Comparison in Chemical Composition of Cell Walls of Glycine-Sensitive- and Resistant Strains of *Bacillus subtilis*

By Daisuke Tsuru, Noshi Minamiura and Juichiro Fukumoto

Faculty of Science, Osaka City University, Osaka

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A glycine-resistant strain of *Bacillus subtilis* was obtained after several time-subcultures of glycine-sensitive parent in a glycine-containing medium, and comparison in the chemical composition of cell wall was made between both strains. The major amino acid components were alanine, glutamic and diaminopimelic acid. Glucose, rhamnose, glucosamine and muramic acid were also found as the common sugar components in both cell walls. However, muramic acid in the cell wall of resistant strain was significantly less than that in the sensitive parent. Also, galactose and galactosamine were found only in the wall preparation of the resistant cells.

In previous papers1-4), the glycine (1~2×10^{-3} M) was shown to inhibit the formation of amylase and proteinase by washed suspensions of *Bacillus subtilis* var. *amyloliquefaciens*. At higher concentrations, glycine markedly delayed the growth rate of cells in a synthetic medium, and also induced a rapid lysis of cells when added to exponentially growing cultures of the bacteria5). However, repeated subcultures of this sensitive parent in a glycine-containing medium induced appearance of glycine-resistant cells which did not lyse even upon addition of 3% glycine to the growing cultures3). In the metabolic activities on various substrate, no remarkable difference was found between both type of cells, though the free amino acid content was higher in the glycine-resistant cells than in normal ones3).

Recently, glycine as well as penicillin have been shown to induce the formation of protoplasts and t-form cells in several gram-negative bacteria and inhibit the synthesis of cell wall of *Staphylococcus aureus*6-8).

The present paper deals with the comparison of chemical composition of cell wall between the glycine-sensitive parent and the resistant strain.

MATERIALS AND METHODS

Organisms and Media. *Bacillus subtilis* var. *amyloliquefaciens* Fukumoto was used as the parent strain. The organism was aerobically grown in a synthetic medium5) at 37°C. The glycine resistant strain was obtained after eight to ten time-subcultures of the parent strain in a synthetic medium containing 1.3% glycine. The resistant strain obtained here was repeatedly subcultured aerobically in a 3% glycine medium3).

Preparation of Cell Wall. The preparation of cell wall was carried out by a modified method9) of Nomura and Hosoda. The cells grown in a synthetic medium were harvested after 24 or 48 hr. cultivation and washed twice with deionized water. A thick

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1) D. Tsuru, This Journal, 26, 288 (1962).
2) D. Tsuru, ibid., 26, 295 (1962).
5) D. Tsuru, ibid., 26, 301 (1962).
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Suspension of the washed cells was boiled for 15 min and centrifuged at 12,000 r.p.m. for 20 min after cooling. The precipitate was suspended in M/30 phosphate buffer of pH 7.8 and incubated with crystalline preparation of bovine pancreas RNAase (20 µg/ml) and bacterial proteinase (100 µg/ml) for 24 hrs. After centrifugal separation, the precipitate was washed once with deionized water and dialyzed against running tap water overnight under a layer of toluene. The dialyzed suspension was centrifuged and the precipitate was washed twice with deionized water and dried under vacuo (yield, 10 to 15% of dry cell weight).

**Acid Hydrolysis and the Chemical Analysis of Cell Wall.**

**Amino Acid.** Twenty mg of cell wall preparation were hydrolyzed with 5 ml of 6 N hydrochloric acid solution at 103°C for 18 hrs. in a sealed tube. The hydrolysate was filtered, and the combined filtrate and washings were evaporated to dryness over potassium hydroxide in vacuo and dissolved in a small amount of deionized water. Total amino acids were determined by the method of Yemm and Cocking10). Aliquots of the hydrolysate were chromatographed two-dimensionally on Toyo-Roshi No. 51 paper with the solvent system of butanol/acetic acid/water (4:1:2) and 75% aqueous phenol solution (saturated with ammonia vapour). After drying, the paper was sprayed with 0.2% ninhydrin solution in butanol saturated with water and each amino acid was identified from its RF value, comparing with that in parallel run of authentic samples.

Semi-quantitative analysis of each amino acid component was performed according to the method described by Katagiri et al.11) Diaminopimelic acid was characterized by the method of Rhuland et al.12), and assayed as described by Fukasawa and Nikaido13).

**Amino Sugar.** Fifty mg of cell wall preparation were hydrolyzed with 5 ml of 2 N hydrochloric acid solution at 100°C for 3 hrs. After filtration, the hydrolysate and washings were combined, evaporated to dryness over sodium hydroxide in a vacuum desiccator to remove hydrochloric acid, and dissolved in a small amount of deionized water. Amino sugars were adsorbed onto an Amberlite IR-120 cation exchange resin column (H+ form, 200 mesh, 1.0X8.0 cm) and eluted with 30 ml of 2 N hydrochloric acid solution. The eluate was treated as described above to remove hydrochloric acid and dissolved in water. After neutralization, the total amino sugars were measured, according to the method of Rondle and Morgan14). Ascending paper chromatography of amino sugar was run on Toyo-Roshi No. 50 paper using the solvent systems of butanol/acetic acid/water (4:1:2 by vol), 80% pyridine, 75% phenol, pyridine/ethyl acetate/water/acetic acid (5:5:3:1 by vol) and butanol/pyridine/water (6:4:3 by vol). Silver nitrate15) and Elson-Morgan reagents16) were used for the detection of amino sugars. Glucosamine and muramic acid were identified from their RF values in various solvent systems and their characteristic spectra obtained by Rondle-Morgan reaction with the water extract of the paper chromatogram.

For the quantitative analysis, the amino sugar fraction was applied as a line (30 cm in length) on a Toyo-Roshi No. 50 paper and chromatographed for 30 hrs. at 25°C with butanol/acetic acid/water (4:1:2) as solvent. The marker spots were revealed by silver nitrate reagent and the corresponding zones on the paper were eluted with water and each amino sugar in the elute was determined by the method of Rondle and Morgan. The amount of muramic acid was calculated by assuming that the colour development at 530 mµ by Rondle-Morgan reaction was equivalent to 27% of that produced by an equal weight of glucosamine17).

Since the separation of glucosamine from galactosamine on paper chromatogram was not satisfactory, an aliquot of glucosamine fraction was treated with ninhydrin in a capillary tube according to the method of Stoffyn and Jeanloz18) with a slight modification of Strominger et al.19), and the descending paper chromatography of the oxidation products was run on Toyo-Roshi No. 51 paper for 48 hrs. using butanol/10)

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acetic acid/water (4:1:2) solvent.

Neutral Sugar. The acid hydrolysate of cell wall was treated with Amberlite IR-120 cation exchange resin as described in the analysis of amino sugar. The effluent obtained there was passed through Amberlite IRA-400 anion exchange resin column (HCO₃ form, 100 mesh, 1.0×10 cm) to adsorb anion impurities. Ascending paper chromatography was carried out with the sample as was done in case of amino sugar. The total neutral sugars were assayed by the anthrone method²⁰) and expressed as glucose.

Enzyme Used. A crystalline preparation of bovine pancreas RNAse was a generous gift from Dr. S. Naono. Crystalline trypsin and bacterial proteinase (Nagarse) were products of Mochida pharmaceutical Co. Ltd. and Nagase Company, respectively.

RESULTS

Tables I and II show the chemical composition of cell walls derived from the glycine-sensitive and resistant cells. The major amino acid components were alanine, glutamic acid, and diaminopimelic acids. Glucosamine, muramic acid, glucose, and rhamnose were also found as the common sugar constituents of cell walls in both type of strains.*

The characteristic differences were that muramic acid content was much less in the resistant cells than in the sensitive ones and that only in the former cells galactose was detected (Fig. 1). As shown in Fig. 2, a spot corresponding to lyxose, an oxidation product of galactosamine, was found on the paper chromatogram obtained with the crude galactosamine fraction from the walls of glycine-resistant cells. This fact indicates that galactosamine is an amino sugar component of

<p>| Table I. Chemical Composition of Cell Walls of the Glycine-Sensitive and Resistant Strains |</p>
<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>G.S.*</th>
<th>G.R.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid, % as alanine</td>
<td>32.2</td>
<td>26.6</td>
</tr>
<tr>
<td>Amino sugar, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>9.0</td>
<td>8.4**</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>15.4</td>
<td>7.7</td>
</tr>
<tr>
<td>Neutral sugar, % as glucose</td>
<td>4.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* G.S. and G.R. indicate the glycine-sensitive and resistant cells, respectively, grown in a synthetic medium for 24 hrs.
** Contained a small amount of galactosamine (see Fig. 3).

* A phosphate compound, which was consisted of glucose, alanine, phosphoric acid and some unidentified components, was found in 10% trichloracetic acid-extracts of the cell wall. This compound seems to be teichoic acid that has been reported by Armstrong et al²¹,²²). The phosphorus content of cell wall was almost the same (1.3%) in both cells.

<p>| Table II. Molar Ratio of Amino Constituents Found in Cell Wall |
|----------------------|----------------|</p>
<table>
<thead>
<tr>
<th>Amino constituent</th>
<th>Mole/mole alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.0 1.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.0 1.0</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>0.6 0.6</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.2 trace</td>
</tr>
<tr>
<td>Serine (glucose)</td>
<td>0.2 0.2</td>
</tr>
<tr>
<td>Valine</td>
<td>0.2 trace</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.2 0.2</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.5 0.5</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.65 0.34</td>
</tr>
</tbody>
</table>

Resistance to water (4:1:2), triple development.
cell wall in the resistant strain, although the quantity is less than that of glucosamine.

Table III shows a comparison of the wall composition of old cells of the glycine-sensitive and resistant strain. Both walls showed almost the same difference in the composition as given by those of young cells. However, an unidentified sugar was detected in the old cell wall preparations. This compound gave positive reaction with ninhydrin, silver nitrate and Elson-Morgan’s reagent, and showed an absorption spectrum almost the same as that produced by muramic acid on Rondle-Morgan reaction.

**TABLE III. CHEMICAL COMPOSITION OF WALLS DERIVED FROM OLD CELLS**

<table>
<thead>
<tr>
<th>Amino sugar</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound A</td>
<td>0.20</td>
<td>0.20</td>
<td>0.50</td>
<td>7.5</td>
<td>0.35</td>
<td>0.53</td>
</tr>
<tr>
<td>Compound B</td>
<td>0.35</td>
<td>0.56</td>
<td>0.54</td>
<td>7.0</td>
<td>0.38</td>
<td>0.46</td>
</tr>
<tr>
<td>Compound C</td>
<td>0.43</td>
<td>0.82</td>
<td>0.86</td>
<td>25.0</td>
<td>0.74</td>
<td>0.64</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.21</td>
<td>0.22</td>
<td>0.50</td>
<td>8.0</td>
<td>0.34</td>
<td>0.54</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.37</td>
<td>0.57</td>
<td>0.53</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.23</td>
<td>0.43</td>
<td>0.58</td>
<td>11.0</td>
<td>0.46</td>
<td>0.45</td>
</tr>
</tbody>
</table>

A: butanol/acetic acid/water (4:1:2);
B: 75% phenol;
C: butanol/pyridine/water (6:4:3), double development;
D: butanol/ethanol/water (4:1:1), descending for 48 hours;
E: pyridine/ethyl acetate/water/acetic acid (5:5:3:1);
F: n-propanol/0.1% NH₄OH (7:3).

Table IV shows the RF values of three amino sugar components present in the wall of old cells. Compounds A and B were identified as glucosamine and muramic acid, respectively, from their RF values and the Rondle-Morgan reaction. RF values of compound C in various solvent system were somewhat different from those found on diamino-hexose, which has been identified as one of the polysaccharide components of Bacillus subtilis(23). The investigation on the chemical structure of compound C is now in progress.

**DISCUSSION**

Recent works concerning the effect of penicillin on various microorganisms have suggested that the antibiotic acts on sensitive strains by interfering with the function of an enzyme which concerns a certain derivative of N-acetylmuramic acid, an intermediate in the

mucoproteptide synthesis\textsuperscript{24-26}. Recently, Wylie and Johnson\textsuperscript{26} have reported that cell walls of penicillin-treated \textit{E. coli} contain less glucosamine, muramic acid and diaminopimelic acid. In \textit{Bacillus subtilis}, however, they did not find any noticeable difference in the amino acids constituents of walls between the normal and penicillin-treated cells, nevertheless, the latter cell absorbed less muramic acid from the medium than normal cells\textsuperscript{27}. As shown in Tables I and II, the cell wall of glycine-resistant strain contained less muramic acid than that of the sensitive parent, as was in case of penicillin-treated \textit{E. coli}. On the other hand, glycine as well as penicillin inhibited the incorporation of lysine into cell wall fraction of \textit{Staphylococcus aureus}\textsuperscript{8} and induced the protoplast formation in several gram-negative bacteria\textsuperscript{6,7}. From these facts, it seems that glycine affects the synthesis of bacterial cell wall in a way similar to that of penicillin.

However, the previous paper\textsuperscript{3} has shown that glycine induces the disruption of protoplasts prepared from the sensitive cell, but does not affect those of the resistant cell. This fact clearly indicates that besides the inhibition of cell wall synthesis, glycine acts on the protoplast membrane of the sensitive cell.

There may be the possibility that certain decrease in the muramic acid content of cell wall results in acquirement of characteristic to resist glycine to the cells. Okada and Fukumoto\textsuperscript{28,29} have compared the lysis of various gram-positive bacterial cells by a certain bacterial lytic enzyme, and concluded that the higher the content of muramic acid, the more the cell be digestible by the enzyme. The relationship between the glycine resistance and digestibility of cells with various lytic enzyme is now under investigation.

\textbf{Acknowledgements.} The authors wish to express their gratitude to Dr. T. Yamamoto for his valuable suggestions and discussions. Thanks are also due to Dr. S. Naono and Dr. H. Nikaido for their generous supply of crystalline RNase preparation and \textit{a,\textit{d}}-di-aminopimelic acid, respectively.

\textsuperscript{25} A.C. Allison et al., \textit{Nature}, 188, 796 (1960).
\textsuperscript{27} J. Roberts and M.J. Johnson, \textit{ibid.}, 59, 458 (1962).
\textsuperscript{29} S. Okada and Fukumoto, \textit{ibid.}, 34, 134 (1960).