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Marine Denitrifying Bacteria from South India

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SUMMARY: Thirty-two isolates from a number obtained from sea water off the South Indian coast, from marine sand and from molluscs, proved to be denitrifiers. The reactions of 20 of these strains on different media show that they fall into five groups; all are considered to be species of *Pseudomonas*, and two of the groups to be new species. It is possible that the observed fluctuations in the nitrate content of sea water are in part due to the activities of denitrifying bacteria.

The true denitrifying bacteria, which reduce nitrates to nitrous oxide and nitrogen, alone cause losses of nitrogen in the nitrogen cycle. The waters of tropical seas appear to be deficient in nitrates (Harvey, 1945; Jayaraman, 1954). Jayaraman believed that the 'seasonal variations in the nitrate content may be due partly to the fluctuations in the number of denitrifiers'. It is possible that tropical waters are poor in nitrates because of denitrification. In this paper, some denitrifying bacteria from South Indian seas are described.

METHODS

The denitrifiers were isolated by plating, usually on sea-water agar, and picking off colonies. The details are given in Table 2. Media for testing denitrifying power were all made up in sea water (see Table 1), as were the media used for classification tests (see Appendix). Aerobic cultures on nitrate and nitrite media were grown in test tubes containing Durham tubes, usually at 37°. For anaerobic cultures, the medium was boiled and cooled before inoculation, and was covered with sterile paraffin.

Nitrite was determined by the Griess-Ilosvay test.

Gas was detected by Durham tubes.

RESULTS

Occurrence of denitrifiers. Denitrifying bacteria could always be isolated from 1 ml. samples of sea water, collected 10 miles off Tuticorin (Madras State), and also collected offshore from Calicut in the Arabian Sea. Of 95 isolates from this locality, 13 were denitrifiers, 9 of which were strictly 'marine' forms (ZoBell, 1948); 2 out of 19 isolates from sea water off Tuticorin were also denitrifiers. The dissolved oxygen content of sea water from which the denitrifiers were isolated was in the range 4·0–6·3 mg./l., and the temperature was 27·1–30·0°. Marine sand collected off Tuticorin contained denitrifiers (3 out of 21 isolates), which could be obtained from 1 g. samples. Denitrifiers were also isolated from green mussels (*Mytilus edulis*), collected inshore off Calicut, and from 1/1000 dilutions made from benthic mollusca such as Chanks (*Turbinella pyrum*) and

Pearl Oysters (*Pinctada vulgaris*) collected off Tuticorin. Some more denitrifiers were isolated on a chitin medium, and three copper-tolerant strains from marine molluscs were also found to be denitrifiers (Sreenivasan, 1956).

Table 1. *Composition of media used*

All media made up in aged autoclaved sea water

(1) Broth media

Ingredient (g./l.)	1 % nitrate broth	0·2 % nitrate broth	0·2 % nitrite broth	0·5 % nitrite–0·2 % nitrate broth
KNO_3	10	2	0	2
$NaNO_2$	0	0	2	5
Meat extract	3	3	3	3
Peptone	5	5	5	5
NaCl	0	0	0	0
pH	$7·0 \pm 0·2$	$7·0 \pm 0·2$	$7·0 \pm 0·2$	$7·0 \pm 0·2$

(2) Other media

	Nitrate and peptone	Ca Lactate (Waksman *et al.*)	Citrate (Giltay)	Glucose (Fred & Waksman)
KNO_3	5	1	2	1
KH_2PO_4	0	0	2	0
K_2HPO_4	1	0·5	0	0·5
$CaCl_2$	0·1	0	0·4	0·5
$MgSO_4$	0·2	0	2	0
$FeCl_3$	0·02	Tr.	Tr.	0
Asparagine	0	0	2	0
Peptone	0·1–1·5	0	0	0
Ca lactate	0	10	0	0
Na citrate	0	0	17	0
Glucose	0	0	0	10
pH	$7·2 \pm 0·2$	—	7·0	7·0

Calcium lactate medium: Waksman, Reuszer, Carey, Hotchkiss & Renn (1933).

Citrate (Giltay): Medium no. 55 in *Laboratory Manual of General Microbiology*, by E. B. Fred & S. A. Waksman (1928).

Glucose: medium no. 56 in the same book. Tr. = trace.

In all, 32 isolations of denitrifying bacteria were made; 20 of these are described in this paper (see Table 2).

Characteristics of the denitrifiers. All the denitrifying bacteria that we were able to isolate appeared to be *Pseudomonas* species. They differed from most other described species of this genus in their ability to grow abundantly in saline media, and at 37°. All of them seemed to need a certain minimum amount of organic matter for denitrification. Increasing amounts of peptone were added to a mineral salt medium containing 0·5 % potassium nitrate; with 1500 p.p.m. peptone all the cultures showed signs of denitrification, but only three isolates were able to denitrify with 1000 p.p.m., though all the cultures could grow with this amount of peptone. Smaller amounts of peptone would support neither growth nor denitrification.

Hydroxylamine, which is a possible intermediate product of nitrate reduction (Tanaka, 1953), was toxic to all the isolates. They all grew in broth containing 0·005 % hydroxylamine, but only one culture (group D) would grow in 0·025 %; it did not, however, produce gas at this concentration.

Table 2. *Source of denitrifying* Pseudomonas *cultures*

Group	No. of isolates	Source	Method of isolation	Presumed identity
A	9	Sea water off Calicut	Sea-water agar plates	*P. marinodenitrificans* n.sp.
	2	Sea-water off Tuticorin	Sea-water agar plates	*P. marinodenitrificans* n.sp.
	1	Marine sand off Tuticorin	Waksman's medium with lactate	*P. marinodenitrificans* n.sp.
	2	Sea water off Calicut	Freshwater agar plates	As above, but able to grow in freshwater media
B	1	Mussels	Sea-water agar	*P. mytili* n.sp.
C1	1	Mussels	Sea-water agar	*P. aeruginosa*
C2	1	Mussels	Sea-water agar	*P. aeruginosa*
D	2	Sea water off Calicut	Sea-water agar at 4°	*P. denitrificans*
E	1	Mussels	Sea-water agar	*Pseudomonas* sp.

Nitrite in 0·2 % concentration was decomposed completely in 48 hr. by all cultures except one (group D). In all cultures except two (groups D and B) gas production was noticed in the presence of 0·5 % sodium nitrite. Only one culture, *Pseudomonas aeruginosa* (group C2) decomposed nitrates with glucose as source of energy and produced gas from the former.

In all cases denitrification took place more rapidly under anaerobic conditions. In open test tubes 0·2 % potassium nitrate was decomposed to gas in 7 days, but the same amount in anaerobic tubes showed gas formation 48–72 hr. after inoculation, and nitrite had completely disappeared in 5 days. The isolates fell into one of five groups (see Table 2).

Group A consisted of 14 strains. Two of them differed from the rest in being able to grow in non-saline media. As will be seen in Table 3, this group is not able to denitrify large amounts of nitrate, for in 1 % nitrate broth it forms nitrite and small amount of gas only; but 0·2 % of nitrate in broth is reduced to gas. The strains in this group can reduce 0·2 and 0·5 % of sodium nitrite to gas; they form gas on Waksman's calcium lactate medium, but do not grow either on Giltay's medium (nitrate, citrate and asparagine) or on the very simple glucose-nitrate medium of Fred & Waksman (see Table 1). As the strains in this group differ from any previously described marine *Pseudomonas* species, we propose to include them in a new species, *P. marinodenitrificans*. The characteristics of this species are given in the Appendix.

Group B consisted of a single culture, isolated from mussels. It was capable of growth in non-saline media, and was a more energetic denitrifier than group A. It reduces 1 % nitrate broth with larger gas volume; 0·2 % nitrate in broth is reduced completely to gas. Though 0·2 % sodium nitrite was reduced to gas, 0·5 % sodium nitrite inhibited gas production. Gas was slowly formed from Waksman's medium and more vigorously from Giltay's medium. But in the glucose + nitrate medium neither growth nor gas production was noted. This is one of the versatile denitrifiers utilizing a large number of amino acids for energy, in contrast to group A (Venkataraman & Sreenivasan, 1955). This culture differs from any hitherto described in the literature, and it is proposed to create for it a new species, *Pseudomonas mytili*. A full description of this is given in the Appendix.

Table 3. *Growth and reactions of groups of strains on different media*

Media	Group A	B	C1	C2	D	I
1 % nitrate broth	Nitrite small gas volume	Gas	Gas	Nitrite small gas volume	Nitrite small gas volume	Gas
0·2 % nitrate broth	Gas	Gas	Gas	Gas	Gas	Gas
	(Reduction takes place in 7 days aerobically, in 2–3 days anaerobically)					
0·2 % nitrite broth	Gas	Gas	Gas	Gas	No reduction: slight growth	Gas
0·5 % NO_2, 0·2 % NO_3 broth	Gas	No reduction: slight growth	Gas	Gas	No growth	Gas
Ca lactate and nitrate	Gas	Gas	Gas	Gas	No reduction: slight growth	No gro
Citrate and nitrate	No growth	Gas	Gas	Gas	Gas	Gas
Glucose and nitrate	No growth	No growth	No growth	Gas	No growth	No red slight

Incubated at 37° (group D at room temperature, 28–30°).

DISCUSSION

The known denitrifying bacteria can be classified in three genera: *Micrococcus* (Kluyver, 1953; Robinson & Gibbons, 1952); *Denitrobacillus* (Verhoeven, 1952); and *Pseudomonas*. Several denitrifying *Pseudomonas* species are known to exist in soil (Christensen, 1903; Meiklejohn, 1940); the most adequately described of these soil forms is *P. stutzeri* (van Niel & Allen, 1952); *P. aeruginosa* and *P. ureae* are also denitrifiers (*Bergey's Manual*, 1948).

But the evidence for the occurrence of denitrifying pseudomonads in the sea is not very clear. There are 13 species of this genus listed as 'sea water to brine' inhabitants in *Bergey's Manual*; the Manual does not mention that any of them produce gas from nitrate, but ZoBell & Upham (1944) say that two of them, *Pseudomonas calcis* and *P. calciprecipitans*, are active denitrifiers. The same authors isolated two new species of marine denitrifiers, *P. azotogena* and *P. perfectomarinus*. Lloyd (1931) described in detail a marine denitrifying organism which she called *Bacillus costatus* (*Vibrio costatus*). Recently, Sreenivasan (1956) has described some marine denitrifiers tolerating high concentrations of copper.

In the present paper we describe 20 isolates, which all reduce nitrate to gas, and all of which we consider to be strains of *Pseudomonas* spp. One isolate we have been unable to allot to a species; others belong to the known species *P. denitrificans* and *P. aeruginosa*; and finally there are several isolates which we consider should be classified in two new species, *P. marinodenitrificans* and *P. mytili*.

It thus appears that denitrifying bacteria are common in the sea off the coasts of South India; this is in striking contrast to the waters of the Gulf of Maine (Waksman, Carey & Reuszer, 1933; Waksman, Hotchkiss & Carey, 1933; Waksman, Reuszer, Carey, Hotchkiss & Renn, 1933). Waksman and his colleagues think that denitrification is not possible at the surface of open seas, as do Thompson & Gilson (1937). These two authors point out that an abundant source of easily oxidizable organic matter is necessary for denitrification, and they suppose that this is not obtainable in the waters of open seas. But ZoBell (1947) cites instances of heterotrophic bacteria in the euphotic zone which get much of their food from organic substances secreted by phytoplankton. Waksman, Carey & Reuszer (1933) noted that *Fucus* material may be used as a source of energy by denitrifying bacteria. Kadota (1951) showed that agar was oxidized by *Vibrio purpureus* during denitrification of potassium nitrate, and symbiotic denitrification was reported by Burgwitz (1935). It thus seems possible that plankton excreting organic matter, decomposing plankton, or other micro-organisms, may provide the organic matter necessary for bacteria to carry out denitrification in the sea. The ubiquitous nature of denitrifiers in marine materials is indeed interesting.

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APPENDIX

Characteristics* of the new species of denitrifying bacteria

	Group A	Group B
Morphological characteristics	Straight rods, 0·4–0·6 × 0·8 μ., single and in pairs, actively motile with a single polar flagellum, Gram-negative, non-sporing, non-capsulated	Rods, straight, 0·5 × 0·8 μ., single and in pairs, actively motile with a single polar flagellum, Gram-negative, non-sporing
Cultural characteristics		
Agar colonies	Circular, grey, translucent flat, smooth, undulate margin	Grey, effuse, thin, smooth circular colonies: entire
Agar stroke	Bluish grey, echinulate, glistening, smooth, adherent, moderate growth	Grey, translucent, moist, glistening, smooth, becoming butyrous; abundant growth
Broth	Heavy turbidity and fragile membranous pellicle and ring. No fluorescent pigment	Uniform turbidity and pellicle but no fluorescent pigment

* All media made up in 'aged' sea water.

APPENDIX (*cont.*)

	Group A	Group B
Biochemical properties	Glucose, sucrose and lactose not fermented; starch not hydrolysed; gelatin not liquefied; litmus milk unchanged; indole and H_2S not produced; nitrates reduced to nitrites and gaseous nitrogen; ammonia not produced from peptone; no growth on potato and on freshwater media without salt; grows on 10% NaCl agar; good growth at 37° and at room temperature (28–32°). No fluorescent pigment. Aerobic, facultative	Acid from glucose, sucrose, maltose, adonitol and sorbitol, but none from glycerol and lactose. Gelatin not liquefied; litmus milk coagulated but not digested; indole and H_2S (peptone iron agar) not produced; nitrates and nitrites vigorously reduced to gaseous nitrogen; fats hydrolysed; grows in fresh water media also; good growth at 37°; no chromogenesis. Aerobic, facultative
Source	Offshore sea water off Calicut and Tuticorin, and marine sand off Tuticorin	Green mussels (*Mytilus edulis*) in inshore sea off Calicut
Name proposed	*Pseudomonas marinodenitrificans* n.sp.	*Pseudomonas mytili* n.sp.

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