Increase in Isoleucine Accumulation by 
$\alpha$-Aminobutyric Acid-Resistant Mutants of 
*Serratia marcescens*

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Several $\alpha$-aminobutyric acid-resistant (Abu-r) mutants of *Serratia marcescens* were found to be superior to the parent strain in converting d-threonine to L-isoleucine. One of them accumulated 1.5 times more L-isoleucine that the parent strain. The level of acetohydroxy acid (AHA) synthetase in this mutant increased twofold above that of the parent strain. In the parent strain, AHA synthetase was repressed and L-isoleucine accumulation was decreased by either L-valine or L-leucine, whereas in the mutant the AHA synthetase level and L-isoleucine accumulation were not affected by these amino acids. AHA synthetase of the Abu-r mutant was feedback-inhibited by L-valine to the same extent as that of the parent strain. The level of d-threonine dehydratase in both strains was only slightly affected by several amino acids tested. L-Threonine dehydratase of the parent strain and of the mutant was almost completely inhibited by L-isoleucine. These results indicate that the increase in L-isoleucine accumulation by Abu-r mutants is due to the genetic derepression of AHA synthetase.

In the course of our investigation of L-isoleucine production from d-threonine by *Serratia marcescens*, it was found that either L-valine or L-leucine inhibited the accumulation of L-isoleucine and caused the formation of $\alpha$-aminobutyric acid in the culture medium (5). A slight formation of L-valine was also observed in the presence of an excess of glucose. These facts indicate that $\alpha$-ketobutyric acid formed from d-threonine is not converted to $\alpha$-acetohydroxybutyric acid but to $\alpha$-aminobutyric acid due to feedback control by L-valine or L-leucine of acetohydroxy acid (AHA) synthetase, the second enzyme in the isoleucine biosynthetic pathway. To decrease the inhibition of L-isoleucine production, we searched for a mutant in which AHA synthetase would be released from feedback inhibition or repression, or both. As a result, it was found that $\alpha$-aminobutyric acid-resistant (Abu-r) mutants were superior to the parent strain in L-isoleucine accumulation. L-Valine accumulation by Abu-r mutants has been described in another report (M. Kisumi et al., J. Bacteriol., in press).

**MATERIALS AND METHODS**

Organisms, medium, and cultural conditions. The organisms used in this study were *S. marcescens* no. 1 (5) and the mutants resistant to $\alpha$-aminobutyric acid, norvaline, and norleucine which were derived from it (M. Kisumi et al., J. Bacteriol. in press). Of the resistant mutants, Abu-r mutant 130-1 was mainly used. A medium containing 2% d-threonine, 2% glucose, 10% dextrin (Matsutani Chemical Co., Ltd.), 0.5% urea, 0.1% K$_2$HPO$_4$, 0.05% MgSO$_4$·7H$_2$O, and 2% CaCO$_3$ was employed as a standard medium. A mixture of glucose and dextrin was autoclaved separately and added aseptically. The organisms were grown in 500-ml shaking flasks containing 15 ml of the medium at 30°C with reciprocal shaking (140 rev/min, 8-cm stroke).

**Analytical methods.** L-Isoleucine and L-valine were measured microbiologically by using *Leuconostoc mesenteroides* P-60. D-Threonine was estimated by paper chromatography. Quantitative determination of total sugar was carried out by the method of Dubois et al. (2). The procedure for estimating growth of organisms was described elsewhere (M. Kisumi et al., J. Bacteriol., in press).

Preparation of cell-free extracts and assay of enzyme activities. The cells cultured for 15, 18, and 24 hr were harvested by centrifugation. The preparation of cell-free extracts and the assay of AHA synthetase and L-threonine dehydratase were as described elsewhere (Kisumi et al., J. Bacteriol., in press), except that AHA synthetase was assayed only at pH 8.0. Assay of d-threonine dehydratase was the same as the assay of L-threonine dehydratase, except that d-threonine was employed as a substrate instead of L-threonine. Specific activities are expressed as micromoles of product formed per milligram of protein per minute.

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RESULTS

L-Isoleucine-accumulating activity in the mutants resistant to analogues of branched-chain amino acids. Several mutants resistant to α-amino-butyric acid, norvaline, and norleucine were tested for L-isoleucine accumulation from D-threonine. Of these mutants, several Abu-r mutants were found to be superior to the parent strain in L-isoleucine accumulation, and mutant 130-1 was selected for further work. Norvaline- or norleucine-resistant mutants were not found to be superior to the parent strain.

Changes during L-isoleucine accumulation by the parent and mutant 130-1. Changes during L-isoleucine accumulation by the parent strain and mutant 130-1 are shown in Fig. 1.

Although the lag phase of growth of the mutant was longer than that of the parent strain, the maximum growth of the mutant strain was considerably higher. This difference in maximum growth may explain the observation that D-threonine was completely consumed by the mutant within 72 hr, whereas some D-threonine remained in the case of the parent strain. The mutant accumulated 15 to 16 mg of L-isoleucine per ml whereas the parent accumulated 10 to 11 mg/ml.

Levels of AHA synthetase and D-threonine dehydratase were highest in the early log phase of both strains and thereafter decreased (Fig. 2). The high rate of L-isoleucine accumulation during the period from 18 to 36 hr is probably due to the high levels of both enzymes at this time. The mutant had approximately a twofold level of AHA synthetase relative to the parent strain, whereas its level of D-threonine dehydratase was only slightly elevated. The increased level of AHA synthetase in the mutant is believed to be the main cause of the increase in L-isoleucine accumulation.

Feedback inhibition of L-threonine dehydratase and AHA synthetase. L-Threonine dehydratase is considered to be the rate-limiting enzyme for isoleucine biosynthesis (7-9). If L-threonine dehydratase of the mutant is insensitive to feedback inhibition by L-isoleucine, the possibility exists that the increase in L-isoleucine accumulation is due to the formation of L-isoleucine from glucose via L-threonine. Therefore, feedback inhibition of L-threonine dehydratase by L-isoleucine was investigated. L-Threonine dehydratase in both strains was found to be almost completely feedback-inhibited by $10^{-4}$ M L-isoleucine (Table 1). This result indicates that the increase in L-isoleucine accumulation cannot be attributed to the formation of L-isoleucine from glucose via L-threonine but to the increase in the conversion of D-threonine to L-isoleucine.

In view of the fact that L-valine strongly inhibits L-isoleucine accumulation (5), AHA synthetase appears to be a second rate-limiting enzyme for the formation of L-isoleucine. Since AHA synthetase has been reported to be feedback-inhibited by L-valine (1, 3, 10), the possibility remains that this enzyme of the mutant is insensitive to feedback inhibition by L-valine. The AHA synthetase of the mutant cells grown in D-threonine was found to be as sensitive as that of the parent, which was inhibited by about 50% in the presence of $10^{-3}$ M L-valine (Table 2). These facts show that the increase in L-isoleucine accumulation was not due to the insensitivity of AHA synthetase to feedback inhibition by L-valine.

Effect of various amino acids on L-isoleucine accumulation and on the formation of D-threonine.
**TABLE 1. Feedback inhibition of L-threonine dehydratase by L-isoleucine**

| L-Isoleucine added (m) | Parent strain | Mutant 130-1 |
|------------------------|---------------|--------------|
|                        | Activity | Inhibition (%) | Activity | Inhibition (%) |
| 0                      | 0.42     | 0             | 0.51     | 0             |
| $10^{-4}$              | 0.38     | 11            | 0.44     | 14            |
| $2 	imes 10^{-4}$      | 0.23     | 47            | 0.26     | 48            |
| $10^{-2}$              | 0.008    | 98            | 0.015    | 97            |

* Cell-free extracts were prepared by using cells cultured for 18 hr.

**TABLE 2. Feedback inhibition of acetoxy acid synthetase by L-valine**

| L-Valine added (m) | Parent strain | Mutant 130-1 |
|--------------------|---------------|--------------|
|                    | Activity | Inhibition (%) | Activity | Inhibition (%) |
| 0                  | 0.20     | 0             | 0.39     | 0             |
| $10^{-2}$          | 0.10     | 45            | 0.22     | 43            |
| $10^{-2}$          | 0.072    | 63            | 0.15     | 62            |
| $10^{-1}$          | 0.063    | 67            | 0.14     | 63            |

* Cell-free extracts were prepared using cells cultured for 18 hr.

dehydratase and AHA synthetase. As described above, no difference in feedback inhibition of L-threonine dehydratase and AHA synthetase was found between the parent and the mutant. Therefore, the possibility exists that the mutant differs from the parent strain in repression of enzymes related to the biosynthesis of L-isoleucine. Accordingly, the effects of various amino acids on the accumulation of L-isoleucine and on the formation of D-threonine dehydratase and AHA synthetase were examined.

As indicated in Table 3, the parent strain accumulated 10 to 11 mg of L-isoleucine per ml from D-threonine in the medium without the addition of amino acids, whereas the addition of 1 mg of L-valine per ml strongly inhibited L-isoleucine accumulation and caused ß-aminobutyric acid formation. L-Valine markedly repressed AHA synthetase but hardly affected the formation of D-threonine dehydratase. These results indicate that ß-ketobutyric acid derived from D-threonine does not condense with pyruvic acid but is converted to ß-aminobutyric acid. L-Leucine also inhibited L-isoleucine accumulation and repressed AHA synthetase to a lesser extent than L-valine. L-Threonine and L-isoleucine hardly affected the formation of two enzymes and the accumulation of L-isoleucine. ß-Aminobutyric acid inhibited the growth of the parent strain and L-isoleucine accumulation, although it had little effect on the formation of D-threonine dehydratase and AHA synthetase.

Table 4 describes the effect of various amino acids on L-isoleucine accumulation and the formation of two enzymes in the mutant. L-isoleucine accumulation and the formation of both enzymes were affected by L-valine, L-leucine, L-isoleucine, and L-threonine, respectively.
L-threonine, and a-aminobutyric acid only slightly. Thus, it was concluded that the mutant differed from the parent strain in the repression of AHA synthetase by L-valine and L-leucine. This fact may account for the insensitivity of L-isoleucine accumulation by the mutant to L-valine and L-leucine. When other Abu-r mutants were tested, L-valine and L-leucine neither inhibited L-isoleucine accumulation nor repressed AHA synthetase.

**Effect of concentration of D-threonine on the formation of D- and L-threonine dehydratases and AHA synthetase.** To examine more critically the differences between the parent strain and the mutant, the effects of the concentration of D-threonine on the formation of D- and L-threonine dehydratases and AHA synthetase were studied (Table 5). As in the case of the parent strain (6), D-threonine dehydratase was inducibly formed by the addition of D-threonine. It is of interest that the levels of L-threonine dehydratase and AHA synthetase in the parent strain were higher in the presence of D-threonine than in the absence of it, although the levels of these enzymes were lower than in the mutant. These observations are discussed below. On the other hand, the levels of these enzymes in the mutant were lower in the presence of D-threonine than in its absence, although the reason for this is unknown. As previously described (M. Kisumi et al., J. Bacteriol., in press), the mutant accumulated a large amount of L-valine in the absence of D-threonine. When 5 mg of D-threonine per ml was added to the culture medium, it accumulated both L-isoleucine and L-valine, whereas the addition of 20 mg/ml caused the accumulation of L-isoleucine only. These observations might be attributed to the difference between the affinities of AHA synthetase for a-ketobutyric acid and for pyruvic acid and to the difference in the amount of these substrates which are formed from D-threonine and glucose.

**DISCUSSION**

L-Isoleucine production from D-threonine by an Abu-r mutant (no. 130-1) was superior to that of the parent strain and was not inhibited by L-valine and L-leucine. From the studies on feedback control of enzymes related to L-isoleucine biosynthesis, it became evident that the increase in L-isoleucine accumulation by this mutant might be mainly due to derepression of AHA synthetase. Moreover, our results also indicate that D-threonine dehydratase [which plays the most important role in the L-isoleucine formation as described previously (6)] and AHA synthetase are rate-limiting for L-isoleucine formation from D-threonine.

It is of interest that a high concentration of D-threonine caused the derepression of L-isoleucine dehydratase and AHA synthetase in the parent strain. Accordingly, repression of AHA synthetase by L-valine and L-leucine must overcome this D-threonine-mediated derepression. Isoleucine-valine biosynthetic enzymes of *S. marcescens* have been found to be multivalently repressed by three branched-chain amino acids (submitted for publication). Therefore, the possibility exists that D-threonine may produce a deficiency of
TABLE 5. Effect of concentration of D-threonine on the accumulation of amino acids and the formation of D- and L-threonine dehydratases and acetohydroxy acid (AHA) synthetase in the parent strain and mutant 130-I

| Strain       | d-Threonine added (mg/ml) | Culture time (hr) | Growth (mg/ml) | Accumulation | Enzyme level |
|--------------|---------------------------|-------------------|----------------|--------------|--------------|
|              |                           |                   |                | L-Threonine (mg/ml) | L-Valine (mg/ml) | D-Threonine dehydratase | L-Threonine dehydratase | AHA synthetase |
| Parent strain| 0                         | 15                | 9.2            | 0            | 0            | 0.001             | 0.15                   | 0.062          |
|              | 18                        | 12.4              | 0              | 0            | 0            | 0.001             | 0.14                   | 0.058          |
|              | 24                        | 14.7              | 0              | 0            | 0            | 0.001             | 0.13                   | 0.043          |
|              | 48                        | 17.6              | 0              | 0.5          | 0            | 0.058             | 0.19                   | 0.070          |
|              | 72                        | 16.7              | 0              | 0.5          | 0            | 0.045             | 0.16                   | 0.075          |
|              | 5                         | 15                | 9.5            | 1.2          | 0            | 0.053             | 0.17                   | 0.058          |
|              | 18                        | 11.8              | 1.1            | 0            | 0            | 0.093             | 0.45                   | 0.17           |
|              | 24                        | 15.1              | 1.6            | 0            | 0            | 0.088             | 0.47                   | 0.15           |
|              | 48                        | 16.7              | 1.8            | 0            | 0            | 0.073             | 0.40                   | 0.093          |
|              | 72                        | 18.5              | 1.7            | 0            | 0            | 0.062             | 0.67                   | 0.60           |
| Mutant 130-I | 0                         | 15                | 9.2            | 0            | 1.6          | 0.001             | 0.67                   | 0.56           |
|              | 18                        | 11.2              | 0              | 2.1          | 0            | 0.001             | 0.67                   | 0.56           |
|              | 24                        | 12.4              | 0              | 4.8          | 0            | 0.001             | 0.63                   | 0.50           |
|              | 48                        | 12.0              | 0              | 6.2          | 0            | 0.053             | 0.60                   | 0.46           |
|              | 72                        | 15.9              | 0              | 7.1          | 0            | 0.048             | 0.57                   | 0.40           |
|              | 5                         | 15                | 8.1            | 1.0          | 1.4          | 0.042             | 0.56                   | 0.38           |
|              | 18                        | 8.9               | 1.6            | 2.5          | 0            | 0.088             | 0.47                   | 0.15           |
|              | 24                        | 10.3              | 3.0            | 4.8          | 0            | 0.073             | 0.40                   | 0.093          |
|              | 48                        | 23.8              | 3.4            | 6.4          | 0            | 0.062             | 0.67                   | 0.60           |
|              | 72                        | 24.5              | 4.1            | 6.8          | 0            | 0.058             | 0.57                   | 0.46           |
|              | 20                        | 15                | 4.8            | 1.3          | 0            | 0.16              | 0.60                   | 0.27           |
|              | 18                        | 7.3               | 2.3            | 0            | 0            | 0.14              | 0.53                   | 0.34           |
|              | 24                        | 11.5              | 8.6            | 0            | 0            | 0.12              | 0.43                   | 0.30           |
|              | 48                        | 27.4              | 13.6           | 0            | 0            | 0.073             | 0.47                   | 0.15           |
|              | 72                        | 29.0              | 14.8           | 0            | 0            | 0.058             | 0.46                   | 0.38           |

* Besides d-threonine, all media contained 2% glucose, 10% dextrin, 0.5% urea, 0.1% K2HPO4, 0.05% MgSO4·7H2O, and 2% CaCO3.  
* Dry cell weight.  
* Specific activity.

L-valine and L-leucine in the cells, i.e., the α-ketobutyric acid which is formed from D-threonine in the absence of feedback control may competitively interfere with α-acetolactate formation. A future report will consider this point more critically.

This paper describes the application of analogue-resistant mutants to L-isoleucine production from D-threonine. The use of analogue-resistant mutants may be considered to be advantageous for the industrial production of amino acids and other useful metabolites for the following reasons. (i) The possibility is great that resistant mutants are released from metabolic controls. (ii) Resistant mutants can be readily isolated from parent strains. (iii) In contrast to amino acid production by auxotrophs which is influenced by the concentration of amino acids required for growth (4), the addition of such supplements is not necessary for production by resistant mutants. (iv) The preservation of resistant mutants is an easy task since back-mutation is prevented by the addition of corresponding analogues to the medium.

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