Effects of Three Environmental Variables on Sulfate Uptake by Aerobic Bacteria

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The effects of various concentrations of sulfate, organic sulfur, and organic carbon on sulfate uptake by aerobic bacteria were studied using pure cultures growing in a defined medium. Cultures of Pseudomonas fluorescens and Corynebacterium striatum took up sulfate faster when young, but sulfate uptake by Serratia marcescens was faster in older cultures. Organic sulfur was found to decrease sulfate uptake, but at concentrations somewhat higher than occurs in most natural freshwater ecosystems. Low levels of sulfate can theoretically directly limit bacterial biomass production but such limitation probably does not occur in natural systems. Evidence is presented which indirectly links the uptake of sulfate and organic carbon, adding credibility to the proposal that sulfate uptake can be used as an indicator of microbial biomass production in freshwater ecosystems.

Most of the transformation mechanisms involved in the environmental sulfur cycle have been qualitatively identified and bacteria are known to be important in many of those transformations. Bacteria are particularly important in anaerobic phases of the sulfur cycle, and therefore most ecological studies involving bacteria-sulfur relationships have been concerned with anaerobic environments. Although the communities of planktonic microorganisms that live in the aerobic portions of aquatic systems are ecologically very important, there is virtually no quantitative information regarding interactions between sulfur and the bacteria present in those aerobic microbial communities.

One of the more obvious aerobic bacteria-mediated aquatic sulfur transformations is the incorporation of ambient sulfur into cellular biomass. It might seem that the sulfur requirement for bacterial biomass production can be satisfied by the utilization of dissolved sulfur-containing organic compounds, but the sulfur cycle literature implies instead that sulfur probably enters into such biomass largely in the form of sulfates (2, 6, 16). Therefore, an understanding of sulfate utilization (uptake) by aerobic bacteria is important to understanding the ecological role(s) of aquatic bacteria and the ecology of the aquatic sulfur cycle.

Sulfate uptake by natural bacterial populations is likely to be a function of many environmental variables, including the ambient concentrations of sulfate, organic sulfur, and organic carbon. Since experimentation using natural (mixed) microbial communities might confuse the concurrent processes of the autotrophic and heterotrophic organisms, pure culture studies were initiated to examine the effect of these three variables on sulfate uptake by bacteria.

MATERIALS AND METHODS

The defined medium used throughout these studies, except where modified as indicated, consisted of 0.4% glucose, 0.4% NaCl, 0.1% urea, 0.1% K2HPO4, and 0.005% MgSO4. Corynebacterium striatum, Pseudomonas fluorescens, and Serratia marcescens, species that might be expected to be present in natural aquatic ecosystems, are capable of growth in this defined medium and were used as the experimental organisms. The particular strains of bacteria used in this study have been identified and characterized previously (9).

The technique for determining bacterial sulfate uptake involved the use of sulfur-35 as a radioisotope tracer. The sulfur-35, which was as carrier-free H35SO4 (New England Nuclear Corp.), was diluted with distilled water to 200 μCi/ml and then sterilized by autoclaving 0.5-ml aliquots in 1-dram vials at 121 C for 15 min. Isotope was added to experimental cultures, and after appropriate incubation the cultures were filtered through 47-mm diameter, 0.45-μm membrane filters (Millipore Corp., type HA). The filters were then washed, as suggested by McMahon (7), by filtering two separate 25-ml portions of isotope-free distilled water. After air drying, the filters were cemented to aluminum planchets, and the isotope retained by each filter
was determined in a gas-flow, thin-window beta counter (Amersham-Searle Corp.). The level of isotope added to each culture was determined by counting triplicate dried 1-ml samples of the isotope-containing culture. Calculation of the sulfate uptake rate assumed that assimilated isotope remained on the filters as particulate organic sulfur. Control cultures containing 5.0% formaldehyde were used so that isotope that absorbed to filters was corrected for by subtracting the counts per minute of the control filters from the counts per minute of the noncontrol filters.

Three different sets of experiments were conducted to examine the effect of the different materials on sulfate uptake. In all of these experiments, 2-liter Erlenmeyer flasks containing 800 ml of defined medium were inoculated with 1.0 ml of 4-day starter cultures. After the appropriate incubation period, cultures were dispensed into four 250-ml centrifuge tubes and centrifuged at 2,800 rpm for 30 min. The supernatant was decanted, and the cells that remained in each tube were resuspended in 200 ml of sterile glucose-free defined medium. The tubes were then centrifuged at 2,800 rpm for 30 min, the supernatant was decanted, and the cells were resuspended in 200 ml of sterile glucose-free defined medium. In those experiments in which sulfate was a test material, sulfate was also excluded from the suspension medium. The contents of all four tubes were pooled into a 2-liter flask, sterile 35SO4 (approximately 80 μCi) was added to this reconstituted culture, and, after mixing, 10-ml aliquots were dispensed into sterile 25-ml Erlenmeyer flasks that contained appropriate levels of the test material(s). These cultures were plugged with cotton and incubated at 25 C for 1 to 1.5 h, at which time each culture was filtered. For each different set of experimental conditions, six replicates were used of which two served as the formaldehyde control cultures. Sulfate uptake as a function of the concentrations of both glucose and sulfate was examined by using a factorial design. The levels of glucose to which the bacteria were subjected were 5, 50, or 500 mg/liter, whereas the sulfate levels, added as MgSO4, were 0.05, 0.5, 5.0, or 50.0 mg/liter. Each species of bacteria was tested at three different ages of their growth cycle: 28 h, 4 days, and 10 days after inoculation with the starter culture.

The influence of organic sulfur on bacterial sulfate uptake was examined by subjecting 3-day cultures to a mixture of DL-methionine (Fisher) and L-cysteine hydrochloride (Fisher). This organic sulfur mixture, made up so that each amino acid contributed 50% of the sulfur, was added to the 25-ml Erlenmeyer flasks so that the final concentration of sulfur was 0.06, 0.6, 6.0, 60.0, or 600.0 μg/liter. This set of experiments was conducted at glucose concentrations of 5 and 500 mg/liter.

Bacterial sulfate uptake as a function of the concentration of organic substrate was examined by subjecting 3-day cultures to glucose levels of 0.5, 1.0, 5.0, 10, 50, 100, 500, or 1,000 mg/liter. No other experimental variables were used.

All incubations were done entirely in the dark, and prior to inoculation all of the experimental flasks were preconditioned to a temperature of 25 C. Also, immediately after the inoculation of each set of experimental flasks, the number of viable cells in the reconstituted cultures was counted by making nutrient agar (Difco) pour plates of 10-fold serial dilutions of the cultures. All experimental results have been corrected to represent sulfate uptake by cultures containing 10^6 viable cells/ml; i.e., 1 liter equals 10⁶ cells.

RESULTS

The data from the factorial experiments are difficult to follow, so they are presented in both tabular (Table 1) and graphical (Fig. 1-3)

### Table 1. Sulfate uptake rates

<table>
<thead>
<tr>
<th>Species</th>
<th>Glucose</th>
<th>Glucose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg/liter</td>
<td>50 mg/liter</td>
<td>500 mg/liter</td>
</tr>
<tr>
<td></td>
<td>28 h 4 day 10 day</td>
<td>28 h 4 day 10 day</td>
<td>28 h 4 day 10 day</td>
</tr>
<tr>
<td>Corynebacterium striatum</td>
<td>0.5 0.0 0.0</td>
<td>2.4 0.0 0.0</td>
<td>2.3 0.0 0.0</td>
</tr>
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<td>Pseudomonas fluorescens</td>
<td>5.9 0.5 0.2</td>
<td>12.2 1.0 0.6</td>
<td>11.9 1.2 0.7</td>
</tr>
<tr>
<td>Serratia marcescens (0.5)</td>
<td>0.3 0.6 0.9</td>
<td>1.1 1.5 2.2</td>
<td>1.1 1.0 1.9</td>
</tr>
<tr>
<td>C. striatum (0.5)</td>
<td>1.9 0.0 0.0</td>
<td>9.2 0.0 0.0</td>
<td>9.1 0.0 0.0</td>
</tr>
<tr>
<td>P. fluorescens (0.5)</td>
<td>20.2 2.2 0.9</td>
<td>37.5 2.7 1.8</td>
<td>36.3 2.9 2.0</td>
</tr>
<tr>
<td>S. marcescens (0.5)</td>
<td>1.0 1.5 2.7</td>
<td>3.8 3.5 5.6</td>
<td>3.6 3.2 4.4</td>
</tr>
<tr>
<td>C. striatum (5.0)</td>
<td>2.5 0.0 0.0</td>
<td>13.9 0.0 0.0</td>
<td>12.6 0.0 0.0</td>
</tr>
<tr>
<td>P. fluorescens (5.0)</td>
<td>25.9 3.8 1.5</td>
<td>53.7 5.3 2.6</td>
<td>49.0 5.3 3.2</td>
</tr>
<tr>
<td>S. marcescens (5.0)</td>
<td>1.3 2.1 3.5</td>
<td>4.4 3.6 5.1</td>
<td>4.3 3.0 3.5</td>
</tr>
<tr>
<td>C. striatum (50)</td>
<td>4.0 0.4 0.2</td>
<td>14.8 1.5 0.3</td>
<td>14.2 2.4 0.5</td>
</tr>
<tr>
<td>P. fluorescens (50)</td>
<td>32.5 4.2 1.9</td>
<td>55.1 4.7 3.0</td>
<td>56.2 5.6 3.1</td>
</tr>
<tr>
<td>S. marcescens (50)</td>
<td>1.8 2.6 2.9</td>
<td>4.1 3.5 3.2</td>
<td>3.7 3.1 3.7</td>
</tr>
</tbody>
</table>

*Measured by three different aged cultures of three bacterial species grown in a defined medium and subjected to different concentrations of glucose and sulfate. Data are expressed as micrograms of sulfur per liter per hour and are corrected to represent 10⁶ cells per liter.

* Sulfate concentration (milligrams per liter).
SULFATE UPTAKE BY AEROBIC BACTERIA

FIG. 1. Sulfate uptake rates of P. fluorescens as a function of glucose and sulfate concentrations. Data are corrected to represent 10⁶ cells/liter.

formats. At all treatment combinations of these experiments, both P. fluorescens (Fig. 1) and C. striatum (Fig. 2) took up sulfate fastest in the 28-h cultures and slowest in the 10-day cultures. The highest rate of sulfate uptake by these two species occurred at the two highest concentrations of both sulfate and glucose, the maximum uptake rate being 56.2 µg of sulfur/liter per h for P. fluorescens and 14.8 µg of sulfur/liter per h for C. striatum. The lowest sulfate uptake rate by P. fluorescens was 0.2 µg of sulfur/liter per h in the 10-day culture at the lowest concentration of both sulfate and glucose. In both the 4- and 10-day cultures of C. striatum, no sulfate was taken up under any experimental treatment that contained sulfate concentrations of either 0.05 or 0.5 mg/liter.

The influence of culture age in the factorial experiments was quite different for S. marcescens. With only one point of exception (50 mg of glucose per liter, 50 mg of sulfate per liter), S. marcescens took up sulfate fastest in the 10-day cultures and slowest in the 28-h cultures (Fig. 3). The highest sulfate uptake rate by S. marcescens was 5.6 µg of sulfur/liter per h at 50 mg of glucose per liter and 0.5 mg of sulfate per liter, whereas the lowest uptake rate, occurring at the lowest concentrations of both glucose and sulfate, was 0.3 µg of sulfur/liter per h. Also unlike C. striatum and P. fluorescens, sulfate uptake rates by S. marcescens were depressed at the higher glucose and sulfate concentrations, with this depression being more pronounced in the older cultures.

The presence of organic sulfur in the growth medium had definite effects upon sulfate uptake by each bacterial species (Fig. 4). As the concentration of organic sulfur increased, the sulfate uptake rate by P. fluorescens decreased in both the 5- and 500-mg/liter glucose-containing cultures. This same trend was evident in the S. marcescens culture that contained only
5 mg of glucose per liter. However, the *S. marcescens* culture that contained 500 mg of glucose per liter, as well as both cultures of *C. striatum*, exhibited increases in the rate of sulfate uptake as the lower concentrations of organic sulfur increased. In each of these three latter cultures, the sulfate uptake rate decreased at the highest concentration(s) of organic sulfur.

In the cultures in which glucose concentration was the only variable, sulfate uptake rates (Fig. 5) were in the range to be expected from the results of the factorial experiments. At glucose concentration up to about 100 mg/liter, as the glucose concentration increased each bacterial species increased the rate at which it took up sulfate. It appears that sulfate uptake rates are not affected by higher glucose concentrations.

**DISCUSSION**

Although this study was intended primarily for ecological interpretation, the results warrant discussion of some of the microbial physiology involved. Some physiological adjustment by the bacteria may have begun soon after each culture was reconstituted, but the short (less than 2 h) incubation time in fresh medium certainly minimized such adjustment, and the measured sulfate uptake rates probably represent a realistic function of the actual physiological age of each culture.

Since 28-h cultures of these bacteria are in exponential growth (9), high rates of sulfur uptake by such cultures would be expected. The 28-h cultures of *C. striatum* and *P. fluorescens* in the factorial experiments did take up sulfate (the only source of sulfur) the fastest. The higher rate of sulfate uptake by the older cultures of *S. marcescens* cannot be easily explained, although this fact illustrates the variability in sulfate uptake between bacterial species. Subsequently, one can infer that there is considerable heterogeneity in the sulfate uptake processes that function in natural multispecies microbial communities.

**Fig. 2.** Sulfate uptake rates of *C. striatum* as a function of glucose and sulfate concentrations. Data are corrected to represent $10^9$ cells/liter.
Fig. 3. Sulfate uptake rates of S. marcescens as a function of glucose and sulfate concentrations. Data are corrected to represent 10^9 cells/liter.

Fig. 4. Sulfate uptake rates of three species of bacteria at different concentrations of organic sulfur (50% methionine-sulfur, 50% cysteine-sulfur). Symbols: O, 5 mg of glucose per liter; ●, 500 mg of glucose per liter; — , S. marcescens; - - - , C. striatum; ····· , P. fluorescens.
Aquatic ecologists do not generally consider sulfur to be a nutrient that limits biotic processes, but the factorial experiments indicate that low sulfate levels could, theoretically, be limiting to bacteria. Some field investigations have shown that the addition of up to 10 mg of sulfate per liter to cultures of natural lake microplankton can stimulate photosynthetic productivity (3, 12). However, low sulfate levels have never been shown to directly limit natural bacterial productivity, and the relatively high concentration of sulfate usually present in the aerobic portion of a lake probably precludes such limitation. Indirect limitation by sulfate might in some cases be inferred, however, in that decreased photosynthesis ultimately leads to lower levels of organic matter that serve as bacterial nutrient.

Although aquatic bacteria certainly occur in many physiological growth phases, any given natural population of aquatic bacteria is probably in or near a state of equilibrium with the environment, i.e., in or near a stationary-growth phase. Since these laboratory studies were established for extrapolation to natural systems and since bacterial sulfate uptake varies with physiological condition, experimentation using cultures in a steady state was considered necessary. Therefore, only 3-day cultures (cells in an early stationary phase) were used in the experiments in which various levels of organic sulfur and glucose were tested.

High levels of organic sulfur tended to decrease the rate of bacterial sulfate uptake. This decrease may be the result of competitive inhibition, although it appears that lower levels of organic sulfur (or specifically cysteine and methionine) stimulated sulfate uptake under the limited nutrient conditions of these experiments. A concentration of about 40 μg of sulfur/liter appears to be a reasonable estimate for the level at which organic sulfur significantly decreases sulfate uptake.

The ecological significance of the occurrence of dissolved organic sulfur in natural aquatic systems is not immediately evident from these studies. Since there is very little published data from which to consider natural concentrations of dissolved organic sulfur, a useful basis for discussing it is the carbon to sulfur ratio found in organic material. Stuiver (15) reported that "the average carbon to sulfur ratios for pure organic materials are of the order of 500:1." Also, ZoBell (16) recognized the wide range of organic sulfur content that occurs among the different types of living organisms, but the values he calculated for the annual photosynthetic conversion of sulfur into organic compounds also reflect the C:S ratio of 500:1. Since this 500:1 is an average ratio to be expected in a heterogeneous mixture of organic compounds, and since dissolved organic compounds (DOC) found in natural aquatic systems will be as a heterogeneous mixture produced by a heterogeneous biotic community, then the DOC in natural aquatic systems should also have a C:S ratio of 500:1.

Although natural concentrations of DOC can range up to about 26 mg of DOC/liter (13), recent investigations have found unpolluted waters to have DOC concentrations considerably less than 15 mg/liter (8, 11, 14). Using the 500:1 C:S ratio, natural dissolved organic sulfur concentrations of less than 30 μg/liter would be expected. Since this level of organic sulfur is probably below the lower concentration at which it can "compete" with sulfate as the source of bacterial sulfur, it can be concluded that organic sulfur is probably not significant as a source of sulfur for most naturally occurring populations of planktonic bacteria.

The uptake of glucose and other organic substrates by bacteria is an active metabolic process, such that, as the concentration of these substrates increase, the rates at which they are taken up also increase but only up to some maximum concentration above which the uptake rates remain constant (1, 4, 5). So, if some metabolic relationship exists between the uptake of sulfate and the uptake of organic carbon, then when bacteria are subjected to a constant sulfate concentration, but increasing concentrations of organic carbon, the rate of sulfate uptake should increase but only up to some maximum organic carbon concentration. This is, in fact, what occurred in this study.
when glucose was the carbon source. Even considering that the low sulfate uptake at the lowest glucose concentrations could result from a depletion of organic carbon rather than the effect of low substrate concentration, these data substantially support the thesis that uptake of sulfate sulfur by bacteria is metabolically linked to the uptake of organic carbon.

The lack of appropriate techniques for measuring naturally occurring heterotrophic biomass production has led to the suggestion that sulfate uptake can be used as a yardstick of such production in aerobic planktonic communities (10). Although this hypothesis may be valid, it is necessary to demonstrate a direct relationship between the uptake of organic carbon and sulfate uptake. The data presented in this paper add considerable credibility to the hypothesis and should provide continued impetus to the pursuit of the unqualified validation of the sulfate uptake method.

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LITERATURE CITED