Threonine Production by Dihydrodipicolinate Synthase-defective Mutants of Brevibacterium flavum

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Threonine-producing mutants of Brevibacterium flavum that lacked dihydrodipicolinate synthase (DPS) activity but had wild-type homoserine dehydrogenase (HD) sensitive to feedback inhibition by threonine were isolated as strains resistant to \( \varepsilon \)-amino-\( \varepsilon \)-hydroxyvaleric acid (AHV). The growth of a mutant, DK131, derived from an aspartate-producing strain was slow and markedly enhanced by diaminopimelic acid (DAP), but not by \( l \)-lysine alone. In a medium containing 10% glucose and the optimum concentration of DAP, strain DK131 produced 13.7 g/l of threonine, which was comparable to that by the previously reported mutant, BB69, with a feedback-resistant HD (HDR). The production was more strongly inhibited by lysine in strain DK131 than in strain BB69. DPS-defective mutants derived from a lysine producer with feedback-insensitive aspartokinase were selected as those which were resistant to AHV and which produced more threonine than lysine. A representative strain, DA105, produced 10.3 g/l of threonine and 3.5 g/l of lysine·HCl, while AHV-resistant mutant, BA68, with an HDR, derived from the same parent, produced 9.7 g/l of threonine and 12.2 g/l of lysine·HCl. Strain DA105 grew well in the absence of DAP.

In the genera Brevibacterium and Corynebacterium, only mutants with HDR's have been reported to produce threonine, and they were selected as mutants resistant to AHV, a threonine analogue\(^1\),\(^2\) (Fig. 1). However, during the screening of AHV-resistant threonine producers, we found a novel type of threonine producers which had normally feedback-sensitive HD, unlike the threonine producers previously obtained, but which were defective in DPS, the first enzyme in the lysine-specific biosynthetic pathway (Fig. 1). The DPS deficiency is considered to be not only novel as a character of threonine producers, but also practically useful. That is, enhancement of threonine productivity is expected on the endowment of HDR type threonine producers with the DPS defect. Furthermore, the byproduction of lysine, an undesirable character often observed in HDR type threonine producers derived from lysine producers, may be depressed by the DPS defect.

The present paper deals with the derivation and characterization of DPS mutants from B. flavum.

Materials and Methods

Bacterial strains and media. Mutant strains of Brevibacterium flavum No. 2247 (ATCC 14067, wild strain), an aspartate producer, No. 70,\(^3\) with a PK defect and a lysine producer, AC6644\(^4\) (No. 664-7 in the previous paper), with an AKR, both of which were derived from strain No. 15-8\(^5\) with a CSL and a PCR, were used. Strain BB69, an AHV-resistant threonine-producing mutant with an HDR, derived from wild strain No. 2247,\(^1\)

Abbreviations : DPS, dihydrodipicolinate synthase; HD, homoserine dehydrogenase; CS, citrate synthase; CSL, low level CS; PC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; AK, aspartokinase; HDR, PC\(^6\) and AK\(^8\), feedback-resistant HD, PC and AK; HK, homoserine kinase; AHV, \( DL-\varepsilon \)-amino-\( DL-\varepsilon \)-hydroxyvaleric acid; HEPES, N-2-hydroxyethylpiperizine-N'-2-ethanesulfonic acid; DAP, diaminopimelic acid; ASA, aspartate-\( \beta \)-semialdehyde; NG, N-methyl-N’-nitro-N-nitrosoguanidine; SMH, soybean-meal hydrolysate.
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Fig. 1. Threonine and Lysine Biosynthesis, and Their Regulation in Brevibacterium flavum.

PEP, phosphoenolpyruvate; HSE, homoserine; DDP, dihydrodipicolinate; PYR, pyruvate; CIT, citrate; ↔, feedback inhibition.

A complete medium, Medium 7, and glucose minimal media, Medium 10 and Medium 13, were described previously.\(^1\) Medium 47, an acetate-pyruvate minimal medium, contained 5g acetic acid, 5g sodium pyruvate sterilized separately by filtration, 5g (NH₄)₂SO₄, 1g KH₂PO₄, 0.4g MgSO₄·7H₂O, 0.5g FeSO₄·7H₂O, 8mg MnSO₄·4H₂O, 500μg d-biotin, 100μg thiamine·HCl, 23.8g HEPES, 20g agar and NaOH to adjust the pH to 7.0, per liter, and was sterilized at 115°C for 10min.

Medium 48 for threonine or lysine production was Medium 36 without L-methionine, and contained 100g glucose per liter. When the (NH₄)₂SO₄ and/or SMH concentrations of this medium were changed, they are indicated in parenthesis after "Medium 48". For example, the medium containing the standard concentrations of (NH₄)₂SO₄ and SMH is designated as Medium 48 (N60 S35). Medium 48 for threonine or lysine production was Medium 36 without l-methionine, and contained 100g glucose per liter. When the (NH₄)₂SO₄ and/or SMH concentrations of this medium were changed, they are indicated in parenthesis after "Medium 48". For example, the medium containing the standard concentrations of (NH₄)₂SO₄ and SMH is designated as Medium 48 (N60 S35). Medium 48 for threonine or lysine production was Medium 36 without l-methionine, and contained 100g glucose per liter. When the (NH₄)₂SO₄ and/or SMH concentrations of this medium were changed, they are indicated in parenthesis after "Medium 48". For example, the medium containing the standard concentrations of (NH₄)₂SO₄ and SMH is designated as Medium 48 (N60 S35).

Chemicals. AHV and dl-DAP were purchased from Sigma. dl-DAP was a mixture of the dd-, ll- and meso-isomers. dl-ASA was prepared as described previously.\(^8\) HEPES was a product of Oriental Yeast Co., Ltd.

Derivation of AHV-resistant mutants. i) From strain No. 70: Cells grown for 24hr on a Medium 7 agar plate supplemented with 10g/l of monosodium L-glutamate were inoculated into 4ml of Medium 13 containing 300μg/l of d-biotin but no agar, and then cultured for 15hr. The cultivation temperature was 30°C throughout this study. The cells were harvested, washed twice with 0.1m sodium phosphate buffer (pH 7.0), resuspended in the same buffer, and then treated with 150μg/ml of NG at 30°C for 15min. The treated cells were washed, and then spread on Medium 10 containing 300μg/l of d-biotin, 10g/l of AHV and 5g/l of l-lysine·HCl, at the inoculum size of about 10⁵ cells per plate (diameter, 9cm). Colonies that appeared during cultivation for 5days were picked up. ii) From strain AC664: Washed cells were prepared as described above except that 300 and 200mg/l of l-histidine·HCl were added to Medium 7 and the modified Medium 13 for the enhancement of growth, respectively.\(^4\) They were treated with 600μg/ml of NG for 15min, washed, and then spread on Medium 47 supplemented with 3 to 5g/l of AHV, at the inoculum size of 10⁵ to 10⁶ cells per plate (diameter, 9cm). As growth enhancers, the following compounds were added to Medium 47: 20mg each of l-alanine and l-cystine, 300mg each of l-arginine·HCl, l-lysine·HCl, l-methionine, l-tryptophan, l-phenylalanine and l-tyrosine, 500mg of l-histidine·HCl and 400mg of yeast extract, per liter. Colonies that appeared during cultivation for 12 days were picked up.

Production of threonine. Method A: This method was used for the screening of threonine producers from AHV-resistant mutants. A loopful of cells grown on Medium 7 supplemented with 10g/l of monosodium L-glutamate for 24hr was inoculated into 3ml of Medium 48 in a test tube, and then cultured for 72hr. Method B: Two loopfuls of cells obtained as described above were inoculated into 20ml of Medium 48 or Medium T-2 in a 500-ml shaking flask, and then cultured for 72hr. In method A and B, 300mg/l of l-histidine·HCl was added to Medium 7 for cultivation of strain AC664 and its mutants. In method B, for cultivation of DPS-defective or -reduced mutants, 1g/l of dl-DAP and 50mg/l of dipicolinic acid were added to Medium 7. Threonine and lysine in the culture broth were detected by thin-layer chromatography as described in the previous paper.\(^8\) L-Threonine,\(^1\) lysine\(^8\) and growth\(^1\) were determined as described previously.

Enzyme assays. Enzyme activities were measured with cells cultured in 20ml of Medium 48 in 500-ml shaking flasks for 40hr. For cultivation of strain No. 70 and its mutants, Medium 48 (N50 S30) was used, whereas Medium 48 (N25 S35) was employed for strain AC664 and its mutants. In the case of DPS-defective or -reduced mutants, the above media were supplemented with 1g/l of dl-DAP and 50mg/l of dipicolinic acid were added to Medium 7. Threonine and lysine in the culture broth were detected by thin-layer chromatography as described in the previous paper.\(^8\) L-Threonine,\(^1\) lysine\(^8\) and growth\(^1\) were determined as described previously.
molecular weight contaminants. The enzyme activity was determined by measuring pyruvate remaining after reaction with dl-ASA. The assay conditions were the same as given previously, except that 1 ml of 0.1 N HCl solution was added to stop the reaction and that 20 μg lactate dehydrogenase was used for determination of the pyruvate, in a total volume of 2 ml. A reaction mixture without dl-ASA was used as a control. The crude extract for HK measurement was prepared by sonic disintegration of washed cells, followed by centrifugation. The extract after passage through a column of Sephadex G-25 was used as the enzyme solution. The enzyme activity was determined as described previously.

Results

Derivation of DPS-defective threonine producer from aspartate-producing strain No. 70

Strain No. 70, which produced 22 g/l of L-aspartic acid in a medium containing 10% glucose, showed much lower sensitivity to AHV than the original wild strain, No. 2247. However, its growth was found to be severely inhibited by AHV plus lysine, which inhibited not only HD but also AK. One hundred and sixty-nine strains of mutants resistant to AHV plus lysine were derived on NG treatment and their threonine productivities were examined by method A with Medium 48 (N70 S30). The best producer, DK131, accumulated 8.9 g/l of threonine in Medium 48 (N50 S30), as shown in Table I. However, the HD of strain DK131 had not been released from feedback inhibition by threonine. Instead, the strain was completely defective in DPS activity. No difference was observed as to either specific activity or feedback inhibition of AK.

Table I. Properties of a Mutant, DK131, Resistant to α-Amino-β-hydroxyvaleric Acid Derived from Strain No. 70

<table>
<thead>
<tr>
<th>Property</th>
<th>No. 70 (Parent)</th>
<th>DK131</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr produced (g/l)</td>
<td>0.0</td>
<td>8.9</td>
</tr>
<tr>
<td>DPS Activity*</td>
<td>63</td>
<td>0.0</td>
</tr>
<tr>
<td>HD Activity*</td>
<td>233</td>
<td>127</td>
</tr>
<tr>
<td>Inhibition(%)b</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>AK Activity*</td>
<td>8.2</td>
<td>10.5</td>
</tr>
<tr>
<td>Inhibition(%)c</td>
<td>94</td>
<td>95</td>
</tr>
</tbody>
</table>

a nmol/min/mg protein.
b Inhibition by 1 mM L-threonine.
c Inhibition by 1 mM L-lysine plus 1 mM L-threonine.

between strain DK131 and its parent strain, No. 70.

Derivation of DPS-defective threonine producers from lysine-producing strain AC664

Strain AC664 produced more than 40 g/l of lysine as its HCl salt in Medium 48 (N70 S35), but, as shown in Table II, 24.5 g/l in Medium 48 (N25 S35), the standard medium used for threonine production in this study. This strain showed low sensitivity to AHV in a glucose minimal medium. However, the growth on acetate plus pyruvate was found to be severely inhibited by AHV, and recovered on further addition of L-threonine (data not shown). Then, 1576 mutants resistant to AHV were derived, and their threonine productivities were examined by method A with Medium 48 (N25 S35). Thirty-one percent of the resistant mutants were threonine producers, but most of these producers accumulated lysine in larger amounts than threonine. A representative strain, BA68, which produced 9.7 g/l of threonine and 12.2 g/l of lysine as its HCl salt, was confirmed to be a conventional HD type threonine producer with a normal level of DPS, as shown in Table II. On the other hand, in strains DA105, DA110, DA417,
Table II. Properties of Mutants Resistant to α-Amino-β-hydroxyvaleric Acid (AHV) Derived from Strain AC664

Amino acid production was examined by method B with Medium 48 (N25 S35).

<table>
<thead>
<tr>
<th>Properties</th>
<th>AC664 (Parent)</th>
<th>BA68</th>
<th>DA105</th>
<th>DA110</th>
<th>DA417</th>
<th>No. 257</th>
<th>No. 415</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHV in selection medium (g/l)</td>
<td>—</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr (g/l)</td>
<td>0.0</td>
<td>9.7</td>
<td>9.0</td>
<td>6.3</td>
<td>6.2</td>
<td>5.2</td>
<td>8.8</td>
</tr>
<tr>
<td>Lys·HCl (g/l)</td>
<td>24.5</td>
<td>12.2</td>
<td>5.3</td>
<td>2.6</td>
<td>4.5</td>
<td>2.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Growth (A₅₆₂)</td>
<td>1.05</td>
<td>1.08</td>
<td>1.04</td>
<td>1.05</td>
<td>1.12</td>
<td>0.61</td>
<td>0.94</td>
</tr>
<tr>
<td>DPS Activity</td>
<td>75.4</td>
<td>59.3</td>
<td>0.0</td>
<td>—0.5</td>
<td>4.2</td>
<td>77.4</td>
<td>94.1</td>
</tr>
<tr>
<td>HD Activity</td>
<td>150</td>
<td>56</td>
<td>74</td>
<td>107</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>98</td>
<td>16</td>
<td>98</td>
<td>99</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK Activity</td>
<td>3.5</td>
<td></td>
<td>5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>—25</td>
<td></td>
<td>—46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK Activity</td>
<td>18.4</td>
<td></td>
<td>14.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>48</td>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a c As in Table I.

**Effects of DAP on the growth of DPS-defective mutants**

As DPS is an enzyme in the pathway for lysine and DAP biosynthesis, the effects of DAP on the growth of DPS-defective strains, DK131 and DA105, were examined in a glucose minimal medium. As shown in Fig. 2, strain DK131 showed slow but substantial growth in the absence of DAP, and thus did not show an absolute requirement for DAP (Fig. 2(A)). However, its growth was markedly enhanced by the addition of DAP, the maximum, which was about four times as much as that in the absence of DAP, being observed in the presence of 6 g/l of D-l-DAP. Moreover, 100 mg/l of L-lysine·HCl in combination with 4 g/l of D-l-DAP further stimulated the growth up to the level in the case of the parent strain. However, L-lysine alone did not promote the growth at a concentration up to 2 g/l (data not shown). The parent strain grew well without DAP and lysine, and did not respond to these compounds. Strain DA105 grew better than Nos. 257 and 415, the amounts of lysine produced were smaller than those of threonine, while the threonine productivities were comparable to that of strain BA68. DPS activity was not detected in strains DA105 and DA110, and it was reduced in strain DA417. The DPS activities of the remaining two strains, Nos. 257 and 415, were comparable to that of the parent strain. Among them, the low lysine productivity of stain No. 257 seemed to be due to its poor growth in the production medium. The results in Table II also show that the HDs of these DPS mutants were not released from feedback inhibition by threonine. The best DPS-defective threonine producer, DA105, was further investigated as to other enzymes in the threonine biosynthetic pathway. No difference was observed between strains DA105 and its parent, AC664, as to either the specific activities or the degrees of feedback inhibition of AK and HK.
Fig. 2. Effects of dl-Diaminopimelic acid on the Growth of Dihydrodipicolinate Synthase (DPS)-defective Mutants.

(A): O, DPS-defective strain, DK131; △, its parent strain, No. 70. (B): O, DPS-defective strain, DA105; △, its parent strain, AC664.

* Diaminopimelic acid.

strain DK131 in the absence of DAP, to an extent comparable to that in the case of the parent strain (Fig. 2(B)). DAP accelerated its growth about 1.5 times, at maximum. However, this acceleration was not due to the DPS defect, because the growth of the parent strain was also accelerated by DAP to a similar extent.

Cultural conditions for threonine production by strain DA105

The cultural conditions for strain DA105 were investigated using Medium 48 (N25 S35) as the standard medium. The optimum concentrations of (NH₄)₂SO₄ and SMH were 20 g/l and 35 ml/l, respectively. Under the optimum conditions, 10.3 g/l of threonine and 3.5 g/l of lysine as its HCl salt were accumulated. The addition of DL-DAP or L-lysine·HCl to the standard medium at concentrations between 0.1 and 5 g/l did not affect either the threonine productivity or the growth.

Cultural conditions for threonine production by strain DK131

The cultural conditions for strain DK131 were examined using Medium T-2 supplemented with 5 g/l of DL-DAP as the standard medium. As shown in Fig. 3(A), (B) and (C), the growth and the threonine production in medium T-2 were markedly affected by the concentration of DL-DAP, the optimum concentrations being between 4 to 6 g/l for production. In the presence of 5 g/l of DL-DAP, the optimum (NH₄)₂SO₄ and MIEKI concentrations were 20 g/l and 2 ml/l, respectively. Then, the effect of the MIEKI concentration was examined in the medium containing 20 g/l of (NH₄)₂SO₄ and 5 g/l of DL-DAP. As shown in Fig. 3(D), the maximum threonine accumulation, 13.7 g/l, was obtained when the concentration of MIEKI was 1 ml/l.

Effects of L-lysine on the threonine production by strains DK131 and BB69

In preliminary experiments, threonine production by strain DK131 in Medium 48 (N50 S30) was found to be markedly depressed by the addition of L-lysine·HCl (data not shown). This result and the previous report¹²) suggested much stronger inhibition of threonine production by lysine in the DPS-defective strain, DK131, than in the HD₇ type threonine producer, BB69. Then the effects of lysine on the two strains were compared using the above established optimum medium for the threonine production by strain DK131 as the
Fig. 3. Cultural Conditions for Threonine Production by Strain DK131.
The standard media used were Medium T-2 supplemented with 5 g/l of DL-diaminopimelic acid for (A), (B) and (C), and the same standard medium but containing 20 g/l of (NH₄)₂SO₄ for (D). The arrows indicate the productivity and growth under the basal conditions. O, threonine produced; □, growth.

Discussion

Strains DK131, DA105 and DA110, isolated as AHV-resistant threonine producers in the present study, lacked DPS activity. There have been no reports on the derivation of DPS-defective mutants of bacteria in the genera Brevibacterium and Corynebacterium. The HDs of these strains were not released from feedback inhibition by threonine. Therefore, they appeared to be novel threonine producers, being distinct from those of the conventional HD⁹ type.

There was no difference between the DPS-defective strain and its parent strain with respect to the key enzymes in threonine and lysine biosynthesis other than DPS, i.e., AK, HD and HK. Therefore, the threonine production by these strains can be considered to be due only to their DPS defects. As to the mechanism of over-production of amino acids by mutants having normal regulatory enzymes, several examples have already been reported,¹³ in which an excess supply of a substrate that competes with the feedback
Fig. 4. Effects of L-Lysine on Threonine Production by Strains BB69 and DK131.

The cultivations were performed for 72 hr by method B in Medium T-2 but containing 20 g/l of (NH₄)₂SO₄, 1 ml/l of MIEKI, 5 g/l of dl-diaminopimelic acid and the indicated concentrations of L-lysine·HCl. ○, threonine production by strain DK131; △, threonine production by strain BB69; ●, growth of strain DK131; ▲, growth of strain BB69.

inhibition of the regulatory enzyme causes over-production. In strains DA105 and DA110, ASA, a substrate of HD, may accumulate in the cells as a result of their DPS defects, as their AKs have been released from feedback inhibition. In strain DK131 with feedback-sensitive AK, excess accumulation of ASA may also be expected to occur with the lysine limitation caused by the DPS defect, where the concerted feedback inhibition of AK would not operate (Fig. 1). For the above proposed mechanism for threonine production by DPS-defective mutants, the excess accumulation of ASA is essential. This was strongly supported by the much severer inhibition of threonine production by lysine in DPS-defective strain, DK131, than in HDₘ type strain, BB69. These DPS-defective strains were obtained as mutants resistant to AHV. As the growth inhibition by AHV has been concluded to be due to its false feedback inhibition of HD,¹² the resistance of these strains may be explained by competitive release of this false feedback inhibition due to their high intracellular concentrations of ASA. Thus, the AHV-resistance of DPS-defective strains also supports the above proposed mechanism for threonine production.

Whereas a DPS-defective mutant of *Escherichia coli* shows an absolute requirement for DAP,¹⁴ DPS-defective strains, DK131 and DA105, showed substantial growth in the absence of DAP. Moreover, strain DA105 produced a small amount of lysine, which is synthesized via DAP.¹⁵ Therefore, another pathway for DAP and lysine biosynthesis that does not involve the DPS reaction may exist in *B. flavum*. Nevertheless, in *B. flavum*, the pathway involving DPS reaction seems to be the main one for DAP and lysine biosynthesis from the following data: The growth of strain DK131 was enhanced 4-fold by DAP compared with that in its absence; and the amount of lysine produced by strain DA105 in Medium 48 (N25 S35), 5.3 g/l, was about one-fifth that by the parent strain, AC664, 24.5 g/l. The results as to the growth response to DAP and the byproduction of lysine indicate that the supply of DAP in strain DA105 is better than that in strain DK131. This difference may be attributed to the AKₘ character of strain DA105, which leads to a better and constant supply of ASA and, therefore, of DAP and lysine through the alternative biosynthetic pathway.

The result that the DPS-defective mutants obtained in the present study did not show an absolute requirement for DAP well explains the fact that DPS-defective strains in the genera *Brevibacterium* and *Corynebacterium* have not been obtained as mutants auxotrophic for DAP.¹⁵ In this study, the resistance to AHV and threonine productivity were used as selection markers for the isolation of DPS-defective mutants. These markers well coincide with the proposed mechanism of threonine production by these strains. For derivation from such a strain as No. 70 with
feedback-sensitive AK, the inhibition of threonine production by L-lysine seems to be another useful marker for distinguishing DPS-defective mutants from conventional HD₅ type strains. For derivation from such a parent strain as AC664 with an AK₅, a decreased level of lysine by production can be an effective selection marker in addition to both the resistance to AHV and threonine production, as found in this study.

Strain DA105 produced 10.3 g/l of threonine. This productivity was almost the same as that of an HD₅ type strain, BA68, derived from the same parent. Strain DK131 showed the maximum productivity of 13.7 g/l, which is comparable to that of a previously reported typical HD₅ type producer, BB69, 13.3 g/l. Therefore, DPS-defective threonine producers seem to possess almost the same productivities as those of HD₅ type producers. It has been reported that in E. coli, a mutant auxotrophic for DAP produced threonine.¹⁶,¹⁷) However, the productivity of the mutant was only 2 g/l, at maximum, and the defective enzyme remains to be elucidated.

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References