**Note**

**Construction of a New Teichuronopeptide-defective Derivative from Alkaliphilic *Bacillus* sp. C-125 by Cell Fusion**

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Cell walls of facultative alkaliphilic *Bacillus* sp. C-125 are composed of peptidoglycan, teichuronic acid, and teichuronopeptide. A mutant lacking teichuronic acid, or defective in both teichuronic acid and teichuronopeptide has been isolated from the organism. We now constructed another type of a cell-wall defective mutant that was defective in teichuronopeptide but had teichuronic acid, by cell fusion using protoplasts prepared from a wild-type strain and a mutant defective in teichuronopeptide and teichuronic acid. This mutant grew more poorly than wild type or a teichuronic acid-defective strain of C-125. The growth, however, was faster than that of the parental strain defective in both teichuronic acid and teichuronopeptide.

Bacilli forms of facultative alkaliphilic *Bacillus* sp. C-125 are shaped by peptidoglycan. The cell surfaces are surrounded with two acidic polymers that bind to the peptidoglycan.1–7) One is teichuronic acid (TUA), which is composed of galacturonic acid, glucuronic acid, and N-acetyl-g-fucosamine.4) The other is teichuronopeptide (TUP), which is a complex composed of polypeptide-α- and β-1,4-o-glucuronic acid and poly-l-glutamic acid.3,5,6) Cell walls of the organism grown at alkaline pH contained higher contents of these acidic polymers than those of the organism grown at neutral pH.1,5) Two kinds of mutants defective in the cell wall components have been isolated (strain C-125-002.8) One (C-125-11) is a TUA-lacking mutant. The other (C-125-90) is a mutant lacking TUA and the poly-l-glutamic acid region of TUP. These mutants grow poorly at alkaline pH. Protoplasts, prepared by complete digestion of cell walls of C-125 with lysozyme, are unstable and labile at alkaline pH. The protoplasts grow and regenerate their cell walls at neutral pH but not at alkaline pH.9,10) These results suggest that these acidic polymers contribute to improve the alkali-tolerance level of the facultative alkaliphilic C-125 in a highly-alkaline environment.

A TUP-defective mutant that has wild-type TUA has not been isolated. Such a TUP-defective derivative has been needed to further investigate biological functions of the acidic polymers in the cell walls. However, isolation of TUP-defective mutants from the wild-type strain C-125-002 had been unsuccessful, because we had no convenient method for selection of such a mutant among the C-125 cells treated with an appropriate mutagen. A genetic recombination system was recently established in the organism by cell fusion using protoplasts prepared from cells treated with lysozyme.11,12) We tried to construct a TUP-defective derivative with wild-type TUA by genetic recombination. Construction of such a mutant was expected by the recombination resulting in complementation of the TUA-defective phenotype of strain C-125-90 with the wild-type gene, or introduction of the TUP-defective phenotype of strain C-125-90 into a wild-type strain. We describe here successful isolation and partial characterization of the mutant.

Bacterial strains used in this study are listed in Table I. Cells of C-125-073 (Met" Na"I) and C-125-90 (Thr" Str" TUA" TUP-Glu") were aerobically grown at 37°C in a complex medium (pH 8.5) consisting of K₂HPO₄, 13.7 g; KH₂PO₄, 5.9 g; citric acid, 0.34 g; MgSO₄·7H₂O, 0.05 g; glucose, 5 g; peptone, 5 g; yeast extract, 1 g; and NaCl, 11.7 g per liter of deionized water.20) This medium is tentatively designated pH 8.5 medium in this report. The cells were harvested at the exponential phase of growth and suspended in SMMD medium (0.5 mM succrose, 20 mM MgCl₂, 20 mM maleic acid, and 0.05% deoxyribonucleic acid; pH 7.0); The cell walls were digested with lysozyme (0.1 mg/ml) at 37°C for 1 h.

The resulting protoplasts were recovered by centrifugation (1000 x g, 30 min, 10°C) and washed once with the SMMD medium. Suspensions of the protoplasts prepared from the two strains were mixed in the presence of 36% (w/v) polyethylene glycol 4000. The mixture was incubated in an ice-water bath for 1 min. Among the protoplasts, Na" Str" fusants were selected on the regeneration medium containing nalidixic acid and streptomycin. The medium consisted of yeast extract, 5 g; casamino acids, 5 g; glucose, 20 g; agar, 10 g; bovine serum albumin, 0.4 g; nalidixic acid, 30 mg; and streptomycin, 3 g; in 1 liter of deionized water containing 30 mM MgCl₂, 1.25 mM CaCl₂, 0.5 mM monosodium succinate, and 30 mM Tris·HCl (pH 6.8).11) Among the Na" Str" fusants, 26 clones were randomly selected, purified by single colony selection, and grown at 37°C in the pH 8.5 medium. Cells in the early stationary phase of growth were harvested by centrifugation (8000 x g, 20 min, 4°C), and incubated in 1% SDS at 80°C for 30 min. The cell walls were prepared from the SDS-treated cells by disruption with a sonic oscillator.11)

The cell walls were assayed for uronic acids by the carboxylate-H₂SO₄ method using glucuronic acid as a standard.12) After hydrolysis of the cell walls in 4 M HCl at 100°C for 16 h, L-alanine and L-glutamic acid were measured with L-alanine and L-glutamate dehydrogenases, respectively.13,14) L-Alanine is a unique constituent of the peptidoglycans.21) L-Glutamic acid is a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>C-125</td>
<td>Wild type</td>
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<tr>
<td>C-125-002</td>
<td>Thr&quot; Str&quot;</td>
<td>8</td>
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<tr>
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<tr>
<td>C-125-90</td>
<td>Thr&quot; Str&quot;TUA&quot;&quot;TUP-Glu&quot;</td>
<td>8</td>
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<tr>
<td>C-125-F19</td>
<td>Met&quot; Na&quot;I TUP-Glu&quot;</td>
<td>This study</td>
</tr>
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* Abbreviations: Met", requirement for methionine; Thr", requirement for threonine; Str", streptomycin-resistance; Na", nalidixic acid-resistance; TUA", loss of TUA in the cell walls; TUP-Glu", loss of poly-L-glutamic acid region in TUP.

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Table II. Components of the Cell Walls of the Parental Strains and the Candidate Fusants

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Molar ratioα of</th>
<th>Presence of FusNβ</th>
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<tr>
<td></td>
<td>l-Ala</td>
<td>l-Glu</td>
</tr>
<tr>
<td>C-125-073</td>
<td>1</td>
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</tr>
<tr>
<td>-90</td>
<td>1</td>
<td>0.11</td>
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<tr>
<td>-F15</td>
<td>1</td>
<td>0.11</td>
</tr>
<tr>
<td>-F17</td>
<td>1</td>
<td>0.24</td>
</tr>
<tr>
<td>-F19</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>-F58</td>
<td>1</td>
<td>0.15</td>
</tr>
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</table>

* The strains C-125-073 and -90 were used as parents. The other strains were the candidates of derivatives defective in TUP but not in TUA. These strains were selected among the 26 fusants because of the low content of l-glutamic acid in the cell walls.

α Ratios of l-glutamic acid (l-Glu) and uronic acids (U.A.) are calculated taking l-alanine (l-Ala) as 1. These components were measured using the cell walls prepared from each strain.

β Presence of fucosamine (FucN) was tested by cellulose thin-layer chromatography of hydrolysates of trichloroacetic acid extracts from the cell walls.

major unique constituent of TUP, and uronic acids are major constituents of TUA and TUP.31

We assumed that cell walls of the desirable mutants should show as high content of uronic acids as the parental strains C-125-073 and -90. In addition, a ratio of the l-glutamic acid content to the l-alanine content should be as low as in C-125-90, which is defective in TUP and TUA. Among the 26 fusants tested, five candidates (C-125-F10, -F15, -F17, -F19, and -F58) agreed with these criteria (Table II). These fusants were considered to lack the polyglutamic acid region of TUP as well as C-125-90, because the cell walls had small amounts of l-glutamic acid. However, it was unclear whether the candidates had wild-type TUA or not.

Non-peptidoglycan components were extracted from the cell walls of the five candidates with 5% (w/v) trichloroacetic acid at 30°C.32 The extracts were dialyzed against running water and hydrolyzed in 4 M HCl at 100°C for 1 h. After removal of HCl in vacuo over NaOH at 50°C, the residues were dissolved in water. Compounds in the hydrolysates were developed by ascending chromatography on an Avicel cellulose thin layer in a solvent of ethyl acetate–pyridine–water–acetic acid (5 : 5 : 3 : 1, by vol.). The compounds were colorized by a ninhydrin spray.15 Fucosamine (Rf 0.47) is one of unique components of TUA, as described above. This sugar was found in the hydrolysates of C-125-073, -F15, -F17, -F19, and -F58 but not in those of C-125-90 and -F10 (Fig. 1 and Table II). These results showed that the four fusants among the candidate strains had TUA in their cell walls. Therefore, these four fusants, C-125-F15, -F17, -F19, and -F58, were the derivatives aimed to be constructed in this study.

The fusant C-125-F19 was selected to characterize the acidic polymers in the cell walls. The trichloroacetic acid extracts from the cell walls of the strain C-125-F19 were separated into two fractions by sequential anion exchange (DE52; Whatman, Maidstone, UK) and gel (Toyopearl HW-55S; Toyo Soda, Tokyo, Japan) chromatographies, as previously described.33 One fraction contained a teichuronic acid whose chemical composition was the same to that of wild-type TUA prepared from C-125 (results not shown). The other contained polyglucuronic acid. This acid corresponded to the polyglucuronic acid region of TUP (results not shown). The polyglucuronic acid is a structural component of the C-125-90 cell walls.34 Therefore, it was concluded that the strain C-125-F19 had wild-type TUA and the polyglucuronic acid region of TUP, but did not the polyglutamic acid region of TUP. Detailed analysis will be published elsewhere.

The fusant C-125-F19 was used to examine the contribution of TUP for the organism to grow at alkaline pH (Fig. 2). C-125, C-125-11, C-125-90, and C-125-F19 were grown in the pH-8.5 medium at 37°C overnight. The culture was diluted with cold saline, and plated on the complex media of which pH was adjusted

Fig. 1. Detection of Fucosamine in Hydrolysates of the Non-peptidoglycan Components.
The strains shown in Table II were aerobically grown in the pH-8.5 medium at 37°C. The cell walls were prepared from the cells harvested during the early stationary phase of growth. The cell walls were extracted with (5% w/v) trichloroacetic acid. The extracts were hydrolyzed in 4 M HCl at 100°C for 1 h. The hydrolysates of the extract were developed on an Avicel cellulose thin layer in a solvent of ethyl acetate–pyridine–water–acetic acid (5 : 5 : 3 : 1, by vol.) at room temperature. Fucosamine was detected by a ninhydrin spray.

Fig. 2. pH-Dependent Growth of Cell-wall-component Defective Derivatives from Alkaliphilic Bacillus sp. C-125.
C-125 (o), C-125-11 (o), C-125-90 (A), and C-125-F19 (h) were grown in the complex medium (pH 8.5) at 37°C overnight. The culture was diluted with cold 0.8% saline, and plated on the complex medium with various pH. The resulting pH of the surface of each agar medium was measured with a flat type of glass electrode immediately before use. The pH values measured are indicated in the Figure. The diameters of 20 colonies grown on each medium were measured after incubation at 37°C for 24 h. The figure shows the square of the average diameter of the colonies grown on the medium.
to various values. After incubation at 37°C for 24 h, growth was measured on the basis of diameters of colonies grown on each agar. The growth optimum of C-125-F19 was pH 7.9. This optimum pH was higher than that of C-125-90 (pH 7.5), but lower than that of C-125-11 (pH 8.7). The strain C-125-F19 grew poorly at alkaline pH above the optimum pH for growth. The growth of C-125-F19 at alkaline pHs was similar to that of C-125-90 but not that of C-125-11. Growth around pH 7 was not greatly different among the strains, C-125, C-125-11, C-125-F19, and C-125-90. In a previous paper, we described that C-125-11 and -90 had lost their alkaliphily although they did not become neutrophilic. Strain C-125-F19 also lost its alkaliphily, but did not become neutrophilic.

The results described here lead to two conclusions. One is that our cell fusion system is convenient and effective for genetic recombination of the facultative alkaliphile C-125. The other is that TUP is more important for the organism to grow at alkaline pH than TUA.

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References