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Capture of Arginine at Low Concentrations by a Marine Psychrophilic Bacterium†

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The cells of the marine bacterium Ant-300 were found to take up arginine when this substrate was at low concentrations. The cells possessed an uptake system(s) that specifically transported L-arginine. The kinetic parameters for uptake appeared to differ when the cells were exposed to nanomolar and micromolar concentrations of the amino acid. Uptake over this concentration range functioned in the absence of an exogenous energy source, even after the cells had been preincubated in unsupplemented artificial seawater. Respiratory activity appeared to be a more important driving force for arginine uptake than adenosine 5'-triphosphate hydrolysis. The cells also exhibited chemotaxis toward L-arginine. The minimum arginine concentration needed to elicit a chemotactic response was between 10^{-5} and 10^{-6} M. It is proposed that the capture of arginine by cells of Ant-300 in nutrient-depleted waters, which are typical of the open ocean, proceeds via high-affinity active transport, whereas in substrate-enriched seawater, capture involves chemotaxis and an active transport mechanism with reduced affinity for the substrate.

Most of the organic matter from the deep sea is considered to be resistant to microbial degradation (2). The concentration of organic matter in the open ocean, particularly below the thermocline, is generally less than 1 mg/liter (20), and the concentrations of specific organic compounds such as amino acids range from 10 to 30 nM (16). Although never directly demonstrated, organic matter in the ocean is thought to be reduced to these low levels via the mineralizing activities of heterotrophic bacteria. Thus, the bacteria are under strong selective pressure to function efficiently at low nutrient (substrate) concentrations. Marine bacteria, in particular, must be well adapted to scavenge the right molecules needed for cellular maintenance.

In this communication we shall use the term "capture" to refer to the mechanisms by which microorganisms obtain nutrients. Kalckar (14) suggested that binding proteins, located in the bacterial cell envelope, may be a means by which microorganisms scavenge specific substrates from the environment. Recently, binding proteins have been implicated both in the chemotactic response and in high-affinity substrate transport by bacteria, two processes which cooperate in effectively sequestering organic molecules from the environment (1, 2, 11).

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While marine bacteria have been shown to exhibit chemotactic responses to organic molecules (3), their capacity for high-affinity substrate transport has not been well documented. No marine bacterial transport systems have yet been described which function at the low substrate concentrations normally observed in the open ocean.

In this communication we describe one mechanism by which marine bacteria capture arginine and reduce the concentration of this amino acid to levels normally observed in the open ocean. Arginine was chosen in view of the fact that it is a building block for cellular material and may also serve as a source of energy for the cell. Furthermore, arginine is rapidly reduced to very low concentrations in seawater, probably because it is rich in nitrogen and therefore a preferred substrate (12).

MATERIALS AND METHODS

Organism. The marine bacterium referred to as Ant-300 has been tentatively identified as a *Vibrio* species. The psychrophilic nature of the organism has been previously characterized (9).

Uptake studies. All procedures subsequently described were performed at 5°C unless otherwise indicated. Cells were cultured in a Casamino Acids medium composed of: vitamin-free Casamino Acids (Difco), 2.4 g; sodium nitrate, 0.5 g; Rila Marine Mix, (Rila Products, Teaneck, N.J.), 30 g; ferrous sulfate, 0.0005 g; distilled water, 1 liter. The pH after autoclaving was 7.8. Cells were harvested during the logarith-

mic phase of growth (optical density = 0.52 at 600 nm) by centrifugation in a Sorvall RC-2B centrifuge at 3,000 × *g*, washed once in artificial seawater (ASW) [4% Rila Marine Mix in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.8], diluted to approximately 10^7 cells per ml with ASW, and allowed to reduce their endogenous reserves by starving for 18 to 24 h (9). Portions of starved cells (5 ml) were transferred to 25-ml flasks containing chloramphenicol (80 μg/ml) and agitated for 5 min. Energy donors, and metabolic inhibitors, when added, were introduced at the time of chloramphenicol addition. The uptake reaction was initiated by the addition of L-[*U*-^{14}C]arginine (277 to 295 μCi/μmol) at the appropriate concentration. The reaction was stopped by filtration through a membrane filter (0.45-μm pore size, Millipore Corp., Bedford, Mass.), which was completed within 20 s, and the filter was washed with 5 ml of ASW solution. That fraction of radioactivity taken up as arginine which was converted to $^{14}CO_2$ was measured in gas-tight reaction vessels (9). The filter with the cells was air dried for 30 min at room temperature and placed in a scintillation vial containing 5 ml of Omnifluor (4 g/liter of toluene). The radioactivity of each sample was determined in a model I Nuclear Chicago liquid scintillation counter.

Uptake kinetics were determined under conditions outlined by Rosen (23). One-minute uptake periods were chosen where initial rates were required. Substrate concentrations were adjusted so that no more than 10% of the substrate added to the medium was accumulated by the cells. Cell densities were used in a range over which substrate accumulation increased linearly with cell concentration.

The procedure of Reid et al. (22) was used to obtain the kinetic constants (K_t, the transport constant, and V_{max}, the maximum velocity of uptake). A nonlinear least-square regression analysis (program BMD 07R, University of California at Los Angeles computer program library) was used to obtain kinetic parameters from the data. A series of values for the transport constants, K_1 and K_2, and the maximum rate constants V_1 and V_2, were substituted in a modified Michaelis-Menten equation for two simultaneous reactions which are described by the expression:

$$V_t = Y_1 + Y_2 = \frac{V_1 \cdot S}{K_1 + S} + \frac{V_2 \cdot S}{K_2 + S} \quad (1)$$

where Y_1 and Y_2 refer to the velocities of uptake and V, K, and S have their usual meanings.

ATP assay. For assay of cellular adenosine 5'-triphosphate (ATP), cells were cultured as described previously, washed, diluted to a cell concentration yielding 2 mg of cell protein per ml, and allowed to reduce endogenous reserves for 24 h in ASW (9). Chloramphenicol and the appropriate metabolic inhibitor were added to 1 ml of cell suspension and agitated for the desired period of time. The reaction was stopped by addition of 1 ml of 12% perchloric acid, the reactants were neutralized to pH 7.4, and the cellular ATP was extracted by the method of Berger and Heppel (5). After extraction, the ATP was measured by the luciferin-luciferase reaction. Desiccated firefly lanterns were frozen in liquid nitrogen pulverized to powder, and stored at −21°C until used. Twenty-four hours before assay, pulverized lanterns at a concentration of 10 mg/ml were extracted with lantern buffer consisting of 50 mM sodium arsenate and 2 mM magnesium sulfate (pH 7.4) and stored at 2°C. The lantern extract was centrifuged at 2,000 × *g*, and 0.04 ml of the supernatant fraction was added to a reaction mixture containing 0.5 ml of ATP standard or extracted sample and 2 ml of buffer composed of 50 mM sodium arsenate, 5 mM potassium phosphate, and 5 mM magnesium choloride (pH 7.4). The mixture was shaken for 5 s on a Vortex mixer, and the luminescence rate was immediately determined over a 10-s period on a Packard TriCarb scintillation counter on optimum tritium setting with the coincidence switch off. Photons emitted over this period are proportional to ATP concentration in the sample (8). Cellular ATP concentrations were determined from a standard curve using equine muscle ATP as a standard.

Chemotaxis. Cells were cultured in Lib-X broth (10) and examined at intervals for motility by phase-contrast microscopy. Cultures used for chemotaxis studies were harvested from this medium by centrifugation and suspended to an optical density of 0.05 at 600 nm in ASW. After 72 h of incubation, the cells were again centrifuged and resuspended in ASW to a cell concentration of approximately 10^7 cells per ml. The cells were then applied to a chemotaxis chamber similar to that described by Adler (1) with a syringe containing a 21-gauge needle. L-Arginine (free base) was diluted to the desired concentration in ASW, the pH was adjusted to 7.6, and the solution was drawn into 1-μl micropipettes (Drummond Scientific Co.). The micropipettes were inserted into the chemotaxis chamber. After incubation for 1 h, the contents of the micropipettes were expelled into a screw-cap tube containing ASW, mixed vigorously, diluted, and plated on Lib-agar medium.

Protein determination. Protein concentration in cell suspensions was determined by the method of Lowry et al. (19). Bovine serum albumin fraction V was used as a standard.

Chemicals. Chloramphenicol, ATP, firefly lanterns, 2,4-dinitrophenol (DNP), *N,N'*-dicyclohexylcarbodiimide, D-lactate, *N,N,N',N'*-tetramethyl *p*-phenylenediamine, phenazine methosulfate, ascorbic acid, and reduced nicotinamide adenine dinucleotide were obtained from Sigma Chemical Co. Disodium succinate, D-glucose, sodium arsenate, and potassium cyanide were obtained from Fisher Scientific. Sodium amytal was obtained from Eli Lilly and Co. L-[*U*-^{14}C]arginine and Omnifluor were obtained from New England Nuclear Corp.

RESULTS

Cells of Ant-300, previously depleted of endogenous reserve material, were found to take up specific substrates that were present in ASW at low concentrations. Radioactivity introduced as L-[*U*-^{14}C]arginine (0.07 μM) in ASW was concentrated 300-fold by the cells after 60 s. This estimation was based on an average cell volume of 2.2 μm^3, which was calculated from size mea-

surements obtained by phase-contrast and electron microscopy. The cells took up arginine at an extremely high rate during the first 15 s of exposure to the substrate (Fig. 1). This was followed by a lower but more constant rate of arginine uptake by the cells. After 10 min, less than 10% of the radioactivity taken up was respired as $^{14}CO_2$.

In view of the rapid rate at which the radioactivity was taken up by the cells, an attempt was made to determine whether the uptake of arginine was mediated by active transport. Under certain conditions (substrate present at less than saturation concentrations) the active transport of specific substrates by bacterial cells exhibits a linear relationship between accumulation rate and external substrate concentration. The Lineweaver-Burk plot illustrated in Fig. 2 revealed a bimodal relationship for arginine uptake over a range of arginine concentrations from 0.034 to 0.59 μM. When the experimental data were substituted in equation 1 (Materials and Methods), assuming two simultaneous reactions, K_t values of 1.7×10^{-8} and 4.5×10^{-6} M and V_{max} values of 12 and 51 pmol/min per 5×10^7 bacteria, respectively, were obtained.

The uptake system(s) was relatively specific for L-arginine. A 20-fold molar excess of L-lysine or L-ornithine, two amino acids with structures similar to arginine which are normally present in seawater, produced at most a 35% inhibition of L-arginine uptake (Table 1).

Since, by definition, active transport requires the expenditure of energy, an effort was made to determine what energy-yielding pathways were coupled to arginine uptake in cells of Ant-300. In general, the addition of respiratory substrates to the uptake reaction mixture produced a moderate stimulation of arginine uptake (Table 2). The order of effectiveness was succinate > D-lactate > *N,N,N',N'*-tetramethyl *p*-phenylenediamine ascorbate > phenazine methosulfate ascorbate. The presence of D-glucose in the external medium produced a stimulation in arginine uptake by the cells, but this stimulation was only observed during the first 60 s of exposure to the amino acid (Fig. 1).

The effect of various metabolic inhibitors on arginine uptake further supported indications that the accumulation of arginine at low external

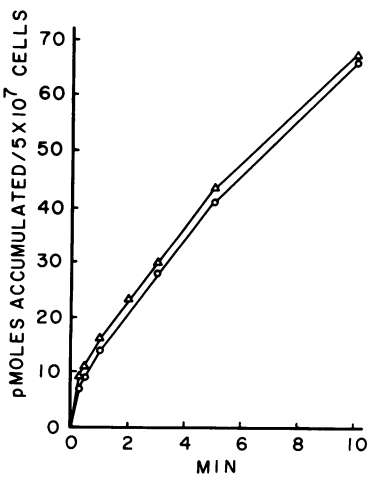

FIG. 1. *Uptake of L-arginine by cells in the presence (△) and absence (○) of glucose. Cells were exposed to L-[U-^{14}C]arginine (0.070 μM) for increased periods of time. Glucose was added to a final concentration of 20 mM.*

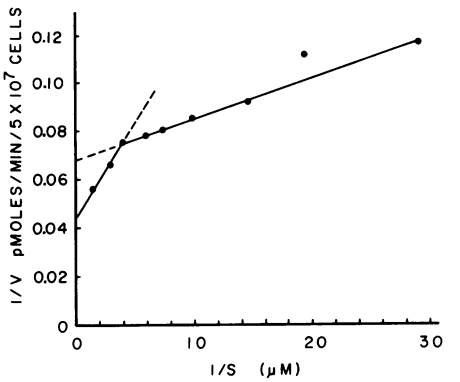

FIG. 2. *Double-reciprocal plot of initial rates of L-arginine uptake by cells of Ant-300. Uptake was carried out as described in the text in the absence of a supplementary carbon source. The points represent the experimental data. The K_t and V_{max} were calculated by substituting experimental data in equation 1.*

TABLE 1. *Specificity of L-arginine uptake systems*[a]

Arginine (μM)	Inhibitor (μM)	Activity[b]	% Inhibition[c]
0.035	None	6.81	0
	L-Lysine (0.72)	5.21	23
	L-Ornithine (0.72)	4.42	35
0.360	None	9.84	0
	L-Lysine (7.20)	8.54	13
	L-Ornithine (7.20)	6.97	29

[a] Cells were prepared as described in the text. The reaction was initiated by simultaneous addition of unlabeled L-lysine or L-ornithine and labeled L-arginine at the final concentrations indicated in parentheses. Arginine uptake was determined for a period of 60 s.

[b] Expressed as picomoles taken up per 5×10^7 cells.

[c] Percent inhibition is based on the amount of L-arginine taken up in the absence of competitor, which is arbitrarily assigned a value of 0.

TABLE 2. *Effect of electron donors on L-arginine uptake*[a]

Electron donor	Activity[b]	
	0.035 μM	0.360 μM
None	6.74	9.01
PMS ascorbate	4.02	8.22
TMPD ascorbate	8.78	13.03
NADH	7.36	10.69
Succinate	10.23	15.78
D-Lactate	8.79	13.75

[a] Arginine uptake was determined at two arginine concentrations (0.035 and 0.360 μM) for 60 s. The final concentrations of the respiratory substrates in the reaction mixture were: phenazine methosulfate (PMS), 150 μM; *N,N,N',N'*-tetramethyl *p*-phenylenediamine (TMPD), 150 μM; ascorbate, 20 mM; reduced nicotinamide adenine dinucleotide (NADH), 4 mM; succinate, 20 mM; D-lactate, 20 mM.

[b] Expressed as picomoles of arginine taken up per 5 × 10^7 cells.

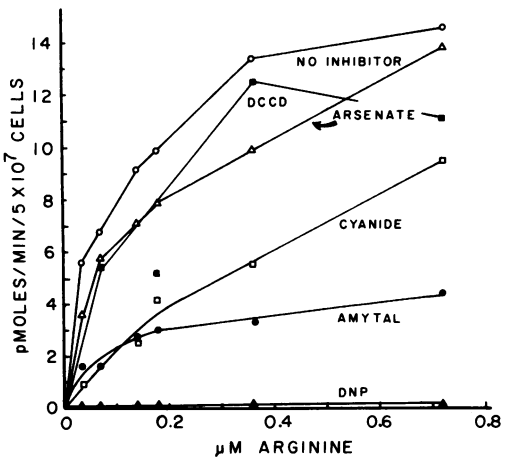

FIG. 3. *Effects of metabolic inhibitors on L-arginine uptake by cells of Ant-300. Cells were treated as described in the text. DNP (2 mM), sodium arsenate (200 mM), N,N'-dicyclohexylcarbodiimide (100 mM), potassium cyanide (20 mM), or sodium amytal (10 mM) was added to the reaction mixture 5 min before the addition of L-[U-^{14}C]arginine in the absence of inhibitor.*

concentrations required the expenditure of energy by the bacteria. The uncoupler, DNP, completely inhibited uptake of arginine by the cells (Fig. 3). The respiratory inhibitors amytal and cyanide also reduced arginine uptake but not to the same extent as DNP (Fig. 3). Chemicals which interfered with the formation or maintenance of ATP, such as arsenate and, *N,N'*-dicyclohexylcarbodiimide had little effect on arginine uptake (Fig. 3). Arsenate, however, was subsequently found to be ineffective in reducing the cellular ATP level below that which existed in cells depleted of endogenous reserve material (Table 3). In contrast, the addition of DNP resulted in a significant reduction in the ATP level of these cells.

Actively growing cells were nonmotile, displaying no detectable chemotactic response to L-arginine when cultured in Lib-X broth. After transfer to ASW, however, the relative number of motile cells increased with time. After 72 h, the majority of cells in suspension were motile. Under these conditions the cells were found to exhibit a positive chemotactic response toward L-arginine. The optimal chemotactic response occurred at an arginine concentration of 10^{-4} M, whereas the threshold concentration (the minimum concentration required for a detectable chemotactic response after 60 min) was between 10^{-5} and 10^{-6} M (Fig. 4).

DISCUSSION

These studies revealed that cells of Ant-300 maintain several mechanisms for the capture of

TABLE 3. *Effects of metabolic inhibitors on intracellular ATP concentration*[a]

Inhibitor	Exposure time (min)	ATP concn (nmol/mg of protein)
None	—	0.865
Arsenate (200 μM)	1	1.103
	5	0.812
	15	0.700
DNP (2 mM)	1	0.091
	5	0.046
	15	0.025

[a] Prior to ATP determination, cells were allowed to reduce endogenous reserves in ASW for 24 h. One-milliliter volumes of cell suspension were exposed to metabolic inhibitors at the concentrations indicated in parentheses for 1-, 5-, and 15-min periods, after which the reaction was stopped and the intracellular ATP levels were determined as described in the text.

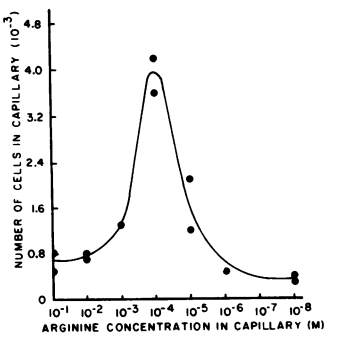

FIG. 4. *Concentration-response curve for L-arginine. Cells were exposed to attractant at 5°C for 1 h.*

nutrients in the marine environment. The kinetic data indicated that high-affinity arginine uptake in this bacterium operated at one-half the maximum velocity (V_{max} = 12 pmol/min per 10^7 bacteria) when the arginine concentration was as low as 17 mM. Since this is within the range of amino acid concentrations found in the open ocean (16), high-affinity substrate uptake is one mechanism by which this marine bacterium can obtain organic molecules for cellular maintenance and growth when nutrient levels are low.

At higher substrate concentrations a different set of kinetic parameters was observed which promoted a greater rate of uptake when the substrate was present in micromolar concentrations. The expression of bimodal kinetics thus enabled cells of this marine bacterium to adapt to a wide range of substrate concentrations. Although the data suggest the operation of at least two independent arginine transport systems, a phenomenon which has been reported in other bacteria (22, 23), it is possible that negative cooperativity (17) or a number of other mechanisms (22) produced the observed bimodal kinetics. Experiments are in progress to investigate this problem in more detail.

One would predict from the results that in the presence of these bacteria, free arginine would disappear approximately four times faster in the micromolar concentration range than in the nanomolar range, following its enrichment in seawater. This is characteristic of the rate of disappearance of dissolved organic carbon from seawater following a phytoplankton bloom in the sense that more than one decay rate has been observed and that these rates vary with concentration of dissolved organic carbon (21).

The inhibitory effects of DNP indicated that L-arginine uptake in cells of Ant-300 was dependent upon an "energized membrane state" formed by a proton gradient across the cytoplasmic membrane. This gradient, which is dissipated by DNP, contributes to a proton motive force believed to drive active transport systems in other bacteria (4). The proton gradient is thought to be generated by the oxidation of substrates along a respiratory pathway or by the hydrolysis of ATP (5).

Several lines of evidence suggested that cells of Ant-300 were more dependent on respiration than on ATP hydrolysis to drive arginine uptake. First, substrates such as succinate and D-lactate were better stimulants of arginine uptake than glucose; oxidation of the former two substrates is closely coupled to respiratory pathways, whereas the latter is primarily coupled to ATP synthesis via substrate-level phosphorylation. Glucose is, however, taken up and metabolized by this bacterium (9). The apparent inhibition of uptake in the presence of the artificial electron donor phenazine methosulfate ascorbate has been observed in other marine bacteria and appears to be an effect produced by phenazine methosulfate specifically (25). Second, greater inhibition of arginine uptake occurred in the presence of chemicals (amytal and cyanide) that blocked various steps of respiration than in the presence of chemicals (arsenate and *N,N'*-dicyclohexylcarbodiimide) that reduced or prevented the formation of intracellular ATP (15). This evidence must be interpreted with caution, though, since arsenate, which is known to deplete some bacteria of their intracellular ATP (5), produced no such effects in cells of Ant-300 that were depleted of endogenous reserve material. The decrease in cellular ATP that occurs in the presence of DNP is thought to be the result of the cells' futile attempt to regenerate a transmembrane potential (13).

Arginine uptake in this marine bacterium was driven, at least initially, by endogenous substrates which were retained by the organism even after 24 h of incubation in unsupplemented ASW. In contrast, enteric bacteria are generally incapable of significant substrate transport activity when subjected to comparable conditions (4). Thus, it is likely that the cells were able to maintain an energy reserve for a limited period under conditions comparable to those of the open ocean. The fact that uptake was sustained for as long as 10 min suggests either that there was substantial energy present in the cells even after the 24-h incubation period or that arginine metabolism was coupled to arginine uptake. It was subsequently shown, however, that the cells did not contain excessive amounts of ATP, but were rather low compared to levels reported elsewhere (18, 24). Further steps to distinguish between these possibilities have not yet been attempted. Nevertheless, this marine bacterium does not appear to require a separate exogenous energy source to drive the uptake of organic nutrients such as arginine. This feature is particularly important since the energy source would likely be present in the same dilute concentrations as, and might even be identical to, the substrate. In this regard, cells of Ant-300 are well adapted to their environment.

Cells of Ant-300 also appeared to possess a mechanism which induced motility and chemotaxis toward arginine only after prolonged incubation in unsupplemented ASW. This type of control represents a potential means for the cells to conserve energy until a time when it becomes advantageous to employ this substrate capture system (i.e., during a period of nutrient enrichment). Chemotaxis appears to be most useful in

guiding the bacteria to areas of nutrient enrichment, since the lowest concentration of arginine able to elicit a detectable tactic response, based on the assay employed, was between 10^{-5} and 10^{-6} M. Since the threshold concentrations fell within the operational range of low-affinity arginine uptake, it seems likely that the two mechanisms cooperate to allow bacteria to locate and utilize arginine when the concentrations of this amino acid are relatively high, such as during phytoplankton blooms (7).

High-affinity uptake appears to be one mechanism these bacteria possess which allows them to take up arginine when the substrate is present in nanomolar concentrations. In view of the normal occurrence of these conditions in the open ocean, this arginine capture mechanism represents one means by which cells of this marine bacterium may obtain organic molecules in between periods of nutrient enrichment.

ACKNOWLEDGMENT

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