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Assimilatory Sulfur Metabolism in Marine Microorganisms: Characteristics and Regulation of Sulfate Transport in *Pseudomonas halodurans* and *Alteromonas luteo-violaceus*†

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Sulfate transport capacity was not regulated by cysteine, methionine, or glutathione in *Pseudomonas halodurans*, but growth on sulfate or thiosulfate suppressed transport. Subsequent sulfur starvation of cultures grown on all sulfur sources except glutathione stimulated uptake. Only methionine failed to regulate sulfate transport in *Alteromonas luteo-violaceus*, and sulfur starvation of all cultures enhanced transport capacity. During sulfur starvation of sulfate-grown cultures of both bacteria, the increase in transport capacity was mirrored by a decrease in the low-molecular-weight organic sulfur pool. Little metabolism of endogenous inorganic sulfate occurred. Cysteine was probably the major regulatory compound in *A. luteo-violaceus*, but an intermediate in sulfate reduction, between sulfate and cysteine, controlled sulfate transport in *P. halodurans*. Kinetic characteristics of sulfate transport in the marine bacteria were similar to those of previously reported nonmarine systems in spite of significant regulatory differences. Sulfate and thiosulfate uptake in *P. halodurans* responded identically to inhibitors, were coordinately regulated by growth on various sulfur compounds and sulfur starvation, and were mutually competitive inhibitors of transport, suggesting that they were transported by the same mechanism. The affinity of *P. halodurans* for thiosulfate was much greater than for sulfate.

Sulfate transport systems of bacteria isolated from marine environments have not been characterized. Since seawater contains over 25 mM sulfate and this saturating concentration has existed for geological time, sulfate transport may be regulated in a different manner in marine bacteria than in terrestrial microorganisms, which experience low and variable environmental sulfate concentrations. Study of the sulfate uptake systems of marine bacteria may therefore reveal features relevant to the understanding of adaptation by microorganisms to the much more dilute terrestrial and freshwater environments. Sulfate is never a limiting nutrient in marine ecosystems, so it is likely that sulfate transport and metabolism are tightly coupled to growth, and mechanisms of transport regulation may be interpretable in terms of intracellular sulfate reduction and metabolism.

Possible regulatory mechanisms for sulfate transport have been suggested through the use of organic sulfur compounds as sole sources of sulfur for growth of microorganisms. Media containing good sulfur sources (e.g., sulfate, cysteine, methionine, and glutathione) produce cultures with a low initial capacity for sulfate uptake (2, 7, 25, 28, 36), but transport rates can be increased substantially by subsequent sulfur starvation (2, 15, 31) or by growth on poor sulfur sources such as djenkolic acid (7, 36). These observations have been interpreted to indicate derepression of permease synthesis resulting from decreased intracellular pools of sulfate or a product of its metabolism which exerts transcriptional control on transport enzyme synthesis. The synthesis of the sulfate-binding protein of *Salmonella typhimurium* during growth on djenkolic acid has been directly demonstrated (22).

Among the structural analogs of sulfate which compete for transport in microorganisms, thiosulfate is of particular interest. As a reduced sulfur compound, it may be responsible for large-scale chemical energy transport from salt marshes and other anoxic water masses (13). Thiosulfate acts not only as an effective inhibitor of sulfate transport (2, 7, 21, 22, 29, 31, 34, 36); it is also an excellent source of sulfur for growth (11, 18, 23, 26). Demonstration of preferential thiosulfate uptake by marine bacteria and algae

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will aid in the determination of the fate of this compound near anoxic sediments and water masses. Furthermore, in the open ocean, where thiosulfate is absent, competition for sulfate uptake and metabolism by added [^{35}S]thiosulfate may provide a means of lowering the isotope dilution barrier which currently hinders the measurement of sulfate uptake. It is therefore desirable to extend the characterization of sulfate transport systems to marine bacteria. If marine sulfate transport systems are indeed similar to previously described transport proteins, generalizations based on these earlier findings can be used with confidence in sulfur metabolism measurements directed towards determination of bacterial growth in marine environments.

This paper presents evidence for the coincident regulation of sulfate and thiosulfate transport in a marine bacterium, *Pseudomonas halodurans*, which is consistent with genetic and nutritional studies of *Salmonella typhimurium* (18) and *Chlorella pyrenoidosa* (11). However, enhancement of transport capacity by growth with cysteine, methionine, and glutathione was in strict contrast to previously reported systems. A second marine isolate, *Alteromonas luteo-violaceus*, also demonstrated uptake stimulation by methionine, but in other respects of regulation was significantly different from the pseudomonad. The observed regulatory features of these bacteria are considered in relation to the environmental conditions experienced in their natural habitat.

MATERIALS AND METHODS

Organisms and culture conditions. *P. halodurans* was obtained from Galen E. Jones, University of New Hampshire, Durham. *A. luteo-violaceus* was isolated over the Puerto Rico Trench from a seawater toilet on R/V *Oceanus* cruise no. 40 (May, 1978) by aerobic enrichment and streaking on seawater medium containing sodium glutamate. It was identified on the basis of its characteristic violet pigment, narrow nutritional capability, and DNA base composition (44 mol% guanine plus cytosine) (9). The organisms were grown in an artificial seawater medium similar to the sulfate-free modification of the Lyman and Fleming formulation (20). However, sulfate contamination from reagent-grade NaCl was sufficient to permit growth of *A. luteo-violaceus* without added sulfate (background concentration, 6.0 μM). The problem was inexpensively overcome through neutralization of NaOH, which is routinely available at one-tenth the sulfate contamination of NaCl. The resulting relatively low-contamination (ca. 1.5 μM sulfate) artificial seawater, designated RLC-water, contained (in grams per liter): NaOH, 16.3; HCl, 14.8; $MgCl_2 \cdot 6H_2O$, 10.6; $CaCl_2 \cdot 2H_2O$, 1.5; KCl, 0.66; $NaHCO_3$, 0.19; KBr, 0.10; H_3BO_3, 0.03; and $SrCl_2 \cdot 6H_2O$, 0.04. Additional sterile supplements to the autoclaved basal salts medium were, with their respective final concentrations: sodium glutamate (10 mM), NH_4Cl (500 μM), KH_2PO_4 (50 μM), and I.M.R. trace elements solution (8) (1 ml/liter). All cultures were grown at pH 7.8 to 8.0 at 20 ± 2°C on a rotary shaker at 250 rpm.

Uptake studies were performed with cultures grown in complete medium containing 1 mM sodium sulfate. Cells were harvested by centrifugation (5,000 × *g*, 10 min) in the late exponential phase of growth (1×10^8 to 3×10^8/ml), washed once with basal salts medium, and suspended to the same density in complete medium minus sulfur. Cultures were starved for 2 h before the assay of transport activity as described below. For derepression experiments, 1,500 ml of complete medium minus sulfur was inoculated at low density (ca. 10^4 cells per ml) and shaken for 10 min to evenly distribute the cells. Samples of 100 ml were aseptically transferred to sterile flasks and supplemented with filter-sterilized solutions of Na_2SO_4, $Na_2S_2O_3$, L-cysteine·hydrochloride (neutralized just before use), DL-methionine, or reduced glutathione at a final sulfur concentration of 500 μM (i.e., 250 μM $Na_2S_2O_3$). Late-exponential-phase cultures were treated as described above.

Sulfate uptake assay. Transport activity was assayed as follows: 9.8 ml of the cell suspension was added to tubes containing 0.1 ml of the inhibitor or appropriate solvent and 0.1 ml of ^{35}S-labeled sulfate or thiosulfate. Carrier-free sulfate (final activity, 1 to 2 μCi/ml) was supplemented with sterile solutions of Na_2SO_4 to achieve the desired final concentration (11.5 to 201.5 μM, including background sulfate). Thiosulfate (sulfane S tagged) was used at concentrations of 2.5 to 25 μM (specific activity, 10 to 30 dpm/pmol). The mixture was blended thoroughly on a Vortex mixer, and 1-ml subsamples were withdrawn at 60-s intervals for 5 min into tubes containing 0.1 ml of 1 M Na_2SO_4 or $Na_2S_2O_3$ as appropriate to dilute the label (final concentration, over 100 mM). Subsamples of 1 ml were filtered through Reeve Angel 984H Ultrafine or Whatman GF/F glass fiber filters and rinsed with 0.5 M NaCl. Termination of the reaction by isotope dilution did not result in loss of label transported into the cells, nor did further detectable uptake of label occur before filtration as determined in separate experiments (data not shown).

Determination of LMW compounds. Cultures were grown as described for transport studies except that [^{35}S]sulfate was included in the medium at 11 to 19 dpm/pmol. After more than 10 generations of growth, cultures were harvested and suspended in sulfur-free medium as described above. Samples (5 ml) were filtered as described above, and the filters were ground in cold 10% trichloroacetic acid, refrigerated for 30 min, and centrifuged (5,000 × *g*, 20 min). The pellets were washed once with cold 10% trichloroacetic acid and centrifuged, and the supernatant fractions were combined. Inorganic sulfate was separated from low-molecular-weight (LMW) organic sulfur compounds by barium precipitation in a mixture containing, in 2.05 ml: 1.75 ml of sample, 0.05 ml of 5 mM Na_2SO_4, and 0.25 ml of 1 M $BaCl_2$ (pH 2). After 2 h at 4°C, the precipitate was removed by centrifugation (5,000 × *g*, 10 min), and 1 ml of the supernatant fluid was counted, giving the LMW organic sulfur fraction.

The pellet was rinsed twice with 1 M $BaCl_2$ and counted as a water suspension, giving the inorganic sulfate fraction.

Filtration procedure. Due to the high activities of radiosulfate required for some experiments, problems with label adsorption to the filters can be considerable (16). We constructed a punch funnel (Fig. 1) which serves to excise only the cell-retaining portion of the filter. Use of the punch funnel reduced adsorption blanks by over 10-fold, which increased the precision of the assays significantly at low uptake rates.

Other methods. Direct cell counts were made using acridine orange epifluorescence microscopy (5). Cell viability was determined by plating dilutions of the culture on complete medium containing 15 g of agar (Difco) per liter. Protein was determined on 10% trichloroacetic acid-precipitable material dissolved in 0.1 N NaOH by the method of Bradford (3). Radioisotopes were counted in Aquasol in a Beckman LS-100C liquid scintillation counter, using the channels ratio method of quench correction.

Radiochemicals. Carrier-free $Na_2{}^{35}SO_4$ and $Na_2{}^{35}S_2O_3$ (10 to 30 dpm/pmol) were obtained from Amersham (Chicago, Ill.).

Other chemicals. Dicyclohexylcarbodiimide and carbonylcyanide *m*-chlorophenylhydrazone were obtained from U.S. Biochemicals (Cleveland, Ohio). *para*-Hydroxymercuribenzoate and 2,4-dinitrophenol were from Sigma (St. Louis, Mo.). Na_2SeO_4 was the gift of I. K. Smith. The protein dye reagent was purchased from Bio-Rad Laboratories (Richmond, Calif.). All other chemicals were reagent grade. Special precautions were taken to obtain components of RLC-water with the lowest possible sulfate contamination.

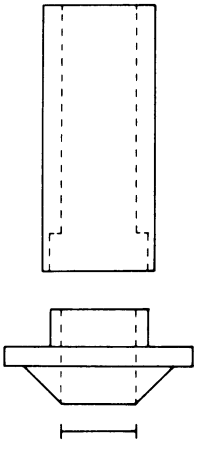

FIG. 1. *Punch funnel used for filtration of radioisotopically labeled samples. The funnel (upper portion) is 0.75-in. (ca. 19-mm) polycarbonate tubing, and the lower portion is 316 stainless steel. The scale bar represents 0.5 in. (ca. 12.7 mm). A small square of Whatman no. 1 or similar filter paper is placed over a 25-mm fritted glass base to preserve the frit surface and to prevent clogging by fibers from the glass filters. A 25-cm Reeve Angel 984H or Whatman GF/F filter is placed on top, followed by the punch funnel. To reduce loss of vacuum for samples larger than 10 ml, a thin piece of rubber punched with a hole slightly larger than 0.5 in. (ca. 12.7 mm) may be placed on top of the filter. This funnel may also be used with membrane filters.*

RESULTS

Cultures of *P. halodurans* grown with 1 mM sulfate as the sole sulfur source exhibited low initial rates of sulfate and thiosulfate uptake which could be enhanced by sulfur starvation (Fig. 2). The rapid increase in transport capacity during the first 2 h of sulfur starvation was mirrored by a decrease in LMW organic sulfur compounds (Fig. 3), but little metabolism of endogenous inorganic sulfate occurred. After 2 h of constant transport activity, a slow increase in uptake rate was accompanied by a linear increase in cell numbers (Fig. 2) and a similarly slow utilization of LMW organic sulfur compounds (Fig. 3). In a similar experiment using cultures grown in medium containing only 250

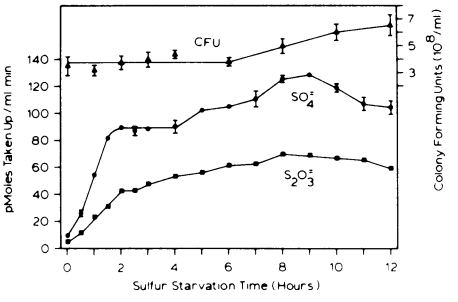

FIG. 2. *Viable cell counts and sulfate and thiosulfate uptake rates during sulfur starvation of P. halodurans. Final assay concentrations and specific activities were 200 μM SO_4^{2-} (17 dpm/pmol) and 20 μM $S_2O_3^{2-}$ (17 dpm/pmol). Uptake rate data are the averages of duplicate determinations; error bars are shown when larger than the symbol. Viable counts are the mean ± 1 standard error of two plates at each of three dilutions.*

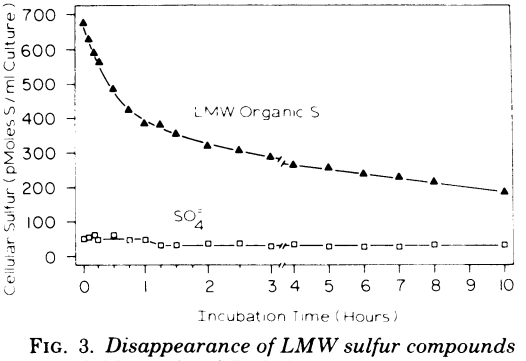

FIG. 3. *Disappearance of LMW sulfur compounds from soluble pools of P. halodurans during sulfur starvation. Experimental details are in the text.*

μM sulfate (a concentration nearing sulfate-limited growth for this bacterium), no increase in cell numbers occurred (data not shown). This indicated that the utilization of endogenous sulfur-containing compounds, rather than the uptake of contaminating sulfate from the medium, was responsible for the cell division shown in Fig. 2.

The low initial rate of sulfate uptake and its enhancement by sulfur starvation as described above were also found for *A. luteo-violaceus* (Fig. 4). This organism did not take up thiosulfate at rates sufficient for assay (4a). As with *P. halodurans*, sulfur starvation resulted in a rapid depletion of the LMW organic sulfur pool (Fig. 5) which was virtually the mirror image of the transport activity time course. However, a different response to prolonged sulfur starvation was manifest in the absence of a plateau and further increase of transport capacity. After 2 h of sulfur starvation the uptake rate began to decrease, accompanied by a loss of cell viability which decreased to 16% of the initial value after 4 h. Viability estimates were complicated by growth of the cells into tangled filaments, as evidenced by the increase in optical density in Fig. 4, but the ultimate decrease in direct counts due to autolysis of this organism (9, 10) supported the lower plating efficiency. Transport activity and apparent viability began to decline as the LMW organic sulfur pool reached the minimum size shown in Fig. 5.

The correlation of transport activity with disappearance of LMW organic sulfur from soluble pools suggested that one or more organic sulfur compounds may repress synthesis of transport proteins. Therefore the transport capacity of

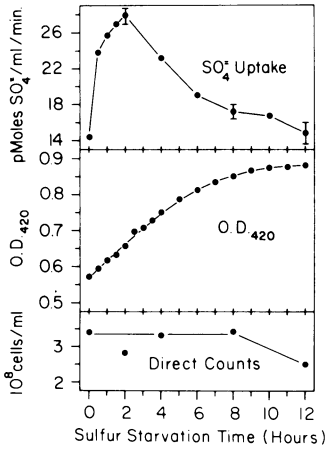

FIG. 4. *Sulfate uptake capacity during sulfur starvation of A. luteo-violaceus. Final assay concentration of $^{35}SO_4^{2-}$ was 26.5 μM (45 dpm/pmol). Cell numbers are single direct count determinations.*

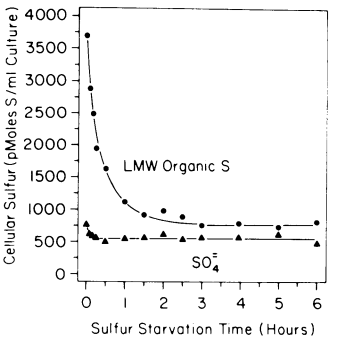

FIG. 5. *Disappearance of LMW sulfur compounds from soluble pools of A. luteo-violaceus. Experimental details are in the text.*

cultures grown on various sole sources of sulfur was investigated. Surprisingly, organic sulfur compounds were poor sources of sulfur for the growth of *P. halodurans*, yielding cultures with growth rates half or less than half of those grown on sulfate or thiosulfate (Table 1). The methionine-grown culture reached 10^7 cells per ml with a lag time of 18 h relative to those grown on sulfate. This lengthy delay cannot be attributed to the slower growth rate alone and suggests that synthesis of enzymes required for the conversion of methionine to cysteine must be induced for growth on methionine to occur. These cells also exhibited a markedly higher protein content. Cultures provided with glutathione grew at the slowest rate; however, extrapolation of the direct count regression line to the time of inoculation indicated a more rapid growth rate before the appearance of turbidity. It is not known whether this was due to dimerization of glutathione and concomitant difficulty of transport or to other effects.

In contrast to previously studied sulfate transport systems (7, 21, 36), growth on all organic sulfur sources stimulated both sulfate and thiosulfate uptake effectively. Uptake rates before sulfur starvation were highest in cultures grown on methionine and glutathione, with transport capacity greater than that observed for sulfate- and thiosulfate-grown cells which had been starved for sulfur for 8 h. However, the characteristically low initial uptake rates for both sulfate and thiosulfate by sulfate-grown cells (Fig. 2, Table 1) were also observed for the culture grown on thiosulfate.

Further enhancement of sulfate transport capacity occurred as a result of sulfur starvation for all cultures except that growth on glutathione. Sulfate and thiosulfate-grown cells showed a primary increase of about sixfold during the first 2 h of sulfur starvation, followed by a small increase. The cysteine-grown culture exhibited

TABLE 1. *Sulfate and thiosulfate transport capacity after growth on various sulfur sources and subsequent sulfur starvation of P. halodurans*[a]

Sulfur source[b] (k)	Starvation (h)	Cells per ml ($\times 10^8$)	Protein per 10^8 cells (μg)	Uptake rate[c]	
				Sulfate	Thiosulfate
Sulfate (0.87 h^{-1})	0	1.45	18.4 ± 0.3	6.1 ± 1.1	5.9 ± 0.0
	2	1.49	21.2 ± 0.8	32.6 ± 0.5	16.7 ± 0.1
	8	2.08	20.7 ± 0.3	49.9 ± 0.1	14.9 ± 2.1
Thiosulfate (0.89 h^{-1})	0	1.40	17.8 ± 1.2	4.8 ± 0.2	5.8 ± 0.3
	2	1.55	19.7 ± 0.5	32.0 ± 0.6	16.5 ± 0.1
	8	2.35	17.9 ± 0.3	47.2 ± 3.3	18.9 ± 0.1
Cystine (0.45 h^{-1})	0	2.01	16.3 ± 0.6	44.1 ± 4.8	23.4 ± 0.2
	2	2.46	19.5 ± 0.1	91.5 ± 0.0	43.4 ± 1.2
	8	4.97	13.9 ± 0.1	45.2 ± 1.2	21.0 ± 1.1
Glutathione (0.32 h^{-1})	0	1.67	18.6 ± 0.2	61.7 ± 0.3	31.5 ± 0.5
	2	2.07	20.2 ± 0.0	61.7 ± 0.4	41.3 ± 0.0
	8	4.43	13.1 ± 0.1	24.9 ± 0.7	14.9 ± 0.2
Methionine (0.46 h^{-1})	0	1.30	23.8 ± 0.4	63.8 ± 2.1	34.8 ± 0.2
	2	2.00	21.1 ± 0.5	89.5 ± 0.6	37.3 ± 0.6
	8	3.31	18.6 ± 0.2	62.2 ± 1.3	22.4 ± 1.0

[a] Cultures were grown, harvested, and assayed as described in the text. Assay concentrations were 100 μM sulfate and 20 μM thiosulfate. The uptake rate is expressed as picomoles of SO_4^{2-} or $S_2O_3^{2-}$ per 10^8 cells per minute. The standard error for duplicate measurements is shown.

[b] The growth rate, k, was determined from direct counts taken at intervals during exponential growth.

[c] The reaction mixture contained, in 10 ml, 9.9 ml of cell suspension and 0.1 ml of either $^{35}SO_4^{2-}$ (final concentration, 100 μM, 32 dpm/pmol) or $^{35}S_2O_3^{2-}$ (final concentration, 20 μM, 8 dpm/pmol). The uptake rate is expressed as picomoles of SO_4^{2-} or $S_2O_3^{2-}$ per 10^8 cells per minute.

a doubling of transport capacity, but an increase of less than 50% accompanied sulfur starvation of the methionine-grown cells; both returned to the initial rate as a result of cell division and dilution of transport proteins among daughter cells. Glutathione-grown cells demonstrated no increase during the first 2 h, but declined by over 50% during the next 6 h as a result of cell division. It should be noted that under no circumstances did the total transport capacity (per milliliter of culture) decrease; all reductions in activity reported in Table 1 were due to increases in cell numbers. This was also true for total protein.

Thiosulfate uptake rates followed the same pattern as those for sulfate. All three organic sulfur sources yielded cultures with enhanced thiosulfate transport capacity. The trends for both sulfate and thiosulfate uptake stimulation by growth on various sulfur sources and subsequent sulfur starvation were the same in 12 of the 15 cases.

In contrast to *P. halodurans*, the three organic sulfur compounds tested were equally good sulfur sources for *A. luteo-violaceus*. The growth rate (i.e., reciprocal doubling time, average 0.27 h^{-1}) varied less than 10% among cultures. Table 2 shows that enhancement of sulfate uptake capacity occurred only in the culture utilizing methionine. Initial uptake rates of cells grown with cysteine or glutathione were lower than in the sulfate-grown culture. Based on initial cell density, all cultures demonstrated increased uptake as a function of sulfur starvation.

The significantly different patterns of regulation of sulfate transport in the marine bacteria compared to previously studied systems warranted the investigation of other characteristics common among them. We therefore tested for substrate specificity, using sulfate analogs (15, 22, 23, 29, 34, 36), for adherence to Michaelis-Menten kinetics, and for the ability to accumulate sulfate in excess of growth requirements (32).

A variety of active transport inhibitors decreased sulfate uptake rates in both marine bacteria (Table 3). Sulfate and thiosulfate uptake by *P. halodurans* were affected identically.

Also consistent with previous studies was the effect of group VI oxyanions on sulfate transport. Table 4 lists the kinetic constants for sulfate uptake by the marine bacteria and for thiosulfate uptake by *P. halodurans*, and Table 5 gives the K'_i values for competitive inhibition of uptake by the sulfate analogs. In both bacteria, the effectiveness of inhibition was inversely propor-

TABLE 2. *Sulfate transport capacity of A. luteo-violaceus after growth on various sulfur sources and subsequent sulfur starvation*[a]

Sulfur starvation (h)	Sulfate transport[b] with sulfur source for growth:			
	Sulfate	Methionine	Cystine	Glutathione
0	4.8 ± 0.4	20.8 ± 4.2	0.9 ± 0.1	0
1	23.3 ± 0.4	68.3 ± 1.9[c]	16.2 ± 0.2	9.4 ± 0.5
2	33.7 ± 0.4	109 ± 0.2[c]	29.6 ± 0	45.4 ± 6.8

[a] Cultures were grown, harvested, and assayed as described in the text. The assay sulfate concentration was 100 μM. Initial cell densities were 1.1×10^8 to 1.7×10^8/ml.

[b] Based on initial cell density and expressed as picomoles of SO_4^{2-} per 10^8 cells per minute.

[c] First 4 min of uptake only.

TABLE 3. *Effects of active transport inhibitors on sulfate uptake by P. halodurans and A. luteo-violaceus and on thiosulfate uptake by P. halodurans*[a]

Addition[b] (concn)	*P. halodurans*				*A. luteo-violaceus* SO_4^{2-} uptake	
	SO_4^{2-} uptake		$S_2O_3^{2-}$ uptake			
	Rate[c]	% Inhibition	Rate[c]	% Inhibition	Rate[c]	% Inhibition
Distilled water	60.7	0	15.0	0	53.4	0
NaN_3 (100 μM)	53.4	12[d]	12.0	20[d]	46.2	14[d]
NaN_3 (1 mM)	29.0	52	6.8	55	10.8	80
Glutamate omitted	6.9	89	4.2	72	20.8	61
Ethanol (1%)	62.0	0	15.7	0	52.8	0
DCCD (100 μM)	48.0	23[e]	13.5	14[e]	23.7	55[e]
2,4-DNP (100 μM)	52.2	16	13.1	17	42.6	19
2,4-DNP (1 mM)	0	100	1.0	94	0	100
CCCP (5 μM)	57.0	8	12.9	18	29.2	45
CCCP (50 μM)	26.5	57	7.4	53	0	100

[a] Starved cell suspensions were prepared as described in the text. Thiosulfate uptake was assayed at 15 μM. Sulfate was assayed at 200 μM (*P. halodurans*) or 100 μM (*A. luteo-violaceus*). The cell density was 4.1×10^8/ml for *P. halodurans* and 1.9×10^8/ml for *A. luteo-violaceus*.

[b] DCCD, Dicyclohexylcarbodiimide; 2,4-DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

[c] Rates are expressed as picomoles of SO_4^{2-} or $S_2O_3^{2-}$ per 10^8 cells per minute.

[d] Inhibition relative to distilled water control for NaN_3 added and glutamate omitted.

[e] Inhibition relative to 1% ethanol control for 2,4-DNP, CCCP, and DCCD.

TABLE 4. *Kinetic constants for sulfate and thiosulfate uptake by P. halodurans and for sulfate uptake by A. luteo-violaceus*[a]

Strain	Compound	K_m (μM)	V_{max} (pmol per 10^8 cells per min)
P. halodurans	Thiosulfate	14.7 ± 1.5 (8)	30.5 ± 3.0 (7)
	Sulfate	214 ± 34 (9)	108 ± 22 (9)
A. luteo-violaceus	Sulfate	186 ± 18 (5)	146

[a] Numbers in parentheses indicate the number of experiments if greater than one.

TABLE 5. *Inhibition constants for competition by sulfate analogs*[a]

Competitor	K'_i (μM)		
	P. halodurans		*A. luteo-violaceus* sulfate
	Thiosulfate	Sulfate	
CrO_4^{2-}	52	3	73
SeO_4^{2-}	1,989 ± 174 (2)	569	238
MoO_4^{2-}	9,109 ± 562 (2)	1,327 ± 120 (3)	12,200
$S_2O_3^{2-}$		16.6 ± 1.8 (2)	ND
SO_4^{2-}	397 ± 75 (3)		

[a] Computed according to the procedure of Webb (35). Numbers in parentheses indicate the number of experiments if greater than one. ND, Not determined.

tional to the molecular weight of the analog, demonstrating size selectivity. Additionally, sulfate and thiosulfate were mutually competitive inhibitors in *P. halodurans*, with the K_m for thiosulfate uptake equal to the K'_i for thiosulfate inhibition of sulfate uptake. Sulfate analogs also competitively inhibited thiosulfate uptake with the same specificity as for sulfate, but higher concentrations of inhibitors were required. This is consistent with the more than 10-fold higher affinity of the transport system for thiosulfate relative to sulfate.

A more subtle regulatory feature was discov-

ered during determination of kinetic constants for uninhibited sulfate uptake. The competitive inhibition studies described above were conducted in the range from 10 to 200 μM added sulfate. In this range, values for K_m and V_{max}, derived from $1/v$ versus $1/S$, v versus v/S, and S/v versus S plots, agreed within 5%. To verify Michaelis-Menten kinetics, it was desirable to extend the range of sulfate concentrations to higher values.

With *P. halodurans* it was found that the sulfate uptake rate ceased to increase in proportion to added sulfate above 250 μM SO_4^{2-}. Indeed, at 1 mM SO_4^{2-} the observed rate was only 12% higher than at 250 μM and only 62% of the rate expected from extrapolation of the $1/v$ versus $1/S$ plot used to derive the kinetic constants in Table 4. Uptake was linear with time at all concentrations. As previously mentioned, 250 μM SO_4^{2-} borders on sulfate-limited growth in this organism. Although the V_{max} calculated for sulfate uptake would permit uptake in excess of growth requirements, the observed maximum rate was just sufficient to meet cellular demands based on the sulfur quota derived from equilibrium labeling studies (R. L. Cuhel, C. D. Taylor, and H. W. Jannasch, Arch. Microbiol., in press).

A close coupling between growth requirements for sulfur and sulfate uptake is achieved through a different mechanism in *A. luteo-violaceus*. Initial sulfate uptake rates increased with added sulfate throughout the range of concentrations tested and were within experimental error of the values predicted from a simple hyperbolic relationship. However, at higher concentrations the initial uptake range was maintained for only a short period (Fig. 6), until 500 to 600 pmol of SO_4^{2-} was taken up per 10^8 cells. This phenomenon was observed whenever initial uptake rates (based on the first 1 to 2 min) were greater than 60 pmol of SO_4^{2-} per 10^8 cells per min regardless of the sulfate concentration, as in the methionine-grown cells in Table 2 after 1 and 2 h of sulfur starvation.

Nonlinear uptake of the type shown in Fig. 6 suggests feedback inhibition of uptake by end products of sulfate metabolism, since the reduction in transport rate is too rapid to be attributable to repression of permease synthesis and subsequent turnover of existing proteins. This was supported by inhibition of sulfate uptake by organic sulfur compounds. When assayed at 100 μM added sulfate, 1 mM methionine had little or no detectable effect, whereas cysteine and glutathione strongly inhibited sulfate uptake (100 and 85%, respectively). *A. luteo-violaceus* is insensitive to the sulfhydryl reagent *para*-hydroxymercuribenzoate with respect to sulfate uptake (4a), so it is not likely that these compounds inhibited transport through binding of cysteine sulfhydryl groups to the active site of the transport protein. It is more likely that the inhibition was a rapid response to an increase in organic sulfur pool size. Additionally, in the presence of glutathione the sulfate uptake rate decreased continuously during the 5-min transport assay, suggesting metabolic conversion to cysteine through the action of a peptidase. These data are consistent with the sulfate uptake rates, tested as a function of the sulfur source for growth, that are presented in Table 2.

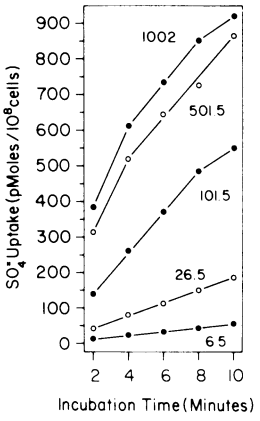

FIG. 6. *Sulfate uptake by A. luteo-violaceus at sulfate concentrations of 6.5 to 1,002 μM. The cell suspension (3.2 × 10^8/ml) was starved for sulfur and assayed as described in the text.*

DISCUSSION

The failure of growth on end products of sulfate metabolism to produce a significant regulatory effect on sulfate transport capacity in *P. halodurans* is extremely unusual if not unique among microorganisms. Some LMW sulfur compound must either repress permease synthesis or exert feedback control on existing enzymes, since cultures grown on sulfate or thiosulfate exhibited low initial uptake capacity. The concurrent enhancement of uptake during sulfur starvation and disappearance of LMW organic sulfur compounds strongly suggest that a component of this fraction is responsible for transport regulation. Sulfate itself is an unlikely candidate because of the relative constancy of its concentration during the development of transport capacity. Growth on cysteine resulted in high initial rates of sulfate uptake, suggesting a compound in the cysteine biosynthetic pathway after sulfate, but not cysteine itself. Since the sulfate reduction sequence occurs via a pathway

of enzyme-bound intermediates (1), it is likely that adenosine 5'-phosphosulfate or adenosine 3'-phosphate 5'-phosphosulfate regulated sulfate transport capacity in *P. halodurans*.

An unambiguous distinction between repression of permease synthesis and feedback inhibition of existing transport proteins cannot readily be made. In vivo inhibition of sulfate reduction enzyme activities by growth on S-containing amino acids (14, 27) cannot be assumed to be due to repression of specific protein synthesis, since the activity of such enzymes has been shown to be regulated in vitro by immediate end products of metabolism (6, 27). For example, *Anacystis* possesses no transcriptional control for synthesis of homoserine-*O*-transsuccinylase, an enzyme of methionine biosynthesis (6). Feedback inhibition by methionine in vitro explained the apparent repression observed in activity assays in cell-free extracts after growth on methionine; passage of the extract through a column to remove LMW material restored enzyme activity. Concentrations of intracellular metabolites can therefore be high enough to control enzyme activity in vivo through feedback inhibition. In addition, both repression of cystathionase synthesis in *Escherichia coli* by growth with methionine and feedback of catalytic activity in vitro by homocysteine have been demonstrated simultaneously (27).

It is probable that feedback inhibition by cysteine is important in sulfate transport regulation in *A. luteo-violaceus*. This was suggested by the non-linearity of uptake when initial rates were greater than growth demands for sulfur and by the rapid inhibition of sulfate uptake in the presence of cysteine and glutathione, which also prevented expression of sulfate transport activity when used as the sole source of sulfur for growth. In both bacteria it is clear that the activity of enzymes catalyzing reactions in the opposite direction from normal cysteine and methionine biosynthetic pathways was sufficiently rate-limiting to prevent build-up of intracellular pools to levels adequate to exert metabolic control on sulfate uptake.

In contrast to regulatory differences, the kinetic characteristics of sulfate uptake in the marine bacteria, i.e., size-selective competitive inhibition by sulfate analogs (29, 34), energy dependence (12, 15, 25, 29, 36), and adherence to Michaelis-Menten kinetics, were very similar to those reported for terrestrial microorganisms. The 5- to 10-fold lower affinity of the marine transport systems for sulfate was not surprising in view of the consistently saturating concentration experienced by marine bacteria in situ, although it suggests that selective pressure has been brought to bear on their nonmarine counterparts.

Thiosulfate was an extremely effective competitive inhibitor of sulfate uptake in *P. halodurans*, with a greater than 10-fold higher affinity for the reduced sulfur compound. The similarity of the K_m for thiosulfate uptake (14.7 μM) to the K'_i for its inhibition of sulfate uptake (16.6 μM), the similar reponse of sulfate and thiosulfate uptake to inhibitors, and the coordinate regulation of sulfate and thiosulfate uptake by growth on various sulfur sources and sulfur starvation leave little doubt that the two compounds are transported by the same system. These data are consistent with genetic and nutritional studies of sulfate metabolism in *Chlorella* (11) and *Salmonella* (18).

The much higher affinity of *P. halodurans* for thiosulfate concurs with the involvement of sulfhydryl groups in the transport mechanism, as demonstrated through inhibition by *para*-hydroxymercuribenzoate (4a, 21, 33). A sulfhydryl group or disulfide bond would provide a site for covalent binding of thiosulfate through the sulfane moiety; such binding is not possible with the unreactive sulfate molecule and would increase the effective concentration of thiosulfate at the transport site. Combined with the identical response of sulfate and thiosulfate transport to the conditions examined in this paper, as well as the virtually unidirectional transport observed in this and other microorganisms (21, 32, 36), the active transport model of Kaback and Barnes (17) is appealing. The absence of effective thiosulfate transport by *A. luteo-violaceus* is the subject of another communication (4a).

The major regulatory difference between *P. halodurans* and *A. luteo-violaceus* was the reduction of sulfate transport capacity by growth using cysteine or glutathione in *A. luteo-violaceus* and its apparent rapid feedback inhibition of transport by these compounds. This organism produces an extracellular protease (9), especially during stationary phases, as evidenced by the rapid decline in transport rates and viability shortly after the cessation of protein synthesis during sulfur starvation. The potency of the protease was confirmed by addition of chloramphenicol to an exponentially growing culture; total protein decreased by 50% in 1 h, less than one-half a generation time. Frequent isolation of *A. luteo-violaceus* from surfaces such as *Sargassum* weed and fish skin suggests that it derives much of its nutrition from proteolysis, which would provide cysteine and methionine at relatively high concentrations compared to seawater.

It should be noted that *A. luteo-violaceus* was

chosen for study on the basis of its unusual nutritional characteristics and distinctive pigmentation and should not be taken as representative of marine bacteria. Unlike most marine bacteria, it can grow on very few organic carbon compounds, and it was the only one of 12 isolates which could not grow on thiosulfate as the sole source of sulfur.

It was stimulating to find that the kinetic characteristics of *P. halodurans* sulfate transport were virtually identical to those previously reported for the more extensively studied nonmarine microorganisms, in spite of the completely different regulatory mechanism. The predictability of sulfate uptake systems indicates that it may still be possible to use competitive inhibition of sulfate uptake by [^{35}S]thiosulfate as a means of lowering the isotope dilution barrier for studies of marine bacterial sulfur metabolism and protein synthesis.

The lack of sulfate transport system regulation by end products of sulfate metabolism in *P. halodurans* is both interesting and gratifying when we consider the use of sulfate metabolism as a measurement of marine bacterial growth. Sulfate transport is energy-requiring, and its reduction is even more so. If *P. halodurans* had been exposed to utilizable concentrations of S-containing amino acids in its recent evolutionary history, it would be expected to take advantage of this by reducing the activity or synthesis of the enzymes of precursor metabolism. The concentrations of total dissolved amino acids in open-ocean seawater are very low (19), but they increase in near-shore and estuarine envornments (4), which are the habitat of *P. halodurans* (A. Rosenberg, Ph.D. thesis, University of New Hampshire, Durham, 1977). Nonetheless, S-containing amino acids, when detectable at all, rarely rise above 5×10^{-8} M (4, 19), scarcely a significant amount. This is largely due to the low to undetectable amounts of these compounds in microbial cells (30) and the consequent small input resulting from death and autolysis of marine organisms. Furthermore, the lag time and slow growth rate of *P. halodurans* when using end products of sulfate metabolism as sole sources of sulfur indicates a poorly developed capability to reverse the pathways of sulfate reduction and S-amino acid biosynthesis.

These observations strongly support the contention that inorganic sulfur, probably sulfate, is the only source of sulfur for marine microorganisms in aerobic waters. Constant, saturating sulfate concentrations and regulation of transport rates to meet but not exceed cellular growth requirements render it likely that sulfate transport and metabolism will provide an accurate measure of marine bacterial growth. Supporting studies on the intermediary metabolism of sulfate and its relation to de novo protein synthesis in *P. halodurans* and *A. luteo-violaceus* have been submitted elsewhere.

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