Production of L-Arginine by Arginine Hydroxamate-Resistant Mutants of *Bacillus subtilis*

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L-Arginine hydroxamate inhibited the growth of various bacteria, and the inhibition was readily reversed by arginine. L-Arginine hydroxamate (10−4 M) completely inhibited the growth of *Bacillus subtilis*. This inhibitory effect was prevented by 2.5 × 10−4 M L-arginine, which was the most effective of all the natural amino acids in reversing the inhibition. L-Arginine hydroxamate-resistant mutants of *Bacillus subtilis* were isolated and found to excrete L-arginine in relatively high yields. One of the mutants, strain AHR-5, produced 4.5 mg of L-arginine per ml in shaken culture in 3 days.

The production of L-arginine is usually carried out by extraction of protein hydrolysates or by chemical synthesis from L-ornithine produced by fermentation. So far, a direct fermentation of L-arginine, although highly desirable, has not been available.

There are eight steps in the pathway of arginine biosynthesis from glutamate, the first precursor of arginine. This pathway has been shown to be regulated by arginine in a wide variety of bacteria (2, 11, 19, 20, 22). Arginine-requiring mutants produce large amounts of ornithine or citrulline when arginine is limiting (7, 12), although their wild-type strains do not accumulate arginine due to these feedback controls by arginine. We considered that arginine would be produced by mutants with a defective regulatory mechanism of arginine, and that such regulatory mutants could be found among a group of analogue-resistant mutants. Teas (16) pointed out that one of his canavanine-resistant mutants of *Neurospora* might excrete arginine; afterwards, Adelberg (1) reported the selection of amino acid analogue-resistant mutants which excrete the corresponding amino acids. We have undertaken the isolation of arginine analogue-resistant mutants, to obtain L-arginine-producing strains, in the same way as in our previous studies on L-isoleucine (8) and L-valine production (9).

Canavanine and homoarginine have been shown to be antagonists of arginine in *Neurospora crassa* (4), lactic acid bacteria (21), yeasts and algae (23), and *Escherichia coli* (11, 13). Very recently, serine hydroxamate has been shown to inhibit the growth of *E. coli* (17, 18). Since we have found isoleucine hydroxamate to be an effective antagonist of isoleucine in *Serratia marcescens* (10; M. Kisumi et al. J. Gen. Microbiol., *in press*), arginine hydroxamate has been examined as an antagonist of arginine. This paper describes the antagonistic action of arginine hydroxamate and the production of L-arginine by arginine hydroxamate-resistant mutants.

**MATERIALS AND METHODS**

**Organisms.** Bacteria used in this study are listed in Table 1, and the isolation of the arginine hydroxamate-resistant mutants of *Bacillus subtilis* is described below.

**Growth experiments.** Spizizen's minimal medium (14) was used and supplemented with biotin (200 μg/liter), thiamine hydrochloride (1 mg/liter), and others as indicated below. The growth experiments were performed in test tubes at 30°C with reciprocal shaking (300 rev/min, 2-cm stroke), and the inocula were taken from fresh overnight cultures. Growth was determined turbidimetrically at 660 nm in a Hitachi photoelectric photometer (EPO-B type). With the instrument employed, an absorbance of 0.10 represented 217 μg of dry cells per ml.

**Fermentation experiments.** Fermentations for the production of L-arginine were carried out as follows. Three milliliters of a medium containing 1% urea, 1% NH₄Cl, 0.7% corn steep liquor, 1% peptone, 2% KH₂PO₄, 0.1% MgSO₄·7H₂O, and 2% CaCO₃ was distributed in test tubes and sterilized by autoclaving. Glucose was autoclaved separately and added at a 10% final concentration aseptically. The tubes were inoculated with a half loopful of resistant mutants. Cultures were incubated at 30°C for 72 hr with reciprocal shaking (300 rev/min, 2-cm stroke).

**Methods of analysis.** Samples of the fermentation broth were chromatographed on Toyo no. 52 paper [solvent system, 1-butanol-acetic acid–water (4:1:1)] and the identification of L-arginine was carried out by
TABLE 1. Inhibition properties of L-arginine hydroxamate in various bacteria

| Bacterium                          | Per cent inhibition of growtha |
|------------------------------------|-------------------------------|
|                                    | 0 M Arginine | 10⁻⁴ M Arginine |
|                                    | Arginine hydroxamate | Arginine hydroxamate |
|                                    | 10⁻⁴ M | 10⁻³ M | 10⁻⁴ M | 10⁻³ M |
| Achromobacter superficialis IAM 1420 | 0      | 0      | 0      | 0      |
| Aerobacter aerogenes OUT 8017       | 17     | 37     | 0      | 4      |
| Alcaligenes faecalis OUT 8029       | 0      | 34     | 0      | 9      |
| Bacillus megaterium OUT 8036       | 18     | 99     | 0      | 100    |
| B. subtilis OUT 8103                | 67     | 94     | 20     | 93     |
| Brevibacterium helvolum IAM 1637    | 83     | 100    | 34     | 90     |
| Corynebacterium fascians IAM 1079   | 0      | 11     | 0      | 0      |
| Escherichia coli ATCC 11303         | 39     | 94     | 16     | 94     |
| Pseudomonas fluorescens IFO 3081    | 16     | 69     | 0      | 60     |
| Sarcina lutea IAM 1099              | 67     | 95     | 3      | 90     |
| Serratia marcescens OUT 8259        | 0      | 0      | 0      | 0      |

a Per cent inhibition of growth was calculated from absorbance at 660 nm in control and in arginine hydroxamate-supplemented cultures at the culture time to attain a half-maximal growth.

Sakaguchi's reagent (5). The quantitative determination of L-arginine was performed with Leuconostoc mesenteroides P-60 (15).

L-Arginine hydroxamate hydrochloride was obtained from the Sigma Chemical Co. (St. Louis, Mo.). Other chemicals used were reagent grade from several sources.

RESULTS

Effect of arginine hydroxamate on the growth of various bacteria. L-Arginine hydroxamate inhibited the growth of a number of bacteria except Achromobacter superficialis and S. marcescens at a concentration of 10⁻⁴ M, as indicated in Table 1. L-Arginine hydroxamate was an extremely potent inhibitor, especially for B. subtilis, Brevibacterium helvolum, E. coli and Sarcina lutea. Growth inhibition resulting from arginine hydroxamate was reversed by L-arginine in all microorganisms, although B. subtilis, B. helvolum, and E. coli were less subject to reversal than the other microorganisms tested.

Since the microorganisms required high levels of arginine for the reversal of the growth inhibition, there was a possibility that arginine hydroxamate-resistant mutants would overproduce L-arginine. B. subtilis was therefore used for
Table 2. Effect of L-arginine on reversal of arginine hydroxamate inhibition

| L-Amino acid (10^{-3} M) | Arginine hydroxamate |
|--------------------------|----------------------|
|                          | Growth (OD)×          |
|                          | 0 M                   | 10^{-4} M                |
| None                     | 0.220                 | 0.060                    |
| Alanine                  | 0.280                 | 0.090                    |
| Arginine                 | 0.280                 | 0.295                    |
| Aspartic acid            | 0.350                 | 0.060                    |
| Citrulline               | 0.200                 | 0.140                    |
| Glutamic acid            | 0.410                 | 0.050                    |
| Glycine                  | 0.100                 | 0.050                    |
| Histidine                | 0.270                 | 0.075                    |
| Isoleucine               | 0.285                 | 0.070                    |
| Leucine                  | 0.490                 | 0.065                    |
| Lysine                   | 0.270                 | 0.185                    |
| Ornithine                | 0.250                 | 0.095                    |
| Methionine               | 0.255                 | 0.060                    |
| Phenylalanine            | 0.260                 | 0.070                    |
| Proline                  | 0.300                 | 0.050                    |
| Serine                   | 0.190                 | 0.065                    |
| Threonine                | 0.105                 | 0.055                    |
| Tryptophan               | 0.260                 | 0.075                    |
| Valine                   | 0.230                 | 0.075                    |

× Growth was measured at 660 nm after 6 hr of incubation. OD, optical density.

detailed growth experiments to verify this possibility.

Effect of L-arginine hydroxamate on the growth of B. subtilis. As seen in Fig. 1, a low concentration of arginine hydroxamate (2.5 × 10^{-3} M) caused only a slight inhibition of the growth rate. The inhibition increased with increasing levels of hydroxamate, and complete inhibition was observed at 10^{-3} M. The initial growth at 10^{-2} M probably is attributable to the intracellular pool of arginine.

Reversal of L-arginine hydroxamate inhibition by L-arginine and its precursors. The inhibitory effect of 10^{-1} M L-arginine hydroxamate was partially prevented by 2.5 × 10^{-4} M L-arginine (Fig. 2). A concentration of L-arginine ten times higher was necessary for complete reversal of the inhibition.

Of the other amino acids tested, L-ornithine, L-citrulline, and L-lysine were able to reverse the inhibitory effect of arginine hydroxamate (Table 2). The effects of ornithine and citrulline on inhibition by arginine hydroxamate were examined in detail, and the results showed that citrulline was more effective than ornithine, but slightly reduced the growth rate at a high concentration (10^{-2} M, Fig. 3). Lysine had a similar effect on the reversal of inhibition of arginine hydroxamate, although not shown. The reversing effect of lysine seems to cause the structural similarity of arginine and lysine, as shown in growth inhibition by canavanine (16).

The finding that the reversal of the growth inhibition decreases in order of arginine, citrulline, and lysine. Growth inhibition may be caused only by ornithine or citrulline, and suggests that the reversal by either ornithine or citrulline is attributable to arginine derived from these amino acids.

Thus, it was shown for the first time that arginine hydroxamate is effective in inhibiting growth as a structural analogue of arginine. The specific reversal by arginine indicated that arginine hydroxamate interferes with arginine biosynthesis and appears, therefore, to be a suitable analogue for the selection of regulatory mutants in the arginine pathway.

Isolation and properties of arginine hydroxamate-resistant mutants. Arginine hydroxamate-resistant mutants were isolated by plating out a heavy inoculum (10^8 cells) of B. subtilis, which had been treated with N-methyl-N'-nitro-N-nitrosoguanidine, at a final concentration of 500 μg/ml, on Spizizen's minimal medium containing 0.2 mg (per ml) of L-arginine hydroxamate. After 3 to 5 days of incubation at 30°C, large colonies appeared on each of the plates. Several colonies from each plate were picked and purified by single-colony isolation on the same medium. In this way, approximately 200 arginine hydroxamate-resistant mutants were obtained and tested for the ability to produce L-arginine. Twelve of them produced more than 2 mg of L-arginine per ml. The typical arginine-producing strains are illustrated in Table 3. It is apparent that arginine hydroxamate-resistant mutants produced large amounts of L-arginine compared with the wild-type strain. In mutant AHR-5, 4.5 mg of L-arginine per ml was observed in 72 hr.

The growth inhibition of AHR-5 by arginine hydroxamate was compared with that of wild-type mutants.
TABLE 3. Accumulation of L-arginine by arginine hydroxamate-resistant mutants

| Strain   | Growth (mg/ml) | L-Arginine accumulated (mg/ml) | L-Arginine accumulated (mg/ml) |
|----------|----------------|--------------------------------|-------------------------------|
|          | 24 hr | 48 hr | 72 hr | 24 hr | 48 hr | 72 hr | 24 hr | 48 hr | 72 hr |
| AHR-5    | 16    | 20    | 19    | 2.3   | 4.3   | 4.5   | 4.0   | 4.3   | 4.5   |
| AHR-26   | 15    | 20    | 20    | 1.0   | 2.9   | 2.8   | 3.2   | 2.9   | 2.8   |
| AHR-114  | 16    | 21    | 20    | 1.4   | 3.4   | 3.4   | 3.8   | 3.5   | 3.5   |
| Wild     | 17    | 21    | 19    | 0.1   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   |

a Dry cell weight.

b Length of incubation.

Fig. 4. Effect of L-arginine hydroxamate on growth of arginine hydroxamate-resistant mutant AHR-5. ●, Wild strain; ○, resistant mutant AHR-5.

The growth of latter strain was completely prevented by 10^{-3} M L-arginine hydroxamate, whereas a concentration of L-arginine hydroxamate as high as 2.5 \times 10^{-2} M was required for complete inhibition of the mutant (Fig. 4). The growth of a regulatory mutant of E. coli isolated on the basis of resistance to the growth-inhibitory action of canavanine was reported to be slowed down under some conditions (3). The fact that the growth rate of this resistant mutant is reduced to 40% of that of the wild type also supports the thesis that metabolic regulation by arginine is abolished in this mutant. To confirm this possibility, the arginine-producing ability of this mutant was investigated in a medium containing high concentrations of L-arginine. As shown in Table 4, the production of L-arginine was not affected by the concentration of L-arginine.

**DISCUSSION**

It has been reported that serine hydroxamate (17, 18) and isoleucine hydroxamate (10) act as growth antagonists against the corresponding amino acids in microorganisms. We observed that arginine hydroxamate inhibits the growth of a number of bacteria and that arginine reverses the inhibition. The action of arginine hydroxamate may be understood by postulating that arginine hydroxamate is a false feedback inhibitor, false co-repressor, or an arginyl-transfer ribonucleic acid synthetase inhibitor. These actions seem to be relaxed in the resistant mutants. This is also supported by the fact that the productivity of arginine was not reduced by L-arginine in the resistant mutant.

It has been shown previously that some mutants resistant to amino acid analogues are suitable as amino acid producers (6). Mutants of this category were isolated with arginine hydroxamate in B. subtilis as well as with isoleucine hydroxamate in S. marcescens (M. Kisumi et al., J. Gen. Microbiol., in press). We succeeded in producing L-arginine by using the arginine hydroxamate-resistant mutant, AHR-5, which was the best arginine-producing strain (Fig. 4, Table 4). As shown above, the developed resistance to the inhibitory effect of arginine hydroxamate resulted in production of arginine. Accordingly, there is the possibility that arginine hydroxamate-resistant mutants of other bacteria would produce large amounts of arginine. Since it is particularly interesting to elucidate the site of action of arginine hydroxamate and the mechanism of overproduction of arginine, these problems are now under investigation.

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