Selective Accumulation of Unsulfated Carbapenem Antibiotics by Sulfate Transport-negative Mutants of *Streptomyces griseus* subsp. *cryophilus* C-19393

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Sulfate transport-negative mutants were derived from *Streptomyces griseus* subsp. *cryophilus* C-19393 which coproduced sulfated and unsulfated carbapenem antibiotics. These mutants were successfully obtained by two approaches: (1) the requirement of thiosulfate or L-cysteine for growth and (2) resistance to selenate. The sulfate transport-negative mutants selectively produced unsulfated carbapenem antibiotics and the titers were almost equivalent to the sum of the titers of the sulfated and unsulfated carbapenems produced by the parent. The characterization of the sulfate transport system and sulfur metabolism in *Streptomyces* are also described.

A series of unique beta-lactam antibiotics, carbapenems, such as thienamycins\(^1\); olivanic acids\(^2,3\); epithienamycins\(^4\); PS-series\(^5,6\) and C-19393-series\(^7\) antibiotics (or carpetimecins\(^8\)); and asparenomycins,\(^9\) have been detected in culture filtrates of various *Streptomyces* species.

A cryophilic streptomycete, *S. griseus* subsp. *cryophilus* C-19393, produces eight sulfated or unsulfated carbapenem antibiotics (Fig. 1).\(^7\) Among these, C-19393 H\(_2\) and S\(_2\), having a dimethyl group at the C-8 position of the nucleus, are chemically stable compared with other carbapenem antibiotics. In particular, C-19393 H\(_2\) exhibits antibacterial activity comparable to that of thienamycin, except against *Pseudomonas aeruginosa*, as well as strong beta-lactamase inhibitory activity.\(^12\)

In a previous paper,\(^13\) we proposed biosynthetic pathways for 5,6-cis carbapenem antibiotics in *S. griseus* subsp. *cryophilus* C-19393 based on the products of three types of mutants blocked in the biosynthesis. Each unsulfated carbapenem was always coproduced with the corresponding sulfated form, and C-19393 H\(_2\) and S\(_2\) were considered to be the final products of the biosynthesis.

Firstly, we tried to selectively accumulate unsulfated carbapenem antibiotics for effective production of C-19393 H\(_2\). We have obtained auxotrophs that require thiosulfate, L-cysteine or L-methionine for growth, and mutants with resistance to selenate, a structural analogue of sulfate, and have examined their products. The present paper deals with the isolation of sulfate transport-negative mutants, the characterization of a sulfate transport system, and sulfur metabolism in *Streptomyces*.

**MATERIALS AND METHODS**

*Organism.* *S. griseus* subsp. *cryophilus* C-19393\(^7\) strain Cy16 and its mutants were used throughout.

*Media.* The minimal agar medium (MMA) contained 5g glycerol, 0.5g L-asparagine, 0.5g K\(_2\)HPO\(_4\), 0.1g MgSO\(_4\).\(_7\)H\(_2\)O, 1ml trace metal solution and 20g agar (Difco) in 1000ml distilled water (pH 7.1). The trace metal solution consisted of 100mg each of FeCl\(_2\):4H\(_2\)O, MnCl\(_2\):4H\(_2\)O, ZnCl\(_2\) and CaCl\(_2\):2H\(_2\)O, in 100ml of distil-

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\(^7\) Presented at the 4th International Symposium on Genetics of Industrial Microorganisms, held in the Kyoto International Conference Hall, June 6\~11, 1982.
led water. To test the assimilability of sulfur compounds, 5 g agarose (Agarose I, Dojin Chemical Labs., Japan) was used instead of 20 g agar (MMAS). Bennett's agar medium was used as the complete medium (CMA). The complete and synthetic media for the fermentation studies were as described previously.13)

**Fermentation.** A loopful of spores or mycelia propagated on T agar was inoculated into a 200-ml Erlenmeyer flask containing 40 ml of the seed medium and incubated at 28°C for 2 days on a rotary shaker (200 rpm). One milliliter of this seed culture was transferred to 40 ml of the fermentation medium in a 200-ml Erlenmeyer flask and the fermentation was carried out at 30°C for 3 to 6 days on a rotary shaker (220 rpm).

**Mutation.** Spores from a slant culture were suspended in 10 ml of saline or 0.05 M Tris-maleate buffer (pH 8.9), and gently ground with a glass homogenizer. After filtering through No. 1 glass filters, the spore suspensions in saline and in the buffer were subjected to mutagenesis by ultraviolet (UV) irradiation (0.1 ~1% survival) or with N-methyl-N’-nitro-N-nitosoguanidine (NTG; final concn., 1 mg/ml) at 30°C for 60 min, respectively. The spores were washed with saline, diluted and plated on agar media.

**Isolation of auxotrophs for sulfur compounds.** The NTG-treated spores were plated on CMA and incubated at 28°C for 4 ~6 days. The colonies on the plate were transferred to MMA and MMA supplemented with 50 mg each of thiosulfate, L-cysteine and L-methionine (SMMAM) by a replica plating technique; the plates were then incubated at 28°C for 3 days. The clones, which showed normal growth on SMMAM but slight or no growth on MMA, were picked up, purified and maintained on T-agar slants.

**Isolation of selenate resistant mutants.** The mutagenized spores were plated on MMA supplemented with 0.5 or 2.0 g/liter of sodium selenate and incubated at 28°C for 5 ~7 days. Colonies, which grew well, were picked up, purified on the same plate and maintained on T-agar slants.

**Assay of sulfate uptake.** The organisms were cultivated in the chemically defined fermentation medium containing 50 mg/liter of Na2SO4 at 28°C for 4 days and then the mycelial pellets were gently ground with a glass homogenizer to obtain homogenous suspensions. After the mycelia were harvested by centrifugation, they were washed twice with a washing medium (the chemically defined medium minus the sulfur source), resuspended in the washing medium and incubated for 2 hr at 28°C to deplete intracellular sulfate in the mycelia. The mycelia were harvested again by centrifugation and resuspended in fresh washing medium (OD at 550 nm = 4.0). Sulfate uptake was measured at 28°C in a reaction mixture containing 1.9 ml of mycelial suspension and 0.1 ml of Na235SO4 (2.3 μCi, 6.7 μg). Samples (0.2 ml) were withdrawn at the indicated times and added to 2.0 ml of ice-cold 10 mM-Na2SO4. After 5 min, the mycelia were harvested by filtration on a membrane filter (Millipore Corp., HA-type, 0.45-μm pore size) and washed with 10 mM-Na2SO4. The filters were placed in scintillation vials and dried; the radioactivity was counted in 5 ml of toluene scintillation fluid. The sulfate uptake rate was expressed as [35S] counts per minute per milligram of dry mycelia.

**Analytical methods.** Mycelial growth was followed by measuring the packed mycelial volume of 10 ml of culture broth after centrifugation at 3000 rpm for 10 min. The concentration of sulfated carbapenem antibiotics was determined by an agar diffusion disc assay employing Klebsiella pneumoniae IFO 3317 as a test organism on nutrient agar containing 2.5 mg/liter of benzylpenicillin as described.15) An agar diffusion disc assay was also used for determining the concentration of unsulfated carbapenem antibiotics with Bacillus sphaericus IFO 12622. The titers of the sulfated and unsulfated carbapenem antibiotics were determined using C-19393 S2 and epithienamycin B as the standards, respectively. One unit of sulfated carbapenem corresponds to the activity of one microgram of C-19393 S2 and epithienamycin B.

**RESULTS**

**Influence of sodium sulfate upon sulfated and unsulfated carbapenem antibiotic production.**

The effect of sodium sulfate on the production of carbapenem antibiotics by S. gri-
Carbapenem Antibiotic Synthesis by Mutants

sals subsp. cryophilus C-19393 Cy16 was first investigated in a chemically defined medium. As shown in Fig. 2, the level of sodium sulfate had a remarkable influence on the ratio of production of sulfated and unsulfated carbapenems: An increase in sulfated forms was accompanied by a decrease in unsulfated forms; unsulfated carbapenems were produced effectively under limited sulfate conditions. It was difficult to control the sulfate concentration in a complex medium and, therefore, it was of utmost interest to isolate mutants capable of selectively producing unsulfated carbapenem antibiotics.

Isolation of sulfur metabolism mutants

Mutants that require thiosulfate, L-cysteine or L-methionine for growth were selected from mutagenized spores of S. griseus subsp. cryophilus C-19393 Cy16 by a replica plating technique and examined as to their growth responses to various sulfur sources as well as their abilities to produce sulfated and unsulfated carbapenem antibiotics. As summarized in Table I, 58 mutants could be divided into 7 classes according to their growth responses and products. The mutants of classes V and VII lost the ability to produce sulfated carbapenems and accumulated increased amounts of unsulfated forms. Class VII mutants were defective in the ability to assimilate sodium sulfate as a sole source of sulfur, whereas class V mutants were defective in the ability to assimilate sodium sulfate, sodium sulfite and sodium sulfide. Though class IV mutants showed the same phenotype as class V mutants, they produced sulfated carbapenems.

Sulfate transport-negative mutants have been selected as strains resistant to toxic analogues, chromate or selenate. We, therefore, selected selenate resistant mutants of this

![Graph](image)

**Fig. 2.** Effect of Sodium Sulfate on Accumulation of Sulfated and Unsulfated Carbapenem Antibiotics by the Parent Strain, C-19393 Cy16.
The concentration of sodium sulfate in the synthetic fermentation medium was changed as indicated and the cultivation was carried out at 30°C for 6 days. ■, sulfated carbapenem antibiotics; □, unsulfated carbapenem antibiotics.

**Table I. Classification of Sulfur Metabolism Mutants of S. griseus subsp. cryophilus C-19393**

<table>
<thead>
<tr>
<th>Class</th>
<th>No. of mutants obtained</th>
<th>Strain example</th>
<th>Assimilability of</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SO₂⁻</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>Parent</td>
<td>Cy16</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>30</td>
<td>MC-11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>MC-15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>MC-17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>MC-36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>5</td>
<td>K-101</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>6</td>
<td>MC-25</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VII</td>
<td>2</td>
<td>K-4</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Spores or mycelia were inoculated on MMAS supplemented with 50 mg/liter of the indicated sulfur sources and incubated at 28°C for 4 days.

Symbols: +, positive; -, negative; S, sulfated carbapenem antibiotics; H, unsulfated carbapenem antibiotics.
Table II. Selenate Resistant Mutants of S. griseus subsp. cryophilus C-19393

<table>
<thead>
<tr>
<th>Selection plate</th>
<th>Type of mutants</th>
<th>No. of mutants obtained</th>
<th>Growth response to sodium selenate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Growth response&lt;sup&gt;b&lt;/sup&gt; to Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1%</td>
<td></td>
</tr>
<tr>
<td>0.05% Sodium selenate</td>
<td>A</td>
<td>45</td>
<td>SR-135</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>122</td>
<td>SR-37</td>
<td>+</td>
</tr>
<tr>
<td>0.2% Sodium selenate</td>
<td>A</td>
<td>10</td>
<td>Se-5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>122</td>
<td>Se-7</td>
<td>+</td>
</tr>
<tr>
<td>Parent</td>
<td>Cy16</td>
<td></td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spores were inoculated on MMA supplemented with the indicated concentrations of sodium selenate and incubated at 28°C for 7 days.

<sup>b</sup> Spores were inoculated on MMAS supplemented with indicated compounds (50 mg/liter) and incubated at 28°C for 4 days.

Symbols: ++++, good growth; ++, moderate growth; +, poor growth; -, no growth. H, sulfated carbapenem antibiotics; S, unsulfated carbapenem antibiotics.

organism and examined their products. The mutants were classified into type A or B according to their growth responses to sulfur compounds. Only type A mutants, which could not grow on sodium sulfate as a sole source of sulfur, lost their ability to produce sulfated carbapenem antibiotics. The frequency of the appearance of type A mutants on selection on 0.05% sodium selenate was higher than that on 0.2% sodium selenate (Table II).

Sulfate transport system in S. griseus subsp. cryophilus C-19393

In microorganisms, sulfate is taken up by

![Fig. 3. Sulfate Uptake in S. griseus subsp. cryophilus C-19393 Cy16.](image)

![Fig. 4. Effect of Various Compounds on Sulfate Uptake by S. griseus subsp. cryophilus C-19393 Cy16.](image)

2,4-Dinitrophenol was added 20 min before [35S]-sulfate was added. Other compounds and [35S]-sulfate were added simultaneously and the reaction was carried out for 10 min.

active transport. The sulfate transport system of S. griseus subsp. cryophilus C-19393 was investigated by using sodium [35S] sulfate. The linear uptake of sulfate by resting cells
Carbapenem Antibiotic Synthesis by Mutants

continued for at least 20 min under our experimental conditions (Fig. 3), and the system was dependent on energy, because the reaction was inhibited by treatment with 2,4-dinitrophenol. It was markedly inhibited by various sulfur compounds, such as sodium sulfite, sodium sulfide, sodium thiosulfate and L-cysteine, but not significantly by L-methionine (Fig. 4). It was also inhibited by sodium selenate and potassium chromate, but not by sodium tungstate.

The effects of various sulfur sources on the formation of enzymes involved in sulfate uptake are illustrated in Fig. 5. Enzyme formation was markedly repressed by exogenous L-cysteine and weakly repressed by sodium thiosulfate.

The activity of sulfate transport in sulfur metabolism mutants

The sulfur metabolism mutants were cultivated in the chemically defined medium and examined as to their sulfate transport activities. As shown in Fig. 6, auxotrophic mutants of classes V and VII and selenate resistant mutants of type A, which lost the ability to produce sulfated carbapenem antibiotics, lacked a sulfate transport system.

Effect of various sulfur compounds on carbapenem antibiotic production by sulfate transport-negative mutants

As the growth of sulfate transport-negative mutants is supported by various sulfur compounds other than sodium sulfate, we examined whether these compounds could also stimulate the production of sulfated carbapenem antibiotics. The sulfur-limited chemically defined medium was used and the results are presented in Table III. Though the pro-

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Fig. 5. Sulfate Uptake by Mycelia of *S. griseus* subsp. *cryophilus* C-19393 Cyl6 Grown on Different Sulfur Sources.

The organism was cultivated in the synthetic medium in which sodium sulfate was replaced by 50 mg/liter of the indicated compounds.

<table>
<thead>
<tr>
<th>Sulfur source</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$SO$_4$</td>
<td>50</td>
</tr>
<tr>
<td>Na$_2$S$_2$O$_3$</td>
<td></td>
</tr>
<tr>
<td>Na$_2$S$_2$O$_5$</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Activity of Sulfate Transport and Productivity of Sulfated Carbapenem Antibiotics in Sulfur Metabolism Mutants.

To measure the activity of sulfate transport, the organisms were cultivated in the synthetic medium in which sodium sulfate was replaced by 50 mg/liter of L-methionine. The productivity of sulfated and unsulfated carbapenem antibiotics was measured using the complete medium.
TABLE III. EFFECT OF VARIOUS SULFUR COMPOUNDS ON CARBAPENEM ANTIBIOTIC PRODUCTION BY THE PARENT AND SULFATE TRANSPORT-NEGATIVE MUTANTS

Fermentation was carried out in the chemically defined medium supplemented with 250 mg/liter of L-histidine and 2,6-diaminopimelic acid together with the indicated sulfur sources (250 mg/liter) at 28°C for 3 days.

<table>
<thead>
<tr>
<th>Sulfur source</th>
<th>Carbapenem antibiotics (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent</td>
</tr>
<tr>
<td></td>
<td>Cy16</td>
</tr>
<tr>
<td></td>
<td>S&lt;sup&gt;a&lt;/sup&gt; H&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>0.3</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>8.3</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;S·9H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>2.4</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;S&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.6</td>
</tr>
<tr>
<td>S-Sulfo-cysteine</td>
<td>2.3</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> S, sulfated carbapenems.

<sup>b</sup> H, unsulfated carbapenems.

Fig. 7. Time Courses of Carbapenem Antibiotic Production by the Parent Strain, C-19393 Cy16, and a Sulfate Transport-negative Mutant, K4.

The fermentation was carried out in the complete fermentation medium at 30°C for 5 days.

- S—·, sulfated carbapenems; O—O, unsulfated carbapenems; △—△, growth (packed cell volume).

Production of sulfated carbapenem antibiotics by the parent strain, Cy16, was stimulated by the addition of these compounds, that by the sulfate transport-negative mutants was not affected by any of these compounds.

**Time course of carbapenem antibiotic production**

Typical time courses for the production of sulfated and unsulfated carbapenem antibiotics by the parent strain, Cy16, and a sulfate transport-negative mutant, K4, are shown in Fig. 7. The parent had produced both sulfated and unsulfated carbapenem in a ratio of 3:2 after 5 days cultivation, while mutant K4 produced unsulfated carbapenem only; the titer of unsulfated carbapenem was about twice that in the case of the parent.
The sulfate transport system in microorganisms has been shown to be regulated by both feedback inhibition and repression. Sulfate transport in *Escherichia coli* is strongly inhibited by L-cysteine, whereas that in *Salmonella typhimurium* is inhibited by sulfite.
and thiosulfate, but not by L-cysteine or sulfide. Sulfate transport in Paracoccus denitrificans was inhibited by sulfite, thiosulfate and sulfide, but not by L-cysteine. On the other hand, sulfate transport in fungi was inhibited by thiosulfate or sulfite. Repression of the sulfate transport system by L-cysteine has been reported in S. typhimurium and that by L-methionine in Neurospora crassa and Saccharomyces cerevisiae.

The sulfate transport system in S. griseus subsp. cryophilus C-19393 was markedly inhibited by L-cysteine, sulfide, sulfite and thiosulfate. The repression by L-cysteine and weak repression by thiosulfate were also observed.

The formation of sulfated carbapenem antibiotics was stimulated by various sulfur compounds when S. griseus subsp. cryophilus C-19393 Cy16 was cultivated in a sulfate-limiting chemically defined medium (Table III). Though cell growth of sulfate transport-negative mutants was supported by various sulfur compounds other than sodium sulfate, sulfated carbapenem antibiotics were not produced from any of these sulfur compounds by the mutants. This suggests that the sulfonate radical in carbapenem antibiotics is derived from sulfate in the medium only and other sulfur compounds may be changed to sulfate, taken up into cells and transferred into antibiotics by the parent strain.

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