APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2963–2966
0099-2240/87/122963-04$02.00/0

Vol. 53, No. 12

NOTES

Characteristics of Cyclic AMP Transport by Marine Bacteria†

JAMES W. AMMERMAN‡* AND FAROOQ AZAM

Institute of Marine Resources, A-018, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093

Received 2 July 1987/Accepted 4 September 1987

Uptake and autoradiography experiments with natural populations of marine bacteria, seawater cultures, and cultured isolates showed that the high-affinity cyclic AMP transport system in marine bacteria has stringent structural requirements, is found in a minority of cells in mixed bacterial assemblages, and appears to be related to the culture growth state.

Natural assemblages of marine bacteria take up intact adenosine 3′,5′-phosphate (3′,5′-AMP or cyclic AMP [cAMP]), which is dissolved in seawater in extremely low concentrations (ca. 10 pM) (1), by an active transport system with picomolar K_m values (2). In contrast, dissolved 5′ nucleotides like ATP are hydrolyzed to nucleosides at the bacterial cell surface by the enzyme 5′-nucleotidase (3). This enzyme does not hydrolyze cAMP (3; J. W. Ammerman, unpublished data). cAMP in seawater is probably too dilute to be a significant carbon source for bacteria (1). cAMP uptake from seawater, however, can increase the intracellular cAMP concentration of the average bacterium by 0.1 to 3 μM h^{-1} (1). Since concentration changes within this range can regulate catabolic enzyme synthesis in bacteria (14), we are interested in the possible ecological implications of cAMP uptake by marine bacteria. The high-affinity cAMP uptake system may be unique to bacteria in the ocean; it is absent in *Escherichia coli* cultures that have a low-affinity uptake system (9) and in several marine isolates that have been tested (2). In this study we wanted to determine whether cAMP transport has stringent structural requirements and varies with growth state, as would be expected if this uptake has regulatory significance. We also wanted to know whether all bacteria in natural marine assemblages take up cAMP.

Surface seawater was collected with a sterile sampler from the pier of the Scripps Institution of Oceanography (32°53′N, 117°15′W), and 5-m-deep samples were collected 2 km offshore with Niskin bottles. [^{3}H]cAMP (27 Ci $mmol^{-1}$) and [^{32}P]cAMP (1,500 Ci $mmol^{-1}$) were obtained from ICN Pharmaceuticals Inc., Irvine, Calif.). [^{32}P]cAMP was obtained from ICN by special order. Radiochemical purity was checked by thin-layer chromatography (17). Uptake of [^{32}P]cAMP (10 pM), alone or in the presence of inhibitors at 1 μM, was determined in 2- to 4-h incubations at 18°C (2). Formalin-killed blanks (2% Formalin, 15-min preincubation) were subtracted.

Grain density autoradiography was done by the MARG-E method (15), except that bacteria were stained with acridine orange (for epifluorescence enumeration) before filtration. Seawater samples were incubated with 2 nM [^{3}H]cAMP or 10 pM [^{32}P]cAMP plus 1 μM each of unlabeled P_i, 5′-AMP, and 3′-AMP to prevent labeling by cAMP hydrolysis products. NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.) was used for ^{3}H, and NTB3 (Kodak) was used for ^{32}P. Clusters of >3 silver grains on ^{3}H-labeled slides and >10 silver grains on ^{32}P-labeled slides (which had higher backgrounds) were counted as labeled cells. Clusters on slides made from Formalin-killed samples were subtracted.

Three types of cultures were used for studies of cAMP transport (by the method described above) under different growth conditions. (i) Natural bacterial assemblages in filtrates (0.6 μm pore size; Nuclepore Corp., Pleasanton, Calif.) of seawater (gentle filtration) were diluted (2%; vol/vol) into unenriched filtered (0.22 μm pore size) seawater (seawater cultures [4]). (ii) Bacteria in another filtrate (0.6 μm pore size) of seawater were allowed to grow without dilution. (iii) Pure cultures of the marine bacterium *Serratia rubidaea* and a recently isolated uncharacterized marine strain, designated P1, were grown overnight in CP medium (7). (iii) Before uptake was measured, these overnight cultures were diluted with unenriched, unfiltered seawater to approximately natural abundance levels and shaken for 2 h. Bacterial abundances in all three culture types were measured by epifluorescence microscopy (10).

Structural requirements for transport substrates were determined from the degree of inhibition of [^{32}P]cAMP uptake by unlabeled P_i, 3′-AMP, 5′-AMP, or 14 different cyclic nucleotides. Results of previous work (2) have shown that increasing concentrations of three different unlabeled cyclic nucleotides (3′,5′-AMP, 3′,5′-GMP, and 2′,3′-AMP) progressively inhibit [^{32}P]cAMP uptake. We infer from this that these nucleotides are competitive inhibitors and, therefore, are substrates of the transport system. There was an absolute requirement for both the cyclic structure and the 2′ oxygen (Table 1). The structurally related noncyclic compounds 3′-AMP and 5′-AMP were not recognized by the

* Corresponding author.

† Contribution no. 4207 from Lamont-Doherty Geological Observatory.

‡ Present address: Lamont-Doherty Geological Observatory of Columbia University, Palisades, NY 10964.

TABLE 1. Uptake of [^{32}P]cAMP in the presence of inhibitors[a]

Inhibitor	% Inhibition in[b]:	
	Expt 1	Expt 2
None	0[c]	0[d]
P_i	2 ± 3	3 ± 2
5′-AMP	3 ± 1	4 ± 0
3′-AMP	6 ± 3	6 ± 2
3′,5′-AMP	100 ± 0	100 ± 0
2′,3′-AMP	100 ± 0	100 ± 0
1,N^6-etheno 3′,5′-AMP	99 ± 0	98 ± 0
N^6-monobutyryl 3′,5′-AMP	99 ± 0	97 ± 0
2′-*O*-monobutyryl 3′,5′-AMP	91 ± 0	91 ± 0
N^6,2′-*O*-dibutyryl 3′,5′-AMP	84 ± 0	82 ± 1
3′,5′-GMP	100 ± 0	100 ± 0
3′,5′-IMP	100 ± 0	99 ± 0
3′,5′-XMP	94 ± 0	92 ± 0
3′,5′-CMP	87 ± 0	83 ± 0
3′,5′-UMP	97 ± 0	94 ± 0
d3′,5′-AMP	14 ± 4	16 ± 3
d3′,5′-GMP	13 ± 2	13 ± 2
d3′,5′-TMP	5 ± 2	7 ± 1

[a] The substrate concentration was 10 pM, and the inhibitor concentration was 1 μM in all cases.
[b] Values are means ± standard deviations (n = 3).
[c] Control substrate turnover rate was 4.15% h^{-1}.
[d] Control substrate turnover rate was 4.06% h^{-1}.

cAMP transport system (poor inhibitors), as shown previously (2). The cyclic deoxynucleotides also were not recognized by the transport system, although cAMP analogs derivatized on oxygen 2′ of the ribose or nitrogen 6 of the adenine were recognized (effective inhibitors). All other cyclic nucleotides were recognized, including 2′,3′-AMP and cyclic nucleotides with other purine and pyrimidine bases. These specific and stringent structural requirements were observed even though we were working with mixed bacterial assemblages. Thus, the responsive components of the assemblage appear to have similar structural requirements for cAMP transport.

The importance of the reactive 2′ hydroxyl is not unique to cAMP transport; it also imparts important properties to nucleotide polymers. For example, RNA undergoes much more rapid hydrolysis than DNA because the 2′ OH acts as an internal nucleophile and results in the formation of a 2′,3′-cyclic intermediate (12, 19). Polymers of ribonucleotides with the 2′ OH (or 2′-O-methyl) groups also have different conformations and optical activities than deoxyribonucleotide polymers, presumably because of the steric effects of the 2′ substituents (6).

Results of the autoradiography experiments showed that only 2 to 7% of the total cells in natural marine bacterial assemblages from Southern California coastal waters took up cAMP by the high-affinity transport system (Table 2). The first experiment, for which data are presented in Table 2, may not represent strictly high-affinity transport since the substrate concentration was 2 nM, but the results were similar in the two experiments. These are minimum estimates because only large clusters of silver grains were counted (see above) and because exposure times were short. In several recent studies (8, 13, 15, 16) similar methods have been used which combine autoradiography with epifluorescence microscopy to determine the fraction of active bacteria in coastal or estuarine waters. The substrates used in these studies were readily metabolized compounds such as tritiated acetate, amino acids, and glucose, as well as tritiated thymidine, which is the nucleoside that is commonly used in bacterial production studies (8). The level of active bacteria in these studies ranged from 2 to 94%, and mean values were from 31% to greater than 50%. If we assume from the results of the studies mentioned above that half of the total cells in our samples were active, then less than 15% of these active bacteria took up cAMP, and the uptake rates per cell were much higher than suggested previously (1). Since we did not determine the fraction of active bacteria ourselves in parallel samples, however, our results must be interpreted with caution.

Figure 1A to C shows sharp contrasts in the magnitude and temporal patterns of cAMP transport capacity among two of the three types of cultures studied; uptake capacity may be inversely correlated with energy availability. The filtrate (0.6 μm pore size undiluted), which had only the organic nutrients found in seawater, was probably the most energy limited. It showed a threefold increase in cAMP uptake per liter of culture (Fig. 1B) because of de novo synthesis or increased expression of uptake capacity and even a slight increase in the uptake rate per cell (Fig. 1C). The two seawater cultures may have increased their metabolic rates because of the 50-fold dilution with seawater. The nutrient concentration would be the same as that of the undiluted culture described above, but the nutrients available per cell would be 50 times greater because of dilution. Although their cAMP uptake capacity per liter of culture increased about 10-fold, the increase in cell number was much faster, resulting in a 10-fold decrease in uptake per cell within 2 or 3 days (Fig. 1A to C). The two pure cultures did not take up cAMP at all (and are therefore not shown in Fig. 1). These cultures were probably still replete with energy, despite brief (2 h) conditioning in unenriched seawater.

In the classical model (14), the intracellular cAMP concentration in bacteria is low when glucose or another readily metabolizable carbon source is abundant and, therefore, cellular energy is high. Recent observations (5, 18) have complicated this picture somewhat, however. Under these conditions, catabolic enzymes for less desirable carbon

TABLE 2. cAMP autoradiography experiments

Sampling site, depth	Bacterial counts (10^6 cells ml^{-1})	Substrate(s), concn	Incubation time (h)	Exposure time (days)	Uptake (cpm ml^{-1})[a]	% Cells labeled[a,b]
Scripps pier, surface	0.92	[^{3}H]cAMP, 2 nM	5.2	4	873 ± 45 (3)	6.7 ± 2.7 (3)
2 km offshore, 5 m	1.50	[^{32}P]cAMP, 10 pM, + 1 μM each of unlabeled P_i, 3′-AMP, and 5′-AMP	3.9	15	1,263 ± 27 (3)	2.5 ± 0.1 (2)

[a] Values are means ± standard deviations (n is shown in parentheses).
[b] Cells with clusters of three or more silver grains on ^{3}H-labeled slides or clusters of 10 or more silver grains on ^{32}P-labeled slides.

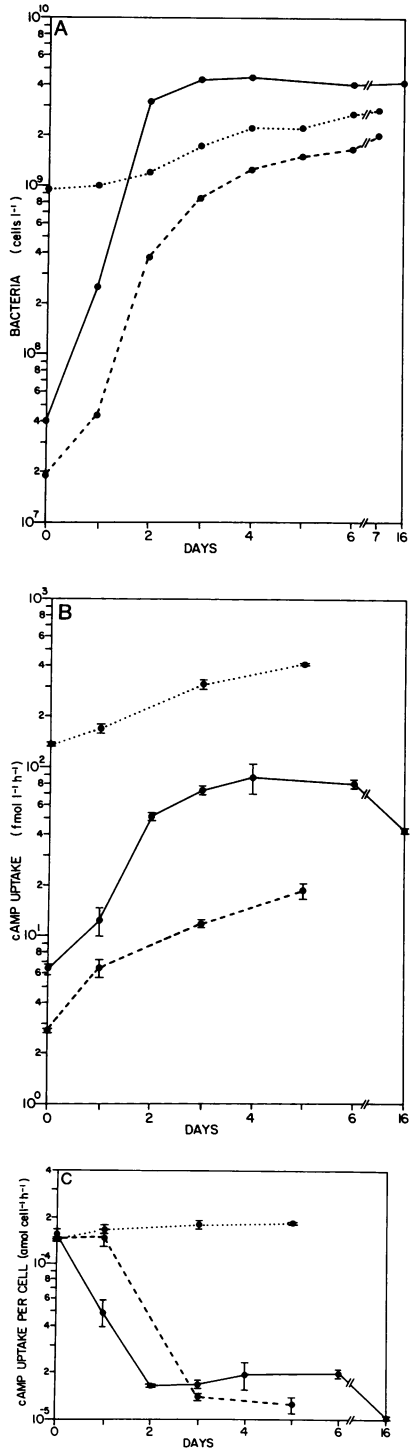

FIG. 1. Variation in bacteria (A), cAMP uptake (B), and cAMP uptake per cell (C) with time in seawater cultures (———, -------), and in an undiluted 0.6-μm filtrate (· · · · ·). Values shown in panels B and C are means ± standard deviations (n = 3), fmol equals 10^{-15} mol, and amol equals 10^{-18} mol. Note the scale breaks in the abscissas.

sources are repressed (catabolite repression). If glucose disappears, then the cells increase their intracellular cAMP concentrations (usually by synthesis from ATP), new catabolic enzymes are made, and other substrates can be used. Our results are consistent with a similar model for natural marine bacterial populations, except that intracellular cAMP concentrations would be increased by uptake. We have no information on cAMP synthesis and did not measure intracellular cAMP concentrations in this study. However, our previous data (2) suggest that natural marine bacteria populations have intracellular cAMP concentrations of approximately 1 μM, which are similar to those reported for *E. coli* and other procaryotes (14). In this scenario, cAMP uptake per cell should increase if energy becomes limiting and causes seawater cultures to enter the stationary phase (Fig. 1C). However, if the stationary phase was caused by nitrogen limitation (11), then no increase in cAMP uptake per cell would be expected.

In conclusion, (i) the cAMP transport system of marine bacteria has stringent structural requirements; only cyclic nucleotides which had a 2′ hydroxyl or derivative were recognized. (ii) Apparently, only a small fraction (2 to 7%) of the bacteria in natural planktonic assemblages rapidly transported cAMP. This, however, still represented 10^4 to 10^5 cells ml^{-1}. Uptake rates were sufficient to increase the intracellular cAMP concentration in these cells by 1 μM within a few minutes. (iii) cAMP uptake capacity varied greatly under different degrees of energy limitation. Therefore, marine bacteria living in the dilute ocean environment may transport and accumulate cAMP for regulatory purposes when energy is limiting.

This study was supported by grants OCE85-18401 (to J.W.A.) and OCE85-01363 (to F.A.) from the National Science Foundation.

LITERATURE CITED

1. **Ammerman, J. W., and F. Azam.** 1981. Dissolved cyclic adenosine monophosphate (cAMP) in the sea and uptake of cAMP by marine bacteria. Mar. Ecol. Prog. Ser. **5**:85–89.
2. **Ammerman, J. W., and F. Azam.** 1982. Uptake of cyclic AMP by natural populations of marine bacteria. Appl. Environ. Microbiol. **43**:869–876.
3. **Ammerman, J. W., and F. Azam.** 1985. Bacterial 5′-nucleotidase in aquatic ecosystems: a novel mechanism of phosphorus regeneration. Science **227**:1338–1340.
4. **Ammerman, J. W., J. A. Fuhrman, Å. Hagström, and F. Azam.** 1984. Bacterioplankton growth in seawater. I. Growth kinetics and cellular characteristics in seawater cultures. Mar. Ecol. Prog. Ser. **18**:31–39.
5. **Botsford, J. L.** 1981. Cyclic nucleotides in procaryotes. Microbiol. Rev. **45**:620–642.
6. **Bush, C. A.** 1974. Ultraviolet spectroscopy, circular dichroism, and optical rotatory dispersion, p. 91–169. *In* P. O. P. Ts'o (ed.), Basic principles in nucleic acid chemistry, vol. II. Academic Press, Inc., New York.
7. **Carlucci, A. F., and D. Pramer.** 1957. Factors influencing the plate method for determining the abundance of bacteria in seawater. Proc. Soc. Exp. Biol. Med. **96**:392–394.
8. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66**:109–120.
9. **Goldenbaum, P. E., and G. A. Hall.** 1979. Transport of cyclic adenosine 3′,5′-monophosphate across *Escherichia coli* vesicle membranes. J. Bacteriol. **140**:459–467.
10. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
11. **Horrigan, S. G., Å. Hagström, F. Azam, and I. Koike.** 1984.

Bacterioplankton growth in seawater may be nitrogen limited. Eos **65**:921.
12. **Mainwaring, W. I. P., J. H. Parish, J. D. Pickering, and N. H. Mann.** 1982. Nucleic acid biochemistry and molecular biology. Blackwell Scientific Publications, Ltd., Oxford.
13. **Meyer-Reil, L.-A.** 1978. Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. **36**:506–512.
14. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5′-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40**:527–551.
15. **Tabor, P. S., and R. A. Neihof.** 1982. Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl. Environ. Microbiol. **44**:945–953.
16. **Tabor, P. S., and R. A. Neihof.** 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl. Environ. Microbiol. **48**:1012–1019.
17. **Tao, M.** 1974. Preparation and properties of adenylate cyclase from *Escherichia coli*. Methods Enzymol. **38**:155–160.
18. **Ullmann, A., and A. Danchin.** 1983. Role of cyclic AMP in bacteria. Adv. Cyclic Nucleotide Res. **15**:1–53.
19. **Westheimer, F. H.** 1987. Why nature chose phosphates. Science **235**:1173–1178.