Growth Inhibition of Streptomyces Species by L-Serine and Its Effect on Tetracycline Biosynthesis

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The addition of serine to minimal medium inhibited the growth of Streptomyces aureofaciens and Streptomyces rimosus. Both the outgrowth of spores and the growth of vegetative cells were inhibited by L-serine. This effect was independent of the carbon source used. In rich nutrient medium, however, the serine effect was not observed. The presence of glycine and methionine in minimal medium reversed the growth inhibition imposed by serine, suggesting that a metabolic block related to the synthesis of these two amino acids was involved. A serine-tolerant mutant of S. aureofaciens isolated after ultraviolet irradiation showed a level of serine deaminase comparable to that of the wild-type strain, which indicated that tolerance to serine was not due to the presence of a more active deaminating enzyme in the mutant. Serine markedly reduced tetracycline and oxytetracycline biosynthesis with the parental strains of Streptomyces spp. The serine-tolerant mutant, however, produced almost the same amount of tetracycline in the presence or absence of serine. The final cell population in fermentation broth was not significantly reduced by L-serine, and the addition of glycine and methionine did not increase the tetracycline yields, which suggested that L-serine inhibition of antibiotic biosynthesis was by a mechanism different from that related to growth inhibition.

Experiments of isotopic competition in Escherichia coli (1) demonstrated that exogenous amino acids are incorporated in high proportion into the cell proteins when added to a minimal glucose-containing medium. The endogenous synthesis of amino acids is reduced by feedback and repression mechanisms (2, 13, 17, 18, 21) which contribute to the economy of the cells.

Although amino acid supplementation of minimal media typically enhances the growth of microorganisms, in a few instances the addition of amino acids to minimal media inhibits their growth. Ingram and Jensen (5) found that growth of the blue-green algae (cyanobacterium) Agmenellum quadruplicatum was inhibited by L-phenylalanine; Johnson and Vishniac (7) found growth inhibition of Thiobacillus neapolitanus by histidine, methionine, phenylalanine, and threonine; and in 1962, Leavit and Umberger (8) demonstrated that valine inhibited the growth of E. coli K-12. In the present study is reported the growth inhibition of Streptomyces aureofaciens and Streptomyces rimosus by the addition of L-serine to a minimal medium, the impairment of tetracycline and oxytetracycline biosynthesis by the addition of L-serine to a complex fermentation medium, and the isolation of an L-serine-tolerant mutant of S. aureofaciens.

MATERIALS AND METHODS

Strains and media. Two strains of S. aureofaciens, PF100 and SP2, and S. rimosus R83, all antibiotic-producing microorganisms, were used during this study.

S. aureofaciens was maintained on Bennet agar slants, and S. rimosus was maintained on Emerson agar slants (American Type Culture Collection, Rockville, Md.). The temperature of incubation was 28°C. These media were also used for viable counts. Cell and spore suspensions in 0.85% NaCl were plated after strong agitation of each dilution tube for 2 min in a Vortex mixer.

The composition in of the minimal medium (ML1) was (per liter): KH2PO4, 2 g; (NH4)2HPO4, 6 g; FeSO4·7H2O, 0.005 g; MgSO4·7H2O, 0.25 g; sucrose, 10 g. The minimal agar medium (MM2) had the same salt composition as ML1 but also contained 18 of agar (Difco) per liter. Media were routinely sterilized for 15 min at 121°C.

Glucose and glycerol were used instead of sucrose as carbon sources at concentrations of 10 and 20 g/liter, respectively.

The A-1 germination medium for S. aureofaciens contained (per liter): glucose, 30 g; molasses, 2 g; corn steep liquor, 20 g; (NH4)2SO4, 4 g; CaCO3, 6 g; MgSO4·7H2O, 0.25 g; MnSO4·H2O, 0.025 g; ZnSO4·7H2O, 0.005 g; CoSO4·7H2O, 0.006 g; soy bean oil, 1 ml (pH 6.3). This medium was sterilized for 30 min at 121°C.

The fermentation A-6 medium for tetracycline pro-

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duction was that of Virgilio and Hengeller (22) modified to contain (per liter): sucrose, 50 g; peanut meal, 30 g; (NH4)2SO4, 4 g; Na2HPO4·7H2O, 0.004 g; CaCO3, 6 g; MgSO4·7H2O, 1 g; MnSO4·H2O, 0.1 g; ZnSO4·7H2O, 0.1 g; CoSO4·7H2O, 0.006 g; Al2(SO4)3·18H2O, 0.015 g; soybean oil, 1 ml (pH 6.5). This medium was sterilized for 30 min at 121°C.

The R-1 germination medium for S. rimosus contained (per liter): lactalbumin hydrolysate (Difco Laboratories), 15 g; glucose, 10 g; yeast extract (Difco), 5 g; sucrose, 2.5 g; blackstrap molasses, 5 g; CaCO3, 1 g (pH 6.5 to 7.0). This medium was sterilized for 30 min at 121°C.

The R-6 fermentation medium for production of oxytetracycline contained (per liter): crude low-viscosity gelatin, 11 g; fish meal, 9 g; blood meal, 12 g; soybean meal, 13 g; corn meal, 15 g; corn steep liquor, 0.4 g; CoSO4·7H2O, 0.0083 g; liquid tallow, 10 g; CaCO3, 2 g (pH 7.5). This medium was sterilized for 75 min at 121°C.

Well-sporulated cultures 10 days old were gently scraped into sterile 0.85% NaCl. For the shake-flask fermentation experiments, 4 ml of spore suspension (approximately 10 spores per ml) was inoculated into 50 ml of germination medium in 300-ml flasks and incubated for 24 h at 28°C on a rotary shaker at 180 rpm. Growing cells (1 ml) were inoculated onto the corresponding fermentation media (50 ml in 300-ml flasks).

The usual period of fermentation was 120 h, and then the antibiotic yields were determined in the broths. Tetracycline was assayed by the method of Virgilio and Hengeller (22) at 430 nm, and the oxytetracycline was assayed on acidified and filtered broths at 353 nm.

Chemicals. Serine and other amino acids as well as guanine, adenine, and thymine were obtained from Sigma Chemical Co.

Mutation. Spores of S. aureofaciens SP2 were irradiated with ultraviolet light (14) (Westinghouse sterilamp) at 25 cm for 60 s, diluted, and plated on MM2 plus 100 μg of serine per ml. The resulting colonies able to grow were streaked again on the same medium. Strain SA3126 was selected in this way as a serine-tolerant mutant and used in further experiments.

Serine deaminase activity. L-Serine deaminase activity was determined in toluene-treated cells by the method of Isenberg and Newman (6). Cells of strains SP2 and SA3126 were grown in 300 ml of ML1 with glycerol as the carbon source and 50 glass beads in each 2.8-liter flask. The inoculum was 10 ml of pregrown 24-h culture in the same medium.

Cells were collected by centrifugation after 48 h, washed with saline solution, and suspended in the same solution. The specific activity was determined in toluene-treated cells and expressed as micromoles of sodium pyruvate produced per milligram of protein in the standard 30-min assay.

Protein was determined with washed cell suspensions by the procedure of Miller and Houghton (11).

RESULTS

Growth inhibition by serine. Addition of serine (50 μg/ml) to MM2 inhibited growth of S. aureofaciens SP2, S. aureofaciens PF100, and S. rimosus R-83. No growth was observed after day 3, and after 7 days of incubation at 28°C only a few (about 15%) of the total viable spores were able to form colonies. In contrast, other amino acids such as alanine, glutamine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, threonine, tryptophan, tyrosine, or valine at the same concentration showed no effect.

Growth inhibition was studied on ML1 by using spores of S. aureofaciens as inoculum and different concentrations of serine, ranging from 10 to 1,000 μg/ml. Growth was visually estimated, and a serine concentration of 100 μg/ml was selected for further experiments because this concentration inhibited growth completely. The same effect was obtained when glycerol or glucose was used as the carbon source instead of sucrose.

The effect of L-serine supplementation on outgrowth of spores and vegetative growth is shown in Table 1.

Spor es free of mycelial fragments were obtained by filtration through a membrane filter (1.2-μm pore size; Millipore Corp.) (15). One milliliter of this spore suspension was inoculated into 50 ml of ML1 and incubated at 28°C for 40 h on a gyratory shaker. Cells were harvested and washed with saline solution. Appropriate dilutions of filtered spore suspension and growing cells were plated on MM2, MM2-serine, Bennet, and Bennet-serine agar plates. Serine inhibited the outgrowth of the spores and also reduced significantly the growth of vegetative cells when it was added to minimal medium; however, on serine-supplemented and non-supplemented Bennet medium, the number of colony-forming units (CFU) per unit volume was similar. These data suggested that compounds in the nutrient-rich medium reverse the growth inhibition imposed by serine.

Addition of 300 μg of Casamino Acids (Difco) per ml to minimal serine medium (1,000 μg/ml)

<table>
<thead>
<tr>
<th>Medium</th>
<th>CFU/ml after inoculation with:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Spores</td>
</tr>
<tr>
<td>MM2</td>
<td>1.6 × 10⁴</td>
</tr>
<tr>
<td>MM2 plus serine (100 μg/ml)</td>
<td>4.0 × 10⁵</td>
</tr>
<tr>
<td>Bennet</td>
<td>1.6 × 10⁴</td>
</tr>
<tr>
<td>Bennet plus serine (100 μg/ml)</td>
<td>2.0 × 10⁴</td>
</tr>
</tbody>
</table>
produced good growth. A mixture of glycine (1,000 µg/ml) and methionine (1,000 µg/ml) was found to replace the Casamino Acids in minimal serine medium. The addition of adenine, guanine, and thymine (50 µg/ml) did not improve the growth obtained in the presence of these two amino acids.

**Genetic approach.** A serine-tolerant mutant (SA 3126) of *S. aureofaciens* was isolated on minimal serine medium after ultraviolet treatment of cells. Spore suspensions of the wild-type strain SP2 and the SA3126 mutant were plated on Bennet, MM2, and serine-supplemented MM2 medium. Table 2 shows the CFU per milliliter on each of the media. The mutant was more resistant to the strong inhibitory effect of 1,000 µg of serine per ml than was the parental strain.

L-Serine deaminase is the enzyme that catalyzes the deamination of serine to pyruvate (16). A high level of this enzyme in the mutant could explain its ability to grow on minimal serine medium. The specific activities of L-serine deaminase determined in toluene-treated cells of the wild-type SP2 strain and the SA3126 mutant were found to be similar. Expressed as micromoles of pyruvate produced in 30 min per milligram of protein, the specific activity was 3.4 µmol/mg for strain SP2 and 3.3 µmol/mg for the tolerant mutant.

**Effect of serine on tetracycline biosynthesis.** The presence of serine in the fermentation medium produced up to 77% reduction of tetracycline biosynthesis by *S. aureofaciens* SP2, whereas in the case of the tolerant mutant SA3126 the reduction was about 10% (Table 3). Glutamate, asparagine, proline, or other amino acids at the same concentration did not significantly reduce the yields. Addition of methionine and glycine to the serine fermentation medium did not improve the low yields.

Serine also caused a drop in oxytetracycline biosynthesis with *S. rimosus*. The presence of 250 and 500 µg of serine per ml in the fermentation broth (Table 4) produced a reduction in antibiotic yields of 41 and 57%, respectively.

Dilution and plating of broths with and without serine at the end of the fermentation showed no significant differences in the total counts.

<table>
<thead>
<tr>
<th>Table 2. Response of <em>S. aureofaciens</em> SA3126 and SP2 to L-serine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
</tr>
<tr>
<td>Bennet</td>
</tr>
<tr>
<td>MM2</td>
</tr>
<tr>
<td>MM2 plus serine (1,000 µg/ml)</td>
</tr>
</tbody>
</table>

**Table 3. Inhibition of *S. aureofaciens* tetracycline synthesis by serine**

<table>
<thead>
<tr>
<th>L-serine (µg/ml)</th>
<th>Tetracycline (µg/ml)</th>
<th>% Yield</th>
<th>Tetracycline (µg/ml)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6,300</td>
<td>100</td>
<td>4,675</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>1,600</td>
<td>25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1,000</td>
<td>1,800</td>
<td>29</td>
<td>3,880</td>
<td>83</td>
</tr>
<tr>
<td>1,500</td>
<td>1,440</td>
<td>23</td>
<td>4,225</td>
<td>90</td>
</tr>
</tbody>
</table>

* After 120 h. ND, Not determined.

**Table 4. Inhibition of *S. rimosus* R83 oxytetracycline synthesis by serine**

<table>
<thead>
<tr>
<th>L-serine (µg/ml)</th>
<th>Oxytetracycline (µg/ml)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12,000</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>7,080</td>
<td>59</td>
</tr>
<tr>
<td>500</td>
<td>5,100</td>
<td>43</td>
</tr>
</tbody>
</table>

* After 120 h.

(Table 5). These results agree with those of Table 1, which indicate that in rich nutrient medium serine does not impose growth restrictions. However, the tetracycline biosynthesis reduction by serine was evident, even when 1,000 µg of glycine and methionine per ml was added to the fermentation broth.

**DISCUSSION**

In most cases the presence of exogenous amino acids in minimal medium promotes balanced growth; however, the results of the present work indicate that serine imposes growth inhibition in the two *Streptomyces* species studied. This phenomenon, produced in minimal serine medium supplemented with sucrose, glycerol, or glucose seemed to be independent of the carbon source.

Serine produced growth restrictions on the spore germination and outgrowth as well as on the growing and branching cells, indicating that this amino acid inhibits some essential metabolic step.

A mixture of glycine and methionine reversed the serine inhibition, suggesting that in these *Streptomyces* species the presence of serine interferes with the biosynthesis of these two amino acids. Staefer et al. (19) found in *Salmonella typhimurium* that the addition of serine to minimal medium repressed the enzyme L-serine transhydroxymethylase, responsible for the synthesis of glycine and 5,10-methylentetrahydrofolate, a methyl donor for methionine.

Among other possibilities, growth inhibition by L-serine in *Streptomyces* species could be produced by a partial repression of L-serine transhydroxymethylase, a key enzyme that par-
The mutant SA3126 produced almost the same amount of antibiotic with and without the addition of serine in the fermentation broth. This is consistent with a mutation that reduces the transport of exogenous serine, allowing normal growth and antibiotic biosynthesis, even though serine appears to be acting by two different mechanisms.

Further investigations of tetracycline biosynthesis and serine-sensitive enzymes will give added insight into the metabolism and antibiotic production of Streptomyces species.

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