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Degradation of Putrescine and Cadaverine in Seawater Cultures by Marine Bacteria

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Marine bacteria removed two diamines, putrescine and cadaverine, from coastal seawater supplemented only with these compounds. Batch cultures of natural bacterial communities were grown in filtered seawater (0.05 μm) supplemented with 500 μg of putrescine or cadaverine per liter. Increases in bacterial cell number were counted with an epifluorescence microscope after acridine orange staining. Removal of diamines from seawater was monitored by high-performance liquid chromatography. Diamines were removed from the seawater cultures within 48 h with no corresponding increase in bacterial yield, growth rate, or viability relative to control (unsupplemented) cultures. Shipboard experiments with open-ocean deep water (1,500 m) showed similar, if slower, removal of putrescine from seawater. Unlike uptake experiments with amino acids, labeled putrescine experiments indicated that most putrescine carbon is mineralized to CO_2 rather than assimilated by the bacteria. After growth in unsupplemented control cultures, the bacteria showed a significant potential to mineralize putrescine, indicating a general degradation potential for this compound by marine bacteria even if the compound was not present during growth. Indicators of metabolic activity such as glucose and glutamic acid uptake and mineralization were not affected by the presence of putrescine. This shows that at the concentrations added, the diamines are not toxic, and therefore detoxification was not the reason for degradation of the diamines by the bacteria.

The diamines putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) are found in high concentrations (millimoles per liter) in many groups of organisms (6, 28). They are part of a group of natural polyamines which serve as stabilizing cations of the macromolecular structure of DNA and RNA and can be expected to be abundant in all living matter (6, 7). The abundance of these diamines is documented in all major groups of marine organisms such as algae (23), invertebrates (20), vertebrates (6), and microorganisms (6). It can therefore be expected to find these compounds in marine environments, especially during phytoplankton blooms in the euphotic zone. Another source for these substances is the degradation of proteins and amino acids. Deamination of lysine or ornithine results in cadaverine or putrescine, respectively (21). These two sources, direct release from dead or living organisms and degradation of proteins, should account for a certain input and flux through marine ecosystems.

The biosynthesis and metabolism of putrescine and cadaverine is well studied in most organisms (6, 25, 28). On the other hand, little is known about their degradation and removal in natural environments. It is known that some polyamines have toxic effects on fish and other aquatic organisms in connection with phycotoxins (24).

The purpose of this study was to investigate bacteria as a possible mechanism for removal of diamines from seawater because marine bacteria are thought to be important in controlling the distribution of organic carbon in the sea (4, 15, 18, 19, 22, 29, 31, 32). Sterile, particle-free, unsupplemented or diamine-spiked seawater from different water bodies was used as an experimental growth medium for uptake studies with marine bacteria. An inoculum of the microbial (<0.8 μm) community from the same water body was used to test the potential influence of this community on putrescine and cadaverine and vice versa. This mixed culture approach with natural water as growth medium has been used by Russian aquatic microbiologists with different modifications for growth rate studies since the late 1940s (26). More recently, Christian et al. (5) and Kirchman et al. (16) used similar approaches for calibration and testing of modern techniques of in situ growth rate measurements. Three different questions will be addressed in this study with the "seawater culture" approach (1). (i) Is there a natural potential in the marine microbial community to degrade diamines? (ii) What is the biochemical fate of the diamines? (iii) Do diamines inhibit or intoxicate microbial growth and metabolic activity?

MATERIALS AND METHODS

Batch culture experiments. Batch cultures for the coastal seawater experiments used water collected during August 1982 and March 1983 from Chemotaxis Dock in Vineyard Sound, Woods Hole, Mass. Seawater was filtered first through combusted glass-fiber (Whatman GF/F) filters and then through sterile 0.05-μm Nuclepore filters and spiked with the appropriate amount of filter-sterilized putrescine or cadaverine solution. Putrescine or cadaverine was added to 100 ml of seawater in glass or polycarbonate 250-ml Erlenmeyer flasks to a final concentration of 500 μg/liter. Each of these cultures was run in duplicate. Two types of controls were run. Two flasks were not supplemented with either diamine to measure bacterial growth in the absence of any added compound. Another flask of seawater was autoclaved before sterile addition of 500 μg each of putrescine and cadaverine per liter. This flask was used to determine the fate of the diamines in the absence of bacteria. All the culture flasks were allowed to equilibrate to a temperature of 22°C on a shaker table (100 rpm) for 30 min. An inoculum of Chemotaxis Dock water equal to 5% of the media volume was added to each culture (time 0). The inoculate had been filtered through a sterile 0.8-μm Nuclepore filter to reduce the concentration of protozoa and larger organisms.

Cultures were sampled every 4 h for 32 h, and final

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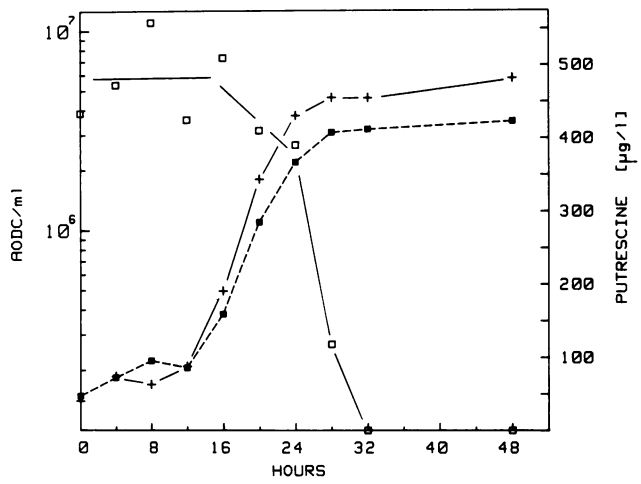

FIG. 1. Bacterial cell counts (AODC) as a function of time during batch cultures with unsupplemented (+) and putrescine-supplemented (500 µg/liter) (■) near-shore seawater. All AODC values are averages of at least two different flasks. Also shown is the putrescine concentration in the spiked cultures, average of duplicate flasks (□).

samples were taken at 48 and 72 h. Stringent sterile techniques were only used for the sterile controls. For the inoculated flasks, semisterile sampling techniques were used to reduce the chances of protozoan contamination. In fact, protozoa were observed (D. Caron, personal observation) only in the later stationary phase of bacterial growth in one set of cultures. Samples for bacterial enumeration were preserved in 2% formaldehyde and refrigerated. Samples for diamine analysis were immediately filtered through 0.2-µm membrane (Amicon Corp.) filters and frozen at −20°C. ^{14}C activity measurements were done immediately after the relevant samples were taken. During all experiments, no attempt was made to keep cultures in the dark; they were exposed to fluorescent light for about 12 h each day. Addition of putrescine or cadaverine did not alter the pH of the seawater. During the experiments, however, the pH of the cultures dropped slightly from 8.1 to 7.9.

A similar batch culture experiment was conducted during cruise 126 aboard R/V Oceanus in September 1982. Seawater was collected by sterile Niskin bag sampler from 1,500 m at 44° 51′ N, 47° 31′ W on the Grand Banks east of Newfoundland in 3,600 m of water. The same experimental scheme was followed as for the coastal water samples, except only polycarbonate Erlenmeyer flasks and a surface inoculum from the collecting site were used.

Bacterial enumeration. Acridine orange direct counts (AODC) were performed with acridine orange staining and epifluorescence microscopy according to the method of Hobbie et al. (13). The number of grids counted was optimized for the number of bacteria on the membrane filter (17).

High-performance liquid chromatography analysis. Putrescine and cadaverine in the culture samples were measured by reverse-phase high-performance liquid chromatography of the fluorescent *o*-phthaldialdehyde (22; C. Lee and L. A. Hicke, personal communication).

[^{14}C]putrescine, glucose, and glutamic acid uptake and mineralization. To determine the turnover potentials of putrescine, glucose, and glutamic acid and the effects of putrescine on the metabolism of bacteria growing in the cultures, the uptakes and mineralizations of [1,4-^{14}C]putrescine (hydrochloride; specific activity, 0.69 mCi/mg; final concentration, 5 µg/liter; New England Nuclear), D-[*U*-^{14}C]glucose (specific activity, 1.5 mCi/mg; final concentration, 92 µg/liter; Amersham), and L-[*U*-^{14}C]glutamic acid (specific activity; 2 mCi/mg; final concentration, 58 µg/liter; New England Nuclear) were measured. A 1-ml portion of culture suspension was taken from the relevant batch culture and added to the labeled substrate in a test tube sealed with a rubber plug to which a CO_2 trap was attached. This CO_2 trap contained 100 µl of a Hyamine hydroxide solution (New England Nuclear) on filter paper (Whatman no. 1) and was installed under the rubber plug (12). The test tubes were incubated for 1 or 2 h on a rotary shaker (100 rpm) at the same temperature as the batch cultures. After this incubation, the reaction was stopped by adding 50 µl of 5 M sulfuric acid. The CO_2 was completely removed from the liquid phase by shaking the test tubes at 200 rpm for another 1 h. Bacterial incorporation was then determined by measuring the radioactivity retained on a 0.2-µm membrane filter (Amicon). Mineralization was estimated by measuring the radioactivity retained by the CO_2 trap. All radioactivity measurements were made with a Beckman LS 100 scintillation counter with Aquassure (New England Nuclear) as scintillation fluid. Quench corrections and corrections for trapping efficiency (94%) were made with internal ^{14}C standards.

Syringe experiment with [^{14}C]putrescine. To determine the fate of the putrescine in seawater, [1,4-^{14}C]putrescine (hydrochloride; specific activity, 0.69 mCi/mg; final concentration, 5 µg/liter; New England Nuclear) was added to Nuclepore membrane-filtered (0.8 µm), unsupplemented coastal seawater. A 250-ml portion of this filtered [^{14}C]putrescine-spiked seawater was sucked into a glass syringe without any gas phase; 10-ml samples were withdrawn with a small syringe connected to the tip of the large syringe with silicone tubing. This method made it possible to take samples without any air contact. The labeled putrescine in the samples was measured in four different fractions. (i) A 3-ml fraction was immediately filtered through a 0.2-µm membrane filter (Amicon) and rinsed twice with 10 ml of filtered, natural seawater to determine the bacterial uptake. (ii) A 5-ml fraction was injected through a rubber seal into a 25-ml

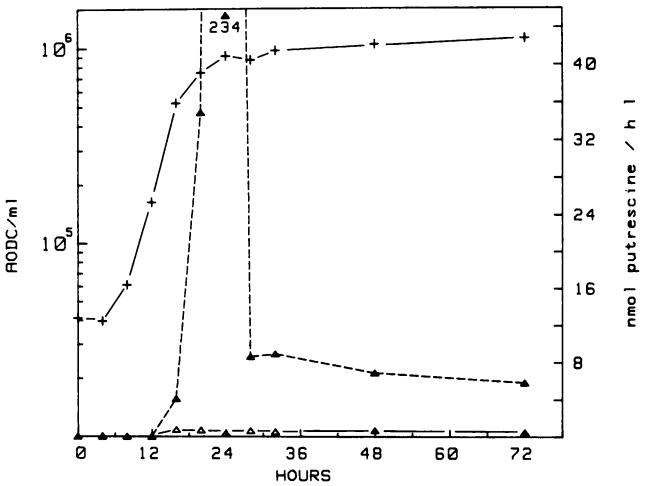

FIG. 2. [^{14}C]putrescine mineralization potential in batch cultures with coastal seawater. Symbols: ▲, putrescine-spiked flask; △, unsupplemented control; +, AODC (mean of all cultures).

Erlenmeyer flask without any air contact. This suspension was then acidified with 100 μl of 5 M sulfuric acid and shaken for 1 h on a rotary shaker (200 rpm). The mineralized $^{14}CO_2$ of this 5-ml culture suspension was also measured by trapping it with a Hyamine hydroxide-soaked filter paper. (iii) The amount of [^{14}C]putrescine incorporated into the macromolecular fraction of the bacteria was determined by filtering 3 ml of the degassed, acidified suspension through a 0.2-μm membrane filter (Amicon). (iv) The radioactivity of the dissolved compounds left in the seawater was determined from the filtrate of this acidified suspension.

RESULTS AND DISCUSSION

Coastal seawater cultures. Bacterial growth curves measured as AODC of all batch cultures with coastal seawater were similar, whether unsupplemented or supplemented with 500 μg of putrescine or cadaverine per liter (Fig. 1). The length of the lag phase was 12 to 16 h in every case, and no significant differences in yield were observed between the cultures. Division rates (0.24 ± 0.04 h^{-1}) and doubling times (2.9 h) calculated for the exponential-growth phase agreed well with results found in experiments with similar approaches (5, 16). All cultures showed no significant differences between any control and supplemented cultures in terms of growth parameters of the AODC.

Toward the end of the growth phase of the bacterial cultures, putrescine decreased to concentrations below 1 μg/liter (Fig. 1). Cadaverine also disappeared, although more slowly (data not shown). Concentrations of both diamines decreased after the concentration of bacteria had increased. This decrease suggests that the presence of larger numbers of bacteria were responsible for the removal of the diamines from solution.

These data suggest that 500 μg of the organic compounds added per liter, about 200 to 300 μg of C per liter for these compounds, did not measurably increase bacterial biomass over the unsupplemented controls. The 500 μg of putrescine added per liter to the cultures represents 270 μg of additional carbon per liter to the system, a 20 to 30% increase in the dissolved organic carbon already present if one assumes a dissolved organic carbon content of 1 to 1.5 mg of C per liter, a low estimate for these waters. If even 25% of this additional carbon were converted to bacterial biomass, final bacterial concentrations would increase by about 5×10^6 cells per ml, assuming a cell density of 0.25 g (dry weight) per cm^3 (9), a cell volume of 0.1 μm^3 per cell (11, 16), and that half of the dry weight of the cell is carbon. Since no difference was observed in cell yield and cell size between control cultures and cultures supplemented with putrescine or cadaverine, one can conclude that the bacteria may not be assimilating putrescine or cadaverine carbon to any great extent.

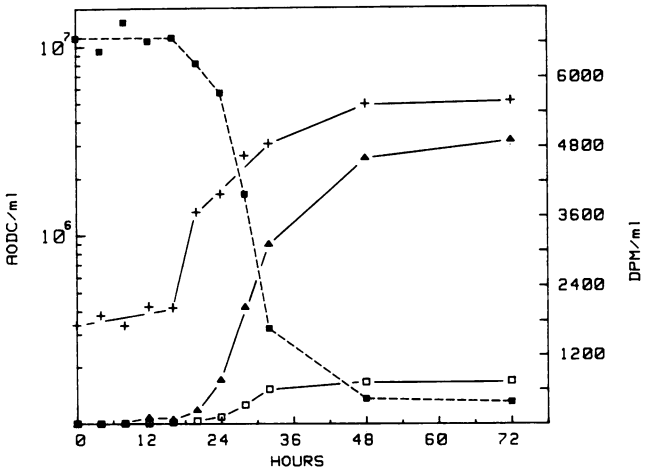

FIG. 3. Degradation of [^{14}C]putrescine in 0.8-μm-filtered coastal seawater during the syringe experiment. Symbols: ■, dissolved ^{14}C; ▲, $^{14}CO_2$; □, ^{14}C taken up by bacteria; +, AODC.

TABLE 1. Amount of [^{14}C]putrescine in various fractions during the syringe experiment with coastal seawater[a]

Time (h)	% Incorporated[b] ± SD	% Uptake[c] ± SD	% Mineralized[c] ± SD	% Recovery[d]
12	5.1 ± 2.8	5.7 ± 1.1	94.3 ± 45.2	IS[e]
16	5.4 ± 1.9	23.5 ± 9.9	76.5 ± 14.5	IS
20	8.9 ± 2.8	16.6 ± 1.7	83.3 ± 8.2	97.6
24	5.6 ± 0.9	12.3 ± 0.2	87.7 ± 12.3	99.2
28	5.4 ± 1.5	13.9 ± 0.2	86.1 ± 5.4	95.1
32	5.2 ± 1.0	16.2 ± 1.7	83.8 ± 15.3	80.7
48	6.2 ± 0.9	13.6 ± 1.2	86.4 ± 2.7	86.4
72	6.6 ± 0.8	13.2 ± 0.6	86.8 ± 4.4	90.5
Mean ± SD	6.1 ± 1.2	14.4 ± 4.6	85.6 ± 4.6	91.7 ± 6.5

[a] Triplicate determinations.
[b] Into the macromolecular fraction, percentage of the total amount of [^{14}C]putrescine turned over as measured by the sum of uptake and mineralization.
[c] Percentage of the total amount of [^{14}C]putrescine turned over as measured by the sum of uptake and mineralization.
[d] Percentage of total available [^{14}C]putrescine, calculated from the sum of the amount turned over and the dissolved part in relation to the total available [^{14}C]putrescine.
[e] IS, Insignificant, <3% of the total available [^{14}C]putrescine.

To determine the biochemical fate of putrescine in nearshore seawater and the rates of its microbial degradation, [^{14}C]putrescine was added (up to 500 μg/liter) to filtered (0.8 μm) coastal seawater. It was not possible to detect a significant uptake or mineralization during incubation times of up to 8 h despite the high putrescine concentrations and the high incubation temperature (22°C). Therefore, the [^{14}C]putrescine uptake and mineralization potentials were followed during a batch culture experiment with coastal seawater spiked with 500 μg of putrescine per liter and unspiked controls. The unsupplemented control in this experiment showed after 16 h a small but easily detectable (four times the blank value) [^{14}C]putrescine mineralization potential of 1.2 nmol per h per liter (Fig. 2). This mineralization potential did not decrease significantly over the next 48 h. The putrescine-spiked cultures showed at the same time a very large increase in [^{14}C]putrescine mineralization potential, which peaked at 24 h at 234 nmol/h per liter. The amount of unlabeled putrescine in these cultures did not decrease significantly during the first 24 h. After 28 h, no putrescine was detectable. All putrescine mineralization potentials measured in these cultures before 28 h were therefore determined at a substrate concentration of 505 μg/liter (labeled and unlabeled), and all the mineralization potentials measured from 28 h on were determined at a concentration of 5 μg/liter (labeled only). This also means that the [^{14}C]putrescine mineralization potentials measured in control and putrescine-spiked cultures were comparable from 28 h on. The mineralization potential for putrescine in the spiked cultures was therefore only seven times greater than that of the control at 28 h, and because of the decrease of the potential in the spiked cultures of one-third during the

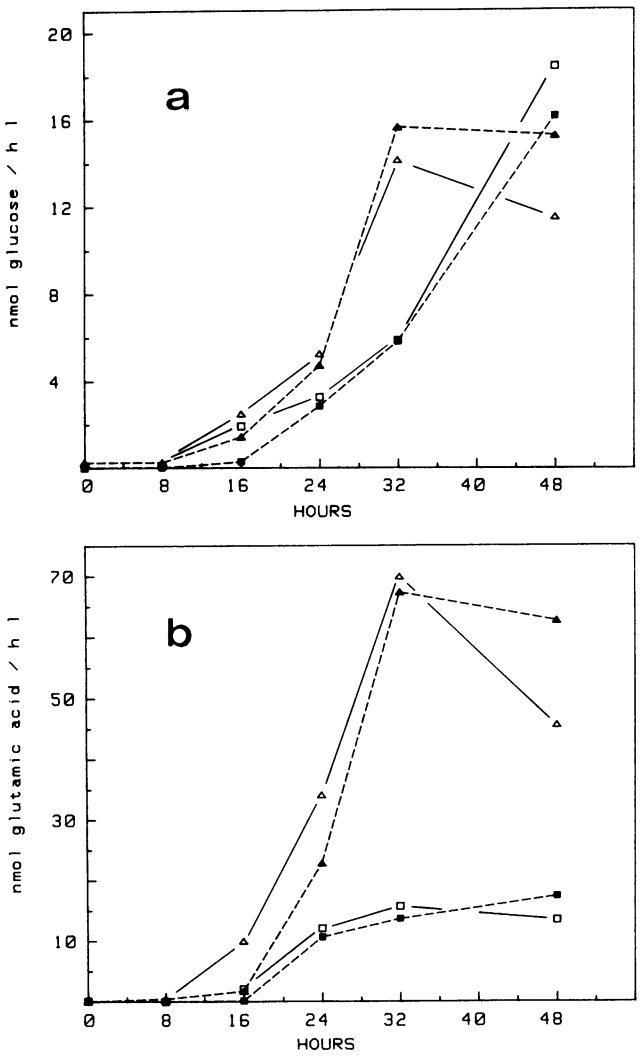

FIG. 4. (a) Uptake (□, ■) and mineralization (△, ▲) potentials of [^{14}C]glucose in filtered, unsupplemented, near-shore water (open symbols) or supplemented with 500 μg of putrescine per liter (closed symbols). (b) Uptake (□, ■) and mineralization (△, ▲) potentials of [^{14}C]glutamic acid in filtered near-shore water unsupplemented (open symbols) or supplemented with 500 μg of putrescine per liter (closed symbols). AODC values are shown in Fig. 1.

following 48 h, it was only five times greater at the end of the experiment. This experiment showed clearly that the bacteria in the control cultures, even if they had never been exposed to putrescine, had a potential to mineralize putrescine after growing for some time in unsupplemented coastal seawater.

During this experiment, the amount of [^{14}C]putrescine incorporated into the macromolecular fraction of the bacteria was also monitored. This amount was rather small (<10% of the mineralized carbon) and most of the time so close to the blank values that a precise determination of the biochemical fate of the putrescine carbon was not possible. To address this question and also to find out how a less-processed sample of coastal seawater degrades putrescine, a syringe experiment was performed. This syringe experiment had the advantage of starting with a high, undiluted natural population and of having no gas phase. Thus, the major biochemical fractions could be better traced and budgeted. Also the amount of metabolized ^{14}C should accumulate and therefore result in higher absolute values. The major biochemical fractions, measured as described above, were: (i) the dissolved putrescine, (ii) the mineralized CO_2, (iii) the amount of putrescine carbon taken up by the bacteria, and (iv) the amount of putrescine carbon incorporated into the acid-precipitable macromolecular fraction of the bacteria. The results of this experiment show that $^{14}CO_2$ was first detectable after 12 h (Fig. 3). Growth of the bacteria during the first 16 h, as indicated by AODC, was either very slow or insignificant. Most of the putrescine was degraded during the second phase of bacterial growth between 24 and 48 h. During this time, the dissolved putrescine decreased to its leftover level (0.3 μg/liter), and a significant fraction of the ^{14}C was found in the bacteria. The budgets, given for any time of significant metabolism of putrescine in Table 1, indicated that the mineralized fraction remained rather constant at 86% and accounted for the bulk of the carbon that disappeared from the dissolved phase. The total amount of ^{14}C found in the bacteria was small (14%) and also constant during the whole time course, except for the first two values in Table 1, when the values of the uptake measurements were close to the blank value of the membrane filter and were therefore subject to a higher systematic error. The amount of ^{14}C incorporated into the macromolecular fraction of the bacteria stayed constant at 6% and accounted for less than half the amount of the total ^{14}C taken up by the bacteria. This indicates that the low-molecular-weight pool of putrescine or its metabolites was responsible for over half of the ^{14}C the bacteria contained. The recovery of the initial ^{14}C in all the measured fractions averaged 92%. This high recovery indicates that hypothetical losses of volatile non-CO_2 compounds must be less than 10%. But its is much more likely that the measured fractions account for all the ^{14}C in the syringe and that small missing fractions and errors in the different quench calibrations total up to the missing percentage. Losses during the CO_2 measurements might be especially responsible for the missing fraction because $^{14}CO_2$ was by far the bulk of the metabolized ^{14}C.

To determine whether the presence of putrescine might be toxic to the microorganisms in the cultures and also to see changes in the biological activity during the experiment, certain catabolic processes were monitored in the presence and absence of putrescine. By adding ^{14}C-labeled glucose and glutamic acid to portions of the relevant cultures, the effect of putrescine on the uptake and mineralization potential of each of these compounds could be measured. There was no significant difference between these metabolic parameters, indicating that the presence of putrescine had no toxic effect on the bacteria (Fig. 4). On the other hand, these heterotrophic potentials showed that the turnover potential for organic substrates increased during bacterial growth and stayed high after the growth. The specific turnover potentials, on a cellular basis, were 20 times greater for glucose and over 100 times greater for glutamic acid at the end of the experiment than at the beginning. This indicates that the bacteria growing in the cultures not only increased their biomass but also their specific heterotrophic activity.

Open-ocean 1,500-m seawater cultures. Similar batch culture experiments were conducted on board R/V Oceanus with oceanic water collected from 1,500 m to test the degradation potential for diamines of a very different microbial community (open-ocean surface) in a seawater poor in organic nutrients. Again, there was no significant difference between bacterial growth rate and yield in unsupplemented

and putrescine- or cadaverine-supplemented seawaters (Fig. 5). In the stationary phase, however, the number of bacteria in the open-ocean water cultures (3×10^5 to 4×10^5 cells per ml) was 10 times lower than in the near-shore water cultures (3×10^6 to 4×10^6 cells per ml). This was probably due to a lower concentration of available substrate, i.e., organic carbon.

The concentration of putrescine decreased in the open-ocean water batch cultures as it had in the experiment with coastal seawater (Fig. 5). The decrease was much more gradual, possibly due to the lower numbers of bacteria present. A lower concentration of bacterial cells does not necessarily indicate lower activity or lower ability on a per-cell basis to take up putrescine. However, the bacteria in the cultures were more likely to be active than bacteria present naturally in seawater since the cells were in the process of growing. Although labeled putrescine was mineralized in the culture experiments with coastal seawater (Fig. 2), it was not mineralized significantly when added to samples taken out of the open ocean. This can be explained by the low numbers of bacteria and therefore low absolute activity of the controls and the high unlabeled background in the putrescine-spiked cultures.

Measurements of ^{14}C-labeled glucose and glutamic acid uptake and mineralization potential in the open-ocean water experiments showed no difference between cultures supplemented with putrescine and those without putrescine (Fig. 6). The absolute uptake and mineralization potentials were two to three times lower in the open-ocean experiments than in the experiments with coastal waters. Since the bacterial cell numbers in the open-ocean water were an order of magnitude lower, this implies that the open-ocean bacteria showed in the deep water a greater average potential per cell for the uptake and mineralization of the organic substrates at the applied substrate concentrations.

Conclusions. The results show that marine bacterial communities from very different locations can remove diamines such as putrescine and cadaverine from different seawaters in mixed-batch cultures. Here the question arises of how representative for in situ heterotrophic potentials are the potentials measured in these cultures. The measured rates

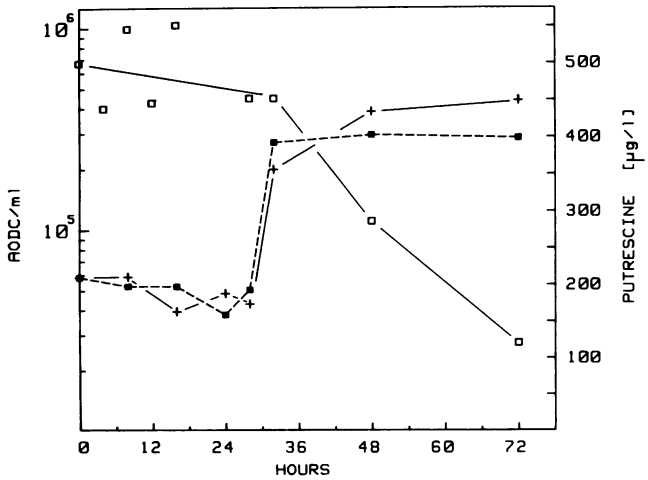

FIG. 5. Bacterial cell counts (AODC) as a function of time during batch cultures with unsupplemented (+) and putrescine-supplemented (500 µg/liter) (■) open-ocean seawater. All AODC values are averages of at least two different flasks. Also shown is the putrescine concentration in the spiked cultures (□).

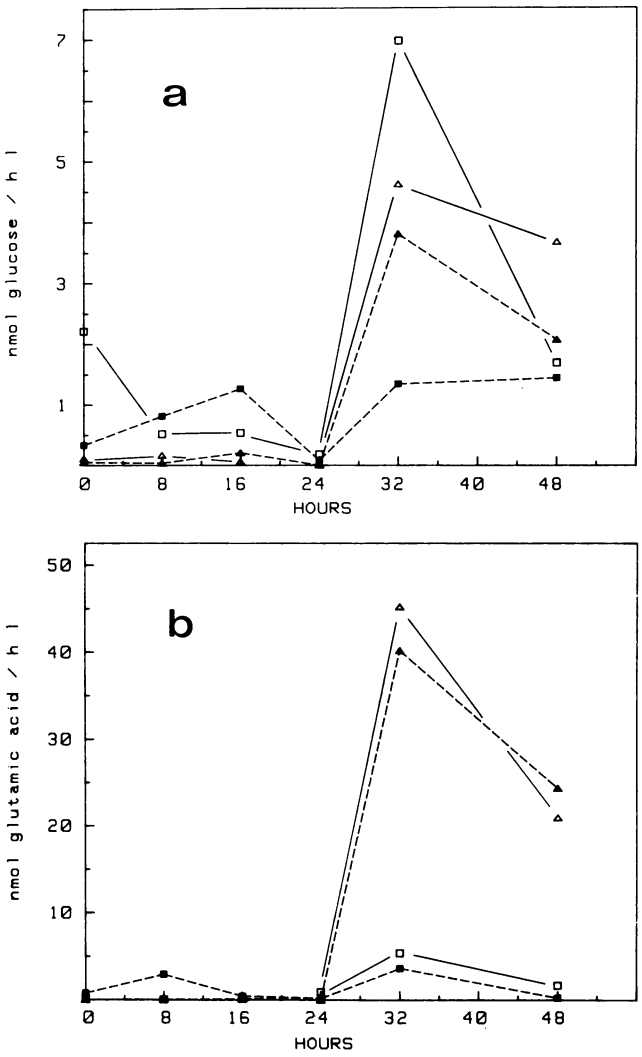

FIG. 6. (a) Uptake (□, ■) and mineralization (△, ▲) potentials of [^{14}C]glucose in filtered open-ocean water unsupplemented (open symbols) or supplemented with 500 µg of putrescine per liter (closed symbols). (b) Uptake (□, ■) and mineralization (△, ▲) potentials of [^{14}C]glutamic acid in filtered open-ocean water unsupplemented (open symbols) or supplemented with 500 µg of putrescine per liter (closed symbols). AODC values are given in Fig. 5.

cannot of course be directly applied to coastal or open-ocean waters, but the general findings should be applicable to marine bacterial communities. Those findings are: (i) an increased mineralization potential for putrescine after its appearance, (ii) a natural background mineralization potential for putrescine in coastal seawater, (iii) a high mineralized fraction of the removed putrescine, and (iv) a resistance of metabolic and growth processes of marine bacteria to high amounts of putrescine and cadaverine.

The high mineralization (86%) of putrescine is unusual. Excretion of by-products cannot be excluded but should play a minor role since over 80% of the ^{14}C ends up in the CO_2 fraction. It is known from pure culture work with *Escherichia coli* (27) that putrescine is taken up by bacteria, but over 95% remained unmetabolized inside the cells. Most other nitrogen-containing compounds like amino acids or purines are also assimilated to a much higher extent (2, 8, 10, 11, 25, 30). An exception are compounds in close relation to

the tricarboxylic acid cycle like malate and glutamic acid (average mineralization of 78% in this study) (11, 12). It is possible that the degradation of putrescine follows a similar pathway. Future work might reveal the biochemical mechanisms involved, with special emphasis on the fate of the nitrogen.

Since the presence of putrescine had no obvious toxic effect on bacterial cultures, it is unlikely that the consumption of these amines was a detoxification reaction. The likely purpose of putrescine uptake and mineralization by marine bacteria is as a source of energy or nitrogen or both (2, 3, 12). If the bacterial community was energy limited and respired putrescine solely to gain energy and not nitrogen, one might expect glucose and glutamic acid to be totally respired also. However, Fig. 4 and 6 showed that glucose and glutamic acid were incorporated into the bacterial cells.

Although the bacteria respired most of the putrescine carbon, the fate of the nitrogen contained in this compound is unknown. It may well have been taken up by the bacteria (14). No increase in growth rate or yield was detectable from the addition of nitrogen to coastal seawater cultures; even the addition of 0.5 mg of ammonia per liter, almost 30 times the background concentration, had no measurable effect on growth (data not shown). Obviously, these cultures were not nitrogen limited.

The mixed batch culture approach described in this study could be used to study these apparent contradictions in the laboratory and help solve some of the questions about heterotrophic potential measurements in situ. On the other hand, this approach can be easily extended to studies of other compounds and other microbial communities. One advantage of this method is that potential effects of the microbial community on compounds available in low or irregular concentration can be studied when bacteria capable of using these compounds are enriched or activated during the growth phase of the culture. Also, effects of microorganisms can be measured as a function of the growth stage of the bacteria rather than during the steady state, which might occur during the incubation of an unfiltered seawater sample.

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