. None of the isolates were able to use glucose or acetate in absence of yeast extract. All grew on pyruvate in presence of sulfate and only 5 grew on pyruvate in absence of sulfate. These 5 isolates were asporogenous and were identified as *Desulfovibrio desulfuricans* by their inability to grow on malate in absence of sulfate and their ability to grow on malate in presence of sulfate. They grew on choline in presence or absence of sulfate. Matching biochemical and cultural properties[6], 15 residual terminal or subterminal spore formers were classified as *Desulfotomaculum orientis*. These bacteria grew

Table 1—Environmental parameters and number of sulfate reducers

St No	Water temp (°C)	*p*H		E_h (mV)		Sal. ($\times 10^{-3}$)	DO ($ml.l^{-1}$)	Organic carbon		Sulfate		Number of sulfate reducers	
		W	S	W	S			W ($mg.l^{-1}$)	S (%)	W ($g.l^{-1}$)	S ($mg.g^{-1}$)	W (ml^{-1})	S (g^{-1})
1	33.0	7.90	6.70	+262	+26	16.49	1.43	134.2	0.61	1.85	3.75	460	2000
2	31.0	7.95	6.40	+272	+16	17.46	1.60	124.8	3.79	1.95	37.50	250	6200
3	32.5	8.25	6.35	+282	+21	16.15	2.13	106.0	5.33	1.43	37.50	950	4200
4	32.5	8.05	6.35	+267	+16	17.27	1.94	124.2	4.67	1.85	37.50	220	4300

W = water and S = sediment

around 30°C only and failed to utilise propionate, formate or ethanol. They were divided into 2 subspecies. All the *Desulfovibrio* spp. were found in sediment only whereas *Desulfotomaculum* spp. were present in both water and sediment.

Ferric phosphate mineralization and N_2–fixing ability of the isolated bacteria are presented in Table 2. In $FePO_4$ broth perfect blackening was noticed in 8 flasks. These bacteria were only able to solubilize $FePO_4$ in excess of their metabolic need. The amount of soluble phosphate accumulated was from 62.5 to 937.5 $\mu g.ml^{-1}$ broth. Other 12 bacteria grew feebly and were unable to produce soluble phosphate in growth medium. Brownish colour was noticed in those flasks while colour in control flasks remained unchanged. E_h came down to as low as -200 to -270 mV in dark blackened sets from an initial E_h of -65 mV before inoculation. The minimum value of E_h recorded from brown sets was -170 mV. *p*H value in those 8 sets varied from 6.7 to 7.35 from an initial *p*H of 6 before inoculation. There was little change in *p*H values in brown coloured sets. Change of *p*H and E_h values in growth media followed no trend corresponding to amount of phosphate mineralized and hence these data are not included in the table. Among these 8 $FePO_4$ mineralizing bacteria 3 were of the genus *Desulfovibrio* while rest 5 were members of the genus *Desulfotomaculum*.

However, all the isolates were able to grow in N–free modified Postgate broth satisfactorily. They were able to fix dinitrogen in growth medium in detectable amounts. Blackening was noticed in all the sets and fall in E_h recorded was as low as -170 to -200 mV from an initial E_h of -70 mV while the reaction of the broth changed a little.

Table 2—Phosphate mineralization and nitrogen fixing ability of sulfate reducing bacteria isolated from water (W) and sediment (S) samples

Number of samples	Isolated genus	In $FePO_4$ broth [μg P. (50 ml)$^{-1}$ broth]	In N–free broth [mg N fixed (100 ml)$^{-1}$ broth]
St 1			
W-1	*Desulfotomaculum*	0	3.66
W-2	do	0	1.96
W-3	do	0	1.96
S-4	do	812.5	3.64
S-5	do	137.5	3.92
St 2			
W-6	do	937.5	3.64
S-7	do	137.5	3.08
S-8	do	0	4.48
S-9	*Desulfovibrio*	62.5	2.24
S-10	*Desulfotomaculum*	0	4.20
S-11	do	0	3.92
St 3			
W-12	do	62.5	2.52
S-13	*Desulfovibrio*	0	2.44
S-14	do	0	3.36
St 4			
W-15	*Desulfotomaculum*	0	3.36
S-16	do	0	3.64
S-17	do	0	4.48
S-18	*Desulfovibrio*	225.0	3.64
S-19	*Desulfotomaculum*	0	3.92
S-20	*Desulfovibrio*	62.5	2.44

Discussion

The environmental conditions, viz. *p*H, organic matter, dissolved oxygen, salinity and sulfate concentrations (Table 1) were favourable for growth and activity of sulfate reducers as described by ZoBell[22]. E_h values were high for sulfate reducing bacteria in both water and sediment and this might be the cause of lower number of sulfate reducing bacterial population in these habitats. How anaerobic sulfate reducing bacteria remained viable in oxidised condition was not understood from this study. Higher number of sulfate reducing bacteria in sediment than in water may be ascribed to prevailing reduced condition in that habitat. Identification of only one species each of 2 genera, viz. *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis*, indicate two possibilities (1) failure of Postgate medium to support the growth of different types of sulfate reducing bacteria, which utilise carbon source other than lactate[17] and (2) only these isolated sulfate reducing bacteria could tolerate the hardship of unfavourable redox potential though they favoured highly reduced condition. Presence of *Desulfovibrio desulfuricans* only in the sediment among the isolates indicate their choice towards higher anaerobeosis. It could be thought that the spore former *Desulfotomaculum* found in relatively oxidised condition, may remain inactive in such condition by formation of inactive endospore and become active only when the condition favoured them.

Why only 8 out of 20 isolates were able to grow better and solubilize $FePO_4$ beyond their metabolic need was not clear. Trace quantities of soluble phosphate was present in the medium due to partial hydrolysis of $FePO_4$ during autoclaving[18] which might have initiated the early stage of growth of the sulfate reducing bacteria in that medium. Slow nature of reduction of sulfate of the residual 12

bacterial cultures might have restricted them to grow at later stage and this was evidenced by brown colour developed in their growth media.

Desulfovibrio has been reported as the principal nitrogen fixing agent in marine environments[4,19]. However, Blake and Liftley[5] found no correlation between number of sulfate reducers and acetylene reduction rate. Masterson and Murphy[20] reported that acetylene reduction method generally gives over estimation of nitrogen fixation and therefore difficult for interpretation of the results. Although, in the present study the amount of N-fixed was low in comparison to that fixed by efficient anaerobic nitrogen fixers, the group of nitrogen fixing sulfate reducing bacteria are ecologically important in marine and estuarine sediments due to the abundant distribution in these habitats[1]. Moreover, mineralization of $FePO_4$ makes the group of bacteria more important ecologically.

Basing on the present data a probable pathway of $FePO_4$ mineralization in marine and estuarine sediments is proposed. As there is a vast store of $FePO_4$ in the seabed[6] and sulfate reducing bacteria are available in the estuarine and marine sediments[1], the chemical reaction between HS^- and Fe^{2+} must produce H^+ in the interaction zones. Near neutral or slightly alkaline nature of the estuarine and marine sediments[22] will definitely favour the formation of soluble HPO_4^{2-} from PO_4^{3-} ions. Keeping this in mind soluble phosphate content in broth was matched with a calibration curve prepared from K_2HPO_4 instead of KH_2PO_4. Once HPO_4^{2-} ion is released fulfilling the metabolic demand of sulfate reducing bacteria in sediment, the excess amount will diffuse through water which in turn will be available to other micro-organisms, phytoplanktons and aquatic weeds.

$$Fe^{3+} + e \xrightarrow[\text{biological}]{\text{chemical or}} Fe^{2+} \quad \ldots\ldots\ldots (1)$$

$$SO_4^{2-} + 9H^+ + 8e \xrightarrow{\text{biological}} HS^- + 4H_2O \quad \ldots\ldots (2)$$

$$Fe^{2+} + HS^- \longrightarrow FeS + H^+ \quad \ldots\ldots (3)$$

$$PO_4^{3-} + H^+ \longrightarrow HPO_4^{2-} \quad \ldots\ldots\ldots (4)$$

$$H_2O \rightleftharpoons H^+ + OH^- \quad \ldots\ldots (5)$$

Reaction (1) takes place chemically or biologically when E_h comes to +300 mV (ref.6) but reaction (2) is entirely biological and takes place when sulfate serves as terminal electron acceptor to anaerobic sulfate reducing bacteria at E_h as low as −150 to −200 mV (ref.21). Reactions (3) and (4) are probable reactions when Fe^{2+} and HS^- ions are available. Insoluble PO_4^{3-} becomes soluble by the action of free H^+ ion. Accumulation of black FeS in artificial medium or in natural sediment supports the possibility of reaction (3). Accumulation of soluble phosphate and near neutral to slightly alkaline *p*H recorded in culture broths support the feasibility of step (4) reaction while step (5) reaction may help in a large extent to maintain *p*H balance and partially in supplying H^+ ions to step (2) reaction.

Acknowledgement

The authors gratefully acknowledge the Director, CMFRI, Cochin for providing facilities needed for the study.

References

1 Fenchel T M & Jorgensen B B, *Adv Microbiol Ecol*, 1 (1977) 1.
2 Fenchel T M, *Soc Gen Microbiol Quart*, 6 (1978) 7.
3 Postgate J R, *The sulfate reducing bacteria*, (Cambridge University Press, Cambridge) 1979.
4 Herbert R A, *J Exp Mar Biol Ecol*, 18 (1975) 215.
5 Blake D & Liftley J W, in *Biology of benthic organisms*, edited by P O Keegan, P O Ceidigh & P J S Boaden, (Pergamon Press, Oxford) 1977, 79.
6 Jones J G, in *Sediment microbiology*, edited by D B Nedwell & C M Brown, (Academic Press, New York) 1982, 107.
7 Hayman D S, in *Soil microbiology*, edited by Walker N, (Butterworths, London) 1975, 67.
8 Cohen B, in *Manual of microbial methods*, (McGraw Hill Book Co, New York) 1957, 72.
9 Strickland J D H & Parsons T R, in *A practical handbook of sea water analysis*, (Fisheries Research Board of Canada, Ottawa) 1968.
10 Bjorndhal H, in *Manual of methods in aquatic environment research (FAO fisheries technical paper No.* 137*)* 1975, 33.
11 APHA, *Standard methods for the examination of water and waste water*, (American Public Health Association, Washington) 1975, pp.1193.
12 Starr M P, Stolp H, Truper H G, Balows A & Schlegel H G (Eds.), *The procaryotes*, Vol. 1, (Springer-Verlag, Berlin) 1981.
13 Ninawe A & Banik S, *J Indian Soc Coastal Agric Res*, 6 (1988) 163.
14 Buchanan R E & Gibbons N E (Eds.), *Bergey's manual of determinative bacteriology*, (Williams and Wilkins, Baltimore) 1974.
15 Rodina A G, in *Methods in aquatic microbiology*, edited by R R Colwell & M S Zambruski, (University Park Press) 1972, 251.
16 ZoBell C E, *Producers Monthly*, 22 (1958) 12.
17 Laanbroek M J & Pfenning N, *Arch Microbiol*, 128 (1981) 330.
18 Banik S & Dey B K, *Zbl Bakt II Abt*, 136 (1981) 478.
19 Blake D, Leftley J W & Brown C M, *J Exp Mar Biol Ecol*, 56 (1982) 115.
20 Masterson C L & Murphy P M, in *Symbiotic nitrogen fixation in plants*, edited by P S Nutman, (Cambridge University Press, Cambridge) 1976, 299.
21 Thauer R K, Jungermann K & Decker K, *Bacteriol Rev*, 41 (1977) 100.
22 ZoBell C E, *Bull Amer Assoc Petrol Geol*, 30 (1946) 477.