Aspartate Decarboxylation Encoded on the Plasmid in the Soy Sauce Lactic Acid Bacterium, *Tetragenococcus halophila* D10

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*Tetragenococcus halophila* D10 decarboxylates aspartate to alanine, but *T. halophila* D10 derivatives generated by a curing treatment could not (Asd− derivatives). We observed by electrophoresis three plasmid bands in *T. halophila* D10; all Asd− derivatives lost the largest of these bands. This plasmid, pD1, has two SalI sites. We cloned and sequenced the 10 kb SalI fragment. The DNA sequence suggests that this fragment contains the aspartate decarboxylating trait.

Key words: *Tetragenococcus halophila*; plasmid; aspartate decarboxylation; soy sauce; curing

Some bacteria are known to decarboxylate amino acids or organic acids. In lactic acid bacteria, the following decarboxylation reactions of amino acids or organic acids are known: decarboxylation of histidine to histamine,1) aspartate to alanine,2) glutamate to γ-aminobutyrate,3) and malate to lactate.4) It has been reported that some lactic acid bacteria obtain energy from these decarboxylation reactions.1,3)

*Tetragenococcus halophila* is a lactic acid bacterium used in soy sauce fermentation. Some strains of *T. halophila* also decarboxylate amino acids such as aspartate5) or organic acids such as malate.6) Aspartate decarboxylation by *T. halophila* in soy sauce mash (moromi) increases the pH of *moromi* and makes the taste of soy sauce milder by the combination of a decrease in the content of sour amino acid aspartate, and an increase in that of the sweet amino acid, alanine.5)

Some aspartate decarboxylating strains (Asd+) become decarboxylation-defective strains (Asd−) after repetitive subculturing. Such genetic instability may be caused by certain unstable genetic factors such as plasmids. Several small plasmids have been reported in *T. halophila*, but all of them are cryptic.7,8) Little is known about plasmids of *T. halophila* and their encoded characters. Thus, we investigated whether the aspartate decarboxylating trait was encoded on a plasmid.

*T. halophila* D10 (Asd+) was obtained from a stock culture in our laboratory. Bacteria were cultured in lactic acid tobacilli MRS broth9) (Difco Laboratories, Detroit, MI) with 5% NaCl (MRS-5) or 15% NaCl (MRS-15) at 30°C. For plate culture, MRS-5 or MRS-15 plates with 1.5% agar were used. The plates were cultured anaerobically at 30°C in a GasPack jar (B. B. L Microbiology Systems, Cockeysville, MD).

For the curing treatment, bacteria were cultured in MRS-5 with 3 μg/ml of ethidium bromide at 30°C for 4d. The cultures were diluted properly in 5% NaCl and spread on MRS-5 plates. After incubation for 4d, the resulting colonies were picked up, inoculated in MRS-5 broth, and cultured as cured strains.

The aspartate decarboxylation ability of the bacteria was detected using aspartate indicator broth, which contained 0.5% beef extract, 0.5% polypeptide, 0.5% yeast extract, 0.5% glucose, 0.1% sodium thioglycolate, 0.0005% pyridoxal hydrochloride, 0.004% bromocresol purple, 15% NaCl, and 2.0% sodium aspartate. The pH of the medium was adjusted to 7.0 with NaOH. The bacteria were inoculated from the previous culture. After 7 days, the color of this medium becomes yellow as a result of lactic acid generation; when aspartate is decarboxylated, the medium color remains purple because the pH increases.

Plasmids were prepared by the method of Anderson and McKay10) except for supplementation of lysis broth with 5% NaCl because *T. halophila* requires NaCl for optimal growth. The plasmid samples were electrophoresed on horizontal 0.7% agarose gels. DNA fragments were purified from agarose gel using Quiaquick Gel Extraction Kit (Quiaigen). Cloning experiments were done using standard DNA recombinant techniques.11)

The involvement of a plasmid in aspartate decarboxylation was examined with a curing treatment of the *T. halophila* D10. Strain D10 was cultured with a curing reagent, ethidium bromide. After 4d of culture, we isolated 96 strains of D10 derivatives and examined their aspartate decarboxylation ability by aspartate indicator broth. Fifty-two strains (54%) were Asd+ and 44 strains (46%) were Asd−. This high rate of loss of aspartate decarboxylating ability suggests that this trait is encoded on a plasmid or other genetically unstable factor.

To confirm whether the strains were Asd+ or Asd−, six strains of each type (Asd+ 1 to 6 and Asd− 1 to 6) were cultured in MRS-5 with 50 mM sodium aspartate. After 7 days, aspartate and alanine concentrations in the cultures were measured with a Hitachi L8500 amino acid analyzer. The consumption of aspartate and production of alanine were observed in all Asd+ strains but not in

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the Asd" strains (Table 1).

We examined the plasmid profiles of Asd" 1 to 6 and Asd" 1 to 6 (Fig. 1). The plasmid profile of the parental strain D10 had three plasmid bands. All Asd" isolates showed the same three plasmid bands, but the largest plasmid band was absent in all Asd" isolates. This suggests that the aspartate decarboxylating trait was encoded on the largest plasmid, named pD1. The size of pD1 is estimated to be approximately 22 kb because the restriction endonuclease SalI digestion of the extracted pD1 fragment from the gel gave 10 and 12 kb fragments.

We cloned the 10 kb SalI fragment in pBluescriptII KS(Stratagene) and sequenced it. The fragment contained two open reading frames (ORFs). As shown in Fig. 2, the alignment of the genes was in the following order; (i) a potential promoter, (ii) first Shine-Dalgaro sequence (SD), (iii) first ORF, the deduced amino acid sequence of which showed a high degree of similarity to known aspartate amino transferases, (iv) second SD, (v) second ORF, the deduced amino acid sequence of which seems to have 12 transmembrane-spanning regions, as is often observed in known carriers[2] according to hydropathy analysis. These data suggest that the first and second ORF encode the genes, aspartate decarboxylase (aspD) and aspartate carrier (aspT), respectively, and that the two genes are transcribed from one promoter upstream of aspD.

Although many examples of decarboxylation of amino acids or organic acids in lactic acid bacteria have been reported, pD1 is the first apparent example of a decarboxylating trait encoded on a plasmid in lactic acid bacteria.

Aspartate decarboxylation is an important trait in soy sauce fermentation because it affects the taste of the sauce. Further study of the aspartate decarboxylation plasmid is thus important for controlling aspartate decarboxylation during soy sauce fermentation.

Expression studies of the genes and functional reconstitution of aspT in proteoliposomes are in progress and will be discussed elsewhere (Abe, K. et al., in prepara-

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**Table 1.** Aspartic Acid and Alanine Concentration in Culture Broth of Asd" and Asd" Derivatives of *T. halophila* D10 after 7 Days Culture

<table>
<thead>
<tr>
<th>Strains</th>
<th>Aspartic acid concentration (mM)</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asd&quot; 1</td>
<td>36.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Asd&quot; 2</td>
<td>37.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Asd&quot; 3</td>
<td>36.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Asd&quot; 4</td>
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<td>Asd&quot; 5</td>
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<td>3.8</td>
</tr>
<tr>
<td>Asd&quot; 6</td>
<td>38.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Asd&quot; 1</td>
<td>6.6</td>
<td>41.3</td>
</tr>
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<tr>
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<tr>
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<td>34.8</td>
</tr>
<tr>
<td>No bacteria</td>
<td>36.8</td>
<td>3.1</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Telagarose Gel electrophoresis of Plasmid from 6 Asd" Strains (Asd" 1 to Asd" 6) and 6 Asd" Strains (Asd" 1 to Asd" 6) of *T. halophila* D10 after Curing Treatment. Lanes; 1 and 14, EcoT14-digested λ phage DNA. Lanes 2-7, Asd" isolates (Asd" 1 to Asd" 6). Lanes 8-13, Asd" isolates (Asd" 1 to Asd" 6).

**Fig. 2.** The Genetic Organization of the Genes Concerned with Aspartate Decarboxylation of *T. halophila* D10 in the 10 kb Fragment of pD1. P, a potential promoter; S, Shine-Dalgaro sequences; aspD, a putative aspartate decarboxylase; aspT, a putative aspartate carrier.

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**References**


Aspartate Decarboxylation Plasmid in *T. halophila*


