Changes in the Nitrate-reducing Community of an Anaerobic Saltmarsh Sediment in Response to Seasonal Selection by Temperature

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Measurement of the nitrate-reducing potential by bacteria in saltmarsh sediment, using a thermal gradient block incubator, revealed seasonal physiological changes in the community. A mesophilic part of the nitrate-reducing community was always present, although it achieved maximum development at the end of the summer and minimum development at the end of the winter. In contrast, a distinct psychrotrophic part of the community achieved maximum development at the end of the winter but disappeared during summer. Chemostat enrichment of nitrate-reducing bacteria at 10°C isolated predominantly Pseudomonas spp., but Vibrio spp. predominated in enrichments at 25°C. The observed seasonal changes in situ might reflect differential seasonal selection of these two groups of bacteria.

INTRODUCTION

The nitrate-reducing bacteria comprise a significant proportion of the general heterotrophic bacterial community in anaerobic environments, including marine sediments. Many facultatively anaerobic bacteria are able to utilize nitrate as a terminal electron acceptor in the absence of oxygen (Painter, 1970), and in freshwater anaerobic sediments nitrate reduction may be quantitatively important in the oxidation of organic carbon (Jones & Simon, 1980). In marine and intertidal anaerobic sediments the contribution to organic carbon mineralization by nitrate reduction tends to be small because of the small amounts of nitrate in both seawater and sediment pore water (Sorensen et al., 1979), but nonetheless there is generally a large potential capacity for nitrate reduction by marine sedimentary bacteria if nitrate becomes available (Nedwell, 1975; Nedwell, 1982).

In the intertidal and coastal environment, bacterial communities are subjected to, and may be modified by, seasonally changing temperature (Sieburth, 1967; Nedwell & Floodgate, 1971; Kaplan et al., 1977). The present work was undertaken in order to examine how seasonal change of temperature influenced and modified the nitrate-reducing bacterial community in an anaerobic saltmarsh sediment, and how the physiological response of the community to environmental temperature varied seasonally.

METHODS

Sampling site. Samples of intertidal sediment were taken from the bottom of a main drainage creek in the Colne Point saltmarsh, Essex, UK (Nedwell & Abram, 1978) at monthly intervals from June 1981 to June 1982. Samples from the 0–2 cm layer of sediment were collected in a completely filled jar which was then stoppered to exclude air. Samples were returned to the laboratory within 2 h. The temperature within the top 2 cm layer of in situ sediment was measured with a mercury thermometer.

Preparation of sediment slurry. Sediment slurry (50%, v/v) was prepared by dilution of the sediment with sodium chloride solution (2-0%, w/v). Sodium chloride solution was used as diluent, rather than seawater, in order to avoid adding nitrate to the sediment. The slurry was mixed thoroughly to ensure homogeneity, and then 18 ml samples of slurry were dispensed into test-tubes, sealed with Subaseals, and gassed out with N2 via hypodermic needles inserted through the septum.

Experimental procedure. For each experiment, 42 replicates of tubes of sediment slurry were prepared and placed
into a thermal gradient aluminium block incubator (Sieburth, 1967). The thermal gradient was maintained between 5 °C and 40 °C by constant temperature water circulators (Churchill Instruments, Greenhill, Middx, UK) at each end of the aluminium block. Holes bored vertically into the block received the test-tubes of slurry. Three parallel rows of 14 holes were bored along the gradient block so that tubes were incubated at approximately 2 °C intervals, with triplicate tubes at each temperature.

The aluminium block of the thermal gradient incubator was insulated on all sides by polystyrene, and kept in a low temperature room (5 °C). A longitudinal row of small vertical holes allowed the temperature of each set of three tubes to be measured with a thermometer. Measurements of temperature with time showed that there was less than ±0.2 °C variation of temperature at any point on the block.

The tubes, after insertion into the block, were left for 15 min to equilibrate to their incubation temperature. Into each tube was then injected 2 ml of a 10 mM-potassium nitrate solution, to give a final nitrate concentration of 1 mM. Duplicate subsamples, each of 5 ml, were withdrawn from tubes at each incubation temperature at intervals of 0, 1, 3 and 5 h, centrifuged at 10000 g for 5 min, and the clear supernatant decanted. The pelleted sediment was then extracted with calcium sulphate solution (Abd. Aziz, 1979) and the extract pooled with the supernatant. Residual nitrate and nitrite were then measured colorimetrically by the method of Strickland & Parsons (1972). Recovery of nitrate was 95.4 ± 0.6%.

Continuous culture enrichment of nitrate-reducing bacteria. The field data indicated (see Discussion) that two communities of nitrate-reducing bacteria, physiologically distinct in response to temperature, coexisted in the saltmarsh sediment at certain times of the year. In order to selectively isolate each community, chemostat enrichments were set up in the laboratory at 10 °C and 25 °C. The medium used was the carbon-limited medium of Brown et al. (1977) amended by the addition of 2% (w/v) sodium chloride and 50 mM-nitrate. This medium contained 0.5 g glucose l⁻¹. The dilution rate was 0.035 h⁻¹, and the culture vessel was stirred to maintain homogeneity. The culture vessel was continuously purged with oxygen-free N₂ (OFN) (British Oxygen Company, UK) and nitrate was the only electron acceptor available.

The medium was initially inoculated with 1 g wet weight of saltmarsh sediment. The chemostats were left for 12 volume changes and the effluents were periodically analysed for residual glucose and nitrate, and for culture density by nephelometer. Glucose was analysed with an enzymic test kit (Boehringer). The chemostats were left running for 14 d (12 volume changes) and had reached steady states by day 11 (9 volume changes), as judged by nephelometer reading and by the residual concentrations of glucose and nitrate. After 14 d the cultures were stopped, and plate-counts of the steady-state cultures were prepared on 1% (w/v) glucose nutrient agar (Oxoid). Triplicate plates were inoculated (0.2 ml inocula) from each dilution prepared, the inocula being spread aseptically with a glass rod over prepared agar plates. One triplicate set of plates was incubated aerobically, and a second triplicate set was incubated anaerobically in anaerobic jars with gas-packs (Oxoid), at the incubation temperature of the continuous culture until it reached the steady states. Further subsamples of the chemostat steady state enrichment cultures were also taken after 14 d in order to determine the response of nitrate reduction by these enrichment cultures to temperature variation. The subsamples of culture (10 ml) were each amended with 1 ml of a sterile solution of glucose to give final concentrations of 10 mM-glucose. The sample tubes were then incubated in the thermal gradient block incubator, and the rates of nitrate reduction at each incubation temperature were determined as for the experiments with sediment slurries.

RESULTS

Experiments with sediment slurry

A plot of ln (residual concentration of nitrate) vs incubation time was made for each incubation temperature and the plot subjected to linear regression analysis. In all cases the plots conformed to straight lines (P < 0.05), indicating that nitrate reduction followed first order kinetics with respect to nitrate concentration. The first order rate constant (k) for nitrate reduction was derived from the calculated slope of the plot for each incubation temperature. For each month's data, the calculated rate constants were then used in an Arrhenius relationship of the form ln k = Ae⁻¹(E/RT). The values of ln k at each temperature were plotted against 1/T, and examples of these plots are shown in Fig. 1, for September 1981, March 1982 and May 1982.
Temperature selection of nitrate-reducers

During September to November, 1981, a single temperature optimum for nitrate reduction was seen at 28 °C (for example, see Fig. 1). After November a second optimum was observed at a temperature slightly lower than 28 °C, and in subsequent months this second optimum increasingly deviated from 28 °C towards lower temperatures (see Fig. 1, for March 1982) The data for May 1982 show the maximum separation between these two optima, at 28 °C and 12.8 °C. In order to illustrate this seasonal variation, the temperature at which the secondary optimum occurred was plotted against time (Fig. 2), together with the temperature measured within the surface layer of the in situ sediment at the time of sampling.

Apart from the optimum temperature, the slope of an Arrhenius plot indicates the physiological response of a population to changing temperature. In a chemical or biochemical system the activation energy can be derived from the slope of the plot, but in a microbiological context the term 'temperature characteristic' has been used for this parameter (Hanus & Morita, 1968). With the present data it was not possible to derive separate temperature characteristics for nitrate reduction by each part of the nitrate dissimilating community because of the overlap of their activities at temperatures between the two optima. However, a straight line relationship was apparent at temperatures below the lower of the two optima. The apparent temperature characteristic was calculated each month from the slope of this part of the Arrhenius plots but there was no seasonal change in the values of the temperature characteristics.

Fig. 1. Arrhenius plots to show effect of temperature in different months upon the rates of nitrate reduction by bacteria in slurries of sediment. Bars indicate SE.

Fig. 2. Seasonal variation in the temperature at which the lower temperature optimum for nitrate reduction occurred. □, In situ sediment temperature; ○, temperature of lower optimum.

Fig. 1

Fig. 2
Fig. 3. Composition of the nitratreducing communities isolated by chemostat enrichment at 10°C and 25°C. Total, total direct count; viable, viable count on glucose nutrient agar; Ps, Pseudomonas spp.; V, Vibrio spp.; Ac, Acinetobacter spp.; Fl, Flavobacterium spp.; En, Enterobacterium spp.; Alc, Alcaligenes spp.

Fig. 4. Arrhenius plots to show effect of temperature upon the rates of nitrate reduction by the nitratreducing communities isolated by chemostat enrichment at 10°C and 25°C. △, 10°C enrichment; □, 25°C enrichment. Bars indicate se.

Continuous culture enrichments

Steady state enrichment cultures were obtained after 9 volume changes in both chemostats, as indicated both by nephelometer readings and by analyses of residual substrate concentrations. Residual glucose was negligible and therefore the culture had to be supplemented by addition of glucose for the subsequent examination of the effect of temperature upon nitrate dissimilation by the enrichment cultures (see Methods). In both chemostats approximately 60% of the nitratre had been removed [residual concentrations 20.6 mM (SD 2.6) at 10°C; 21.7 mM (SD 2.7) at 25°C]. However, the steady state nitrite concentrations showed a marked difference, with over twice the amount in the 25°C culture as compared to the 10°C culture [concentrations 0.395 mM (SD 0.025) at 10°C; 0.938 mM (SD 0.008) at 25°C].

The viable counts obtained anaerobically for the steady state cultures were 4.25 x 10⁶ cells ml⁻¹ at 10°C, and 4.35 x 10⁷ cells ml⁻¹ at 25°C. The aerobic plate counts were not significantly different from the anaerobic counts, indicating a facultatively anaerobic community. Six distinct colony forms were present in the 10°C enrichment and eight in the 25°C enrichment. All were Gram-negative rods, and up to five examples of each colony form were picked off and identified as far as possible by the taxonomic scheme of Gibson et al. (1977). In some cases, fewer than five examples of a colony type were present, and then all examples of that colony type were isolated. In all cases, all examples of a colony type keyed out to the same genus, indicating that the differentiation of the community on colony form was acceptable. The log density of each genus present in the two enrichment cultures is shown in Fig. 3. In the 10°C enrichment 98% of the community was Pseudomonas spp. comprised of two distinct populations. A large yellow-pigmented colony form was 79% and a small white colony form was 19% of the total steady-state community density. Vibrio spp. comprised 3.3% of the total community at this temperature. At 25°C 94.4% of the total community was Vibrio spp. with negligible numbers of Pseudomonas spp.

Examination of the ability of the two enrichment cultures to dissipitate nitratre over a range of temperature revealed distinct temperature optima; at 13.4°C for the 10°C enrichment, and 30.7°C for the 25°C enrichment (Fig. 4). The temperature characteristics for the two enrichments were 97.9 kJ mol⁻¹ at 10°C, and 59.7 kJ mol⁻¹ at 25°C.
DISCUSSION

Nitrate reducers comprise a taxonomically diverse group of micro-organisms (Painter, 1970), many facultatively anaerobic bacteria being able to respire nitrate in the absence of oxygen. Previous work has shown the presence of nitrate-reducing bacteria in many marine sediments, together with high capacities for nitrate reduction within such sediments (Nedwell, 1975; Kaplan et al., 1977; Oren & Blackburn, 1978; Sorensen, 1978; Nedwell, 1982). Specifically, in saltmarsh sediments, there is a considerable capacity for nitrate reduction (Nedwell, 1982) although the actual rate may be limited by low concentrations of nitrate in these predominantly anaerobic sediments (Abd. Aziz, 1979). In the present work our techniques did not examine the in situ rates of nitrate reduction, but rather were an examination of the potential physiological capacity of the in situ nitrate-reducing microbial community to reduce nitrate in relation to environmental temperature and seasonal temperature change.

The experiments with slurries of sediment showed that nitrate reduction was always first order with regard to nitrate concentration, and the change in nitrate reduction rates with temperature revealed the presence throughout the year of an optimum temperature for nitrate reduction at about 28 °C (range 27–29 °C). This showed the permanent presence of a mesophilically adapted part of the nitrate-reducing community. However, during December 1981, a second distinct temperature optimum became apparent at 21-5°C, and during succeeding winter months this second optimum increasingly diverted from 28 °C, reaching a minimum value at 12-8 °C during May 1982 (Fig. 2). This second, lower temperature optimum implied the emergence during the colder winter of a distinct psychrophilic or psychrotolerant community which was physiologically better adapted to reduce nitrate at lower environmental temperatures than was the mesophilic community. Kaplan et al. (1977) have also previously deduced the seasonal selection by temperature of two distinct denitrifying communities in the sediment of a North American saltmarsh. The lack of significant seasonal changes in the measured temperature characteristics of the psychrotrophic part of the community supports the conclusion of other workers (Harder & Veldkamp, 1971; Morita, 1975; Abdollahi & Nedwell, 1978; Reichardt & Morita, 1982) that this parameter does not reflect physiological adaptation. Maximum development of the psychrotrophic nitrate-reducing community was observed during May 1982, at the end of the cold winter period and, in contrast, at the end of the warm summer period during October 1981, the psychrotrophic community was apparently absent. The seasonal change in the temperature of the lower optimum that occurred demonstrated seasonal physiological adaptation to changing temperature, although, as shown in Fig. 2, the adaptation was 2-3 months out of phase with the seasonal cycle of sedimentary temperature. Similar 2-3 months lag periods for physiological adaptation to seasonally changing temperature have also been reported for the heterotrophic bacterial communities of an intertidal sediment (Nedwell & Floodgate, 1971) and of sea water (Sieburth, 1967). The secondary temperature optimum, indicating development of a psychrotrophic community, was first observed in December 1981, some 2 months after the sediment temperature had decreased to about 10 °C (Fig. 2). Both Sieberth (1967) and Nedwell & Floodgate (1971) have previously shown that the environmental temperature must fall below 10 °C before a psychrophilic or psychrotolerant microflora will develop.

Although the mesophilic nitrate-reducers were present throughout the year, their relative importance to overall nitrate-reducing capacity by the total nitrate-reducing community might nonetheless change seasonally. Any seasonal variation of the mesophiles could be examined by comparing the monthly rates of nitrate reduction at 28 °C, where the contribution of the mesophiles was maximum while that of the psychrophilic was only small (Fig. 5). Variations in the rates of activity at this constant temperature must largely reflect seasonal changes in relative activity of the mesophilic community, due to variations in factors including population size. These data clearly show that although the mesophiles were permanently present throughout the year the mesophilic community appeared to achieve maximum development at the end of summer when the psychrotrophic community, in contrast, has disappeared. The mesophilic community then decreased in significance during the winter, the period when the psychrotrophic community developed.
The experiments with continuous culture enrichments, at two temperatures chosen to select for the two parts of the nitrate-reducing community, confirmed our previous conclusions. Steady state cultures were obtained whose physiological characteristics with respect to nitrate reduction reflected those deduced from the slurry experiments. The steady state culture selected at 10°C had an optimum at 13.4°C, closely reflecting the minimum temperature optimum detected in the sedimentary community at the end of the winter period, while the 25°C enrichment had a temperature optimum at 30.7°C which was similar to that for the mesophilic nitrate-reducing community in the sediment. Examination of the bacteria present revealed the predominance of Pseudomonas spp. at 10°C, whereas Vibrio spp. predominated at 25°C. This implies that the psychotrophic community which became apparent during the winter in the saltmarsh sediment was likely to have been Pseudomonas spp., while the mesophilic community was predominantly Vibrio spp. Further work to count and identify the in situ nitrate-reducing community is needed to confirm this.

Dunn et al. (1980) isolated nitrate-reducing bacteria from the sediments of the River Tay, Scotland, using an MPN technique with incubation at 15°C. The Aeromonas/Vibrio group predominated. With C-limited chemostat enrichments, the carbon substrate influenced the organisms selected and with 0.4 M-sodium chloride (seawater concentration, as in our study) fermentative enterobacteria were in the majority, whereas on acetate medium pseudomonads were the major group. Interestingly, pseudomonads appear to denitrify nitrate to gaseous products, and little nitrite is produced (Herbert et al., 1980; McFarlane & Herbert, 1982). In our work, the chemostat enrichment at 10°C, which was dominated by pseudomonads, produced both a smaller concentration and a smaller proportion of nitrite at steady state than the 25°C enrichment where vibrios dominated. This tended to support the difference of products of nitrate reduction with metabolically oxidative pseudomonads denitrifying nitrate, and metabolically fermentative bacteria such as vibrios reducing nitrate to nitrite and perhaps ammonium (Cole & Brown, 1980).

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REFERENCES


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Temperature selection of nitratereducers


