Characterization of the Presumed Peptide Cross-Links in the Soluble Peptidoglycan Fragments Synthesized by Protoplasts of *Streptococcus faecalis*

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Protoplasts of *Streptococcus faecalis* ATCC 9790 were produced with the aid of lysozyme, and the ability of these bodies to synthesize soluble, peptide cross-linked peptidoglycan (PG) fragments was examined. Lysozyme digests of PG isolated using gel filtration from the supernatant medium of protoplasts grown in the presence of \[^{14}C\]acetate and L-[^3H]lysine contained small amounts of PG having \(K_p\) expected for peptide cross-linked dimers and trimers. Addition of benzyl penicillin (300 \(\mu g/ml\)) to growing protoplast cultures did not affect the net amount of PG fragments synthesized but resulted in inhibition of synthesis of dimer and trimer fractions by 27 and 59%, respectively. Failure of penicillin to completely inhibit the accumulation of the dimer fraction was attributed to the presence of atypical forms of dimer. In fact, the supernatant medium of penicillin-treated cultures did not contain detectable amounts of typical peptide cross-linked dimer. The degree of peptide cross-linkage of protoplast PG was at most only 13% of that found in walls isolated from intact streptococci. The relative amounts of monomers, dimers, and trimers synthesized during early and late stages of protoplast growth was approximately the same. Protoplasts synthesized soluble PG fragments in amounts which were of the same order of magnitude as that expected for insoluble PG produced by an equivalent amount of intact streptococci.

Among the pivotal biochemical reactions in the synthesis of the rigid peptidoglycan (PG) layer in bacterial cell walls are the reactions which involve the addition of membrane-bound intermediates to established acceptor wall. These events occur outside the cellular permeability barrier. Consequently, effective integration with all the other events in the bacterial cell cycle is thought to require maintenance of a favorable spatial relationship between membrane-bound enzymes, substrates and intermediates, and the exocellular final acceptor (i.e., the wall: 6).

Protoplasts of *Streptococcus faecalis* can be prepared with the aid of lysozyme, or by the action of native autolytic muramidase in the absence of added PG hydrolases, and grown in osmotically stabilized medium (9, 14). The preceding report (12) presents evidence which suggests that, as the amount of intact acceptor wall remaining on streptococci decreases, the ability of these bodies to synthesize acid-precipitable (macromolecular) PG decreases to the point of being below detectable levels. Protoplasts prepared with the aid of lysozyme do, however, synthesize large quantities of soluble PG fragments which are released into the culture medium (12).

After treatment of the supernatant medium with hen egg-white lysozyme, several molecular weight species of PG fragments were isolated using gel filtration. The majority of this material was recovered as disaccharide-peptide monomer, but approximately 20% of the soluble PG fragments was in the molecular weight range of bisdisaccharide-peptide dimers and trisdisaccharide-peptide trimers. The gel filtration profiles of the peaks corresponding to these substances were symmetrical and contained \[^{14}C\]acetate:[^3H]lysine at a molar ratio of 2.0:1.0 throughout the peaks (12). Such a ratio was found in the cell wall PG of *S. faecalis* (P. Dezeele and G. D. Shockman, J. Biol. Chem., in press). Furthermore, the synthesis of materials present in both the dimer and trimer peaks was inhibited by cycloserine and vancomycin to the
same extent as disaccharide-peptide monomer (12). The intent of this investigation was to characterize these presumed peptide cross-linked fragments and thereby to define more completely the ability of protoplasts to synthesize cross-linked PG fragments.

**MATERIALS AND METHODS**

Preparation of protoplasts of *S. faecalis* ATCC 9790 with the aid of lysozyme, growth of protoplasts in osmotically stabilizing medium containing L-[H]lysine, L-[\textsuperscript{14}C]lysine, and [\textsuperscript{14}C]acetate (added individually or in combination), and isolation of soluble PG fragments from the supernatant medium of protoplast cultures or from cell walls of intact streptococci using gel filtration were performed as previously described (12). High-voltage paper electrophoresis was also carried out as described earlier (12). Treatment of PG samples using *Chalaropsis* B muramidase (0.5 \( \mu \text{g/ml} \)) was performed in 0.05 M sodium acetate, pH 4.6, for 5 h at 37 C.

**\( \beta \)-Elimination.** Desalted samples were treated with 2.0 ml of 0.05 N NaOH or 4 N NH\(_4\)OH at 37 C for periods of time from 2 to 16 h. This treatment is known to catalyze the \( \beta \)-elimination of the lactyl-peptide moiety from N-acetylglycosaminyl-N-acetyl-

muramyl-peptide resulting in the liberation of the reduced disaccharide and, in this species, a mixture of N\(^\alpha\)- (\( \alpha \) and \( \beta \)-aspartyllactyl-peptides (5, 18). Elimination of \( \delta \)-lactate or lactyl-peptides occurs only from free, reducing residues of muramic acid (5, 18). NaOH-treated samples, destined for electrophoresis, were desalted on a Sephadex G-10 column, whereas the NH\(_4\)OH-treated preparations were desalted by evaporation in vacuo over H\(_2\)SO\(_4\) and P\(_2\)O\(_5\).

The disaccharide-peptide structural unit of the cell wall of *S. faecalis* consists of \( \beta \)-1,4,\( \beta \)-acetylglycosaminyln-N-acetyl-

muramyl-N\(^\alpha\)= (l-alanyl-N-isoglutaminyl)-N\(^\alpha\)=(\( \delta \)-isopasargamyl)-l-lysyl-b-alanyl-\( \delta \)-alanine) (5). The presence of the terminal \( \delta \)-alanine residue is variable and is dependent on the extent of the action of carboxypeptidase activity present in membranes of this species (4). Thus, the lactyl-peptide which will result from \( \beta \)-elimination could be either a lactylpen-ta- or a hexapeptide, and \( \beta \)-elimination of peptide cross-linked dimer could result in release of deca- or undecapeptides. For the purpose of this investigation, the presence of either hexa- and undecapeptide in protoplast monomer and dimer, respectively, is not pertinent. Thus, for the sake of simplicity, the peptide moieties of protoplast monomer and dimer will be referred to as pentapeptide and decapetide, respectively.

**RESULTS**

Effect of penicillin on the synthesis of soluble PG fragments by protoplasts. A single preparation of protoplasts was inoculated into two flasks of osmotically stabilized medium. One flask contained \( \text{l-}[\text{\textsuperscript{14}C}]\)lysine (0.83 \( \mu \text{Ci/ml} \)) and 300 \( \mu \text{g} \) of penicillin G potassium per ml (Wyeth Laboratories, Radnor, Pa.), whereas the other flask contained only \( \text{l-}[\text{\textsuperscript{14}C}]\)lysine (2.5 \( \mu \text{Ci/ml} \)). In replicate experiments the procedure was the same except that the radioactive labels were reversed. After incubation until culture turbidities leveled off (about 140 min), the supernatants from control and penicillin-treated cultures were combined, digested with hen egg-white lysozyme (E.C. 3.2.1.17; Boehringer-Mannheim), and subjected to gel filtration on connected columns of Sephadex G-50, G-50, and G-25. Quantitation of soluble PG fragments was calculated on the basis of the specific activity of \( \text{\textsuperscript{14}C} \)- (control) and \( \text{\textsuperscript{3}H} \)- (plus penicillin) lysine-labeled PG fragments and expressed as equivalents of PG disaccharide-pentapeptide monomer. The presence of penicillin in the protoplast growth medium had no effect on either the rate or extent of turbidity increase of the cultures or on the total amount of soluble PG fragments synthesized (Table 1). Also, the total amount of soluble PG fragments synthesized by protoplast cultures was of the same order of magnitude as that expected for insoluble PG synthesized by an equivalent amount of whole cells (Table 1). In the presence of penicillin, the relative amounts of radioactive material in the dimer and trimer fractions were

<table>
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<th>Component</th>
<th>Untreated control</th>
<th>Penicillin treated</th>
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<tr>
<td></td>
<td>PG (nequiv/ml)*</td>
<td>% of sum</td>
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<tr>
<td>Monomer</td>
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<tr>
<td>Dimer</td>
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<td>Trimer</td>
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<tr>
<td>Higher oligomer</td>
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*Untreated protoplasts were grown in the presence of \( \text{\textsuperscript{14}C} \)lysine (2.5 \( \mu \text{Ci/ml} \)), penicillin treated in \( \text{\textsuperscript{3}H} \)lysine (0.83 \( \mu \text{Ci/ml} \)). Supernatants from control and penicillin cultures were combined, treated with Boehringer lysozyme, and fractionated on Sephadex G-50, G-50, G-25.

*Experimental values for amounts of PG are based on the specific activity of \( \text{\textsuperscript{3}H} \)- or \( \text{\textsuperscript{14}C} \)lysine in PG fractions, expressed in terms of equivalents (equiv) of disaccharide-pentapeptide/milliliter. Approximate amount of PG produced during growth for a similar increase in cellular mass by whole cells is 18 nano-equivalents/ml. This estimate is based on the presence of 0.25 mg of wall/mg of cellular dry weight (15) and 450 nano-equivalents of disaccharide pentapeptide per mg of walls. (Dezeele and Shockman, in press).
only 73 and 41% of the amounts found in comparable fractions from the untreated control. The apparent net loss of labeled material from the dimer and trimer peaks of penicillin-treated cultures was recovered as additional label incorporated into material in the monomer peak (Table 1).

Since penicillin is known to inhibit the final peptide cross-linking reaction of PG biosynthesis in intact cells (6), it was not surprising that addition of this antibiotic to the medium reduced the already low levels of dimers and trimers produced by growing protoplasts (Table 1). Considering the relatively high concentration of penicillin used, the reduction in presumed peptide cross-linked material was not nearly as great as expected. Due to these unexpected results it was necessary to study the chemical nature of the dimer fraction.

Digestion of protoplast dimer using mild alkali (the β-elimination reaction). Purified PG dimer, labeled in the disaccharide with [14C]acetate and in the peptide with L-[3H]lysine, was isolated from the supernatant of lysozyme-induced protoplast cultures using gel filtration as described in the preceding report (12). Material present in the dimer fraction was desalted on a Sephadex G-10 column and treated with 0.05 N NaOH at 37°C for 14 h to cause the β-elimination of lactyl-peptides from reducing moieties of muramic acid, and the alkali-treated material was filtered on the series of columns of Sephadex G-50, G-50, G-25. The elution pattern of both [14C]acetate- and [3H]lysine-containing products was complex (Fig. 1A), especially when compared to the products of β-elimination of isolated and purified peptide cross-linked dimer isolated from muramidase hydrolysis products of walls of S. faecalis (Fig. 1B). Gel filtration of the products of β-elimination of wall dimer yielded three peaks: the first contained [3H]lysine and very little [14C]acetate and the other two contained [14C]acetate and very little [3H]lysine. The first peak (the dilactyldecapetide) and the latter two peaks (lactyl-less disaccharide) (Dezelee and Shockman, in press) were isolated, desalted, and used as markers for the experiments described below.

The comparative complexity of the gel filtration pattern (Fig. 1) of β-elimination products of protoplast dimer could be due to the presence of more than one compound in the initial dimer fraction. The structure of peptide cross-linked dimer and two possible isomers is shown in Fig. 2. Peptide dimer contains a peptide cross-bridge between nonadjacent disaccharides. When peptide dimer, labeled in the disaccharide with [14C]acetate and in the peptide with [3H]lysine, is treated with alkali under conditions known to cause the β-elimination reaction, complete separation of label occurs. Such is the case for the dimer peak from walls (Fig. 1B) which yields peaks identifiable as disaccharides and dilactyl-decapeptide. The glycan dimer does not contain a peptide cross-linkage, but is linked via a
glycosidic bond. If this component is treated with mild alkali, only the lactyl-peptide on the reducing muramic acid residue is removed, leaving the other peptide linked to the nonreducing muramic acid residue of the tetrasaccharide. "Bi-linked" dimer is a cyclic compound, linked through both a glycosidic and a peptide cross-linkage. β-Elimination of such a compound would not result in a complete separation of [3H]lysine-labeled peptide and [14C]acetate-labeled disaccharide. Other possible structures of dimer, which could result in either the absence of or modification of the alkali-catalyzed β-elimination, include the absence of a reducing end on one (or both) of the N-acetyl-muramic acid residues. Such a situation has recently been encountered in walls of *Escherichia coli* (17).

β-Elimination of the three possible dimer isomers (Fig. 2) would yield distinct products which can be separated by gel filtration and high-voltage paper electrophoresis, and which can be presumptively identified by their (i) content of [3H]lysine and/or [14C]acetate, (ii) molecular size, and (iii) charge. In addition, the relative amounts of each product can be quantitated by their content of ^1H and/or ^14C. Thus, analysis of β-elimination products provides a means to quantitate components in a mixture of dimers.

Gel filtration of the β-elimination products of protoplast dimer (Fig. 1A) yielded only two peaks (D and E) which were identical to peaks obtained from β-elimination of purified dimer obtained from intact walls. The same two [14C]acetate-containing peaks were also found in NaOH digests of purified protoplast (or wall) monomer, and in all cases peak E contained about four to six times more ^14C than peak D. When these two [14C]acetate peaks isolated from NaOH-treated wall dimer or protoplast monomer or dimer were combined and run on electrophoresis at pH 2 or 4.2, all samples gave a single [14C]acetate peak very close to the origin. This was consistent with the movement of the fluorescent spot which was detected after NaOH treatment of unlabeled monomer standard (5). From these results it was concluded that peaks D and E (Fig. 1A) contained free disaccharide resulting from β-elimination of peptide dimer (Fig. 2A). Peak E probably contained the lactyl-less disaccharide, whereas the minor peak D, which appears to have slightly higher molecular weight based on gel filtration, may contain an O-acetylated disaccharide (7). Based on the expected β-elimination products of various dimer isomers (Fig. 2), the only possible source of free disaccharide is peptide cross-linked dimer, and this form of dimer would also be expected to yield a [3H]lysine peak having an identical Kᵥ as the [3H]lysine peak of dilactyl-decapeptide standard obtained from peptide cross-linked wall dimer (Fig. 1B). Peak B (Fig. 1A) contained [3H]lysine and has a Kᵥ expected for the decapetide, but this peak also contained [14C]acetate. When the material isolated from peak B was run on electrophoresis at pH 2, a single cationic peak containing both labels was observed. Electrophoresis at pH 4.2, however, yielded an anionic [3H]lysine-rich peak which had an electrophoretic mobility identical to that of the dilactyl-decapeptide standard and a separate acetate-rich peak which was slightly anionic and contained some of the [3H]lysine label. It was concluded that peak B (Fig. 1A) contained a mixture of at least two products of β-elimination: the dilactyl-decapeptide from β-elimination of peptide dimer and tetrasaccharide-pentapeptide from glycan dimer (Fig. 2B). Failure to separate these components using Sephadex G-50, G-50, G-25 columns is not surprising since the expected molecular sizes would be quite similar. Since the tetrasaccharide-pentapeptide is a β-elimination product peculiar to glycan dimer (Fig. 2) then one could predict that the other product (lactyl-pentapeptide) should also be present.

Peak C (Fig. 1) had the same elution volume on the Sephadex G-50, G-50, G-25 columns and the same electrophoretic mobilities at pH 2 and 4.2 as the [3H]lysine-containing peak obtained from NaOH-treated protoplast monomer and the ninhydrin-reactive material from NaOH-treated wall monomer. Thus it was concluded that peak C contained the lactyl-pentapeptide which arose from β-elimination of glycan dimer (Fig. 2B). Peak A (Fig. 1A) eluted at a Kᵥ value and ratio of [3H]:^14C very similar to that of untreated dimer. A second treatment with alkali, under the same conditions, did not change the relative amount of radioactive label found in peak A, indicating that peak A was not merely the result of incomplete digestion of dimer. However, "bi-linked dimer" (Fig. 2C) would yield a product of the observed molecular size containing both labels.

Based on the above tentative identifications, the relative amounts of the three forms of protoplast dimer in a particular sample were calculated from the types and amounts of products present after β-elimination. When samples of protoplast dimer were labeled in both the peptide and the sugar moiety, inde-
pendent measurements based on $[^{1}H]$lysine- or
$[^{14}C]$acetate-containing products after $\beta$-elimina-
tion gave similar results. Analyses were hand-
led in the following manner. (i) Amounts of
$[^{1}H]$lysine or $[^{14}C]$acetate in peak A were
considered as estimates of bi-linked dimer. (ii) The
amount of $[^{14}C]$acetate in peak B was consid-
ered an estimate of glycanc dimer. Since peak B
contained $^{3}H$ in both lactyl-decapeptide (from
$\beta$-elimination of peptide dimer) and tetrascar-
charide-pentapeptide (from $\beta$-elimination of
glycan dimer), the amount of $^{3}H$ in this peak
could not be used as a direct estimate of glycanc
dimer. The amount of $[^{1}H]$lysine in peak C (the
lactyl-pentapeptide from $\beta$-elimination of
glycan dimer) represents half the total peptide
content of glycanc dimer, so that twice this value
gave an approximation of glycanc dimer. (iii) The
total amount of label in peaks D and E was consid-
ered an estimate of peptide dimer based on
$[^{14}C]$acetate content. The amount of $[^{1}H]$ly-
sine in peak B minus $[^{1}H]$lysine in peak C was
considered an approximation of peptide dimer
based on $[^{1}H]$lysine content.

In some experiments the only radioactive
label present was in lysine so that only the
peptide moiety contained radioactivity. In these
instances, the method of analysis for various
components of the dimer fraction was the same
except that it was based on content of $[^{1}H]$- or
$[^{14}C]$lysine only.

**Comparison of $\beta$-elimination products of
dimers made in the presence and absence of
penicillin.** A mixture of $[^{1}H]$lysine- and $[^{14}C]$ly-
sine-labeled dimers, isolated from the superna-
tant of untreated and penicillin-treated protoplas-
ts, respectively, was treated with NaOH and
the amounts of various dimer components were
analyzed (Table 2) on the basis of their
$\beta$-elimination products as described above. Gly-
can dimer accounted for about half of the total
dimer in the supernatant medium after growth of
untreated protoplasts, whereas the peptide-
linked components (peptide and bi-linked di-
mer) accounted for about 20 and 30%, respec-
tively. Dimer isolated from penicillin-treated
protoplasts, on the other hand, contained no
detectable peptide dimer and yielded reduced
levels of the presumed bi-linked component, as
compared to the control. In light of these data
(Table 2) it is possible to interpret the failure of
penicillin to completely inhibit the synthesis of
protoplast dimer (Table 1). Under the condi-
tions used for these experiments, the protoplas-
ts produced relatively large amounts of
glycan dimer which did not contain peptide
cross-linkages and whose formation was not

<table>
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<th>Component</th>
<th>Untreated control</th>
<th>Penicillin treated</th>
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<tbody>
<tr>
<td></td>
<td>nequiv/ ml$^c$</td>
<td>% of total dimer</td>
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<tr>
<td>Glycan dimer</td>
<td>0.84</td>
<td>49.2</td>
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<tr>
<td>Peptide dimer</td>
<td>0.34</td>
<td>19.7</td>
</tr>
<tr>
<td>Bi-linked dimer</td>
<td>0.53</td>
<td>31.1</td>
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</table>

* Untreated protoplasts were grown in $[^{1}H]$lysine; penicillin treated in $[^{14}C]$lysine. Culture supernatants were combined and digested with lysozyme and the dimer fraction was isolated by gel filtration on Sepha-
dex G-50, G-50, G-25. Components of dimer were identified and quantitated on the basis of their products after treatment with 0.05 N NaOH as described.

$^{c}$ Nanoequivalents per milliliter; based on incorpo-
ration of $[^{1}H]$lysine.

Based on incorporation of $[^{14}C]$lysine.

sensitive to inhibition by the antibiotic. The
observed reduction in amount of dimer fraction
was apparently due to complete inhibition of
synthesis of peptide dimer and partial inhibi-
tion of synthesis of bi-linked dimer.

**Components of protoplast dimer: products of in vivo synthesis or artifacts?** The evidence
for the presence of at least three components in
the dimer fractions of protoplasts seems clear.
However, it seemed possible that at least some
of these products were artifacts produced by the
experimental conditions used rather than ac-
tual products synthesized by the growing protoplas-
ts. Evidence which suggests that each of the
three products was produced in vivo was ob-
tained.

Complete inhibition of synthesis of peptide
cross-linked dimer by penicillin suggests that
this substance is an actual product of protoplast
biosynthesis.

The presence of glycanc dimer could occur
because of incomplete hydrolysis of glycanc
chains by hen egg-white lysozyme. The action of
lysozyme is product inhibited and this enzyme
hydrolyzes low-molecular-weight substrates
only poorly (2, 3). However, another murami-
dase (Chalaropsis B muramidase), which di-
gests all susceptible $\beta$-1,4-glycosidic bonds of
PG obtained from walls of exponential-phase
cells and leaves only peptide cross-linked pro-
ducts (Dezelee and Shockman, in press), failed to
reduce the relative amount of glycanc dimer
found in the supernatant medium after growth of
protoplasts.
Hen egg-white lysozyme is also known to catalyze the reverse of its normal hydrolytic action and therefore to act as a transglycosidase (2, 3). Thus, it was possible that bi-linked dimer was an artifact formed by a lysozyme-catalyzed transglycosidation of peptide dimer. To examine this possibility, authentic \(^[^1]^C\)acetate- and \(^[^1]^H\)lysine-labeled peptide cross-linked dimer from walls of \(S.\) faecalis was added to protoplast growth medium containing no additional radiologically labeled material. All procedures for growth of protoplasts, treatment of culture supernatants with lysozyme, fractionation of PG fragments, and isolation of the dimer fraction followed by treatment with NaOH were performed exactly as described above. Transglycosidation of peptide dimer at any stage of the experimental procedure to form bi-linked dimer would result in the production of bi-linked dimer from the exogenously added, labeled peptide dimer. This was not the case since only the predicted products of \(\beta\)-elimination of typical peptide cross-linked dimer were detected. Furthermore, lysozyme-catalyzed transglycosidation reactions leading to the formation of labeled products with a molecular size larger or smaller than dimer were not observed. This (negative) control suggests that the NaOH-resistant (presumably bi-linked) component of dimer is an actual biosynthetic product.

The partial inhibition of the synthesis of bi-linked dimer by penicillin (Table 2) is further indirect evidence that this component is a product of in vivo synthesis.

**Percentage of cross-linking of protoplast PG.** The degree of cross-linking of the PG products found in the supernatant medium after protoplast growth was calculated using the following formula (Dezelee and Shockman, in press): percentage of cross-linkage \(= 0.5 \times \%\) peptide cross-linked dimer + 0.67 \(\times \%\) peptide cross-linked trimer. The dimer obtained from untreated protoplasts after digestion of supernatants with lysozyme contained glycan dimer and the presumed bi-linked dimer in addition to peptide-cross-linked dimer (Table 2), and presumably the small trimer peak contained even more possible forms. Consequently, estimates of the degree to which PG produced by protoplasts was cross-linked are only approximations and would be indicative of the maximum degree of cross-linking.

Approximately half of the total dimer was glycan dimer and thus made no actual contribution to the overall cross-linking. The remaining half of the dimer (peptide dimer plus bi-linked dimer), representing about 8% of the total PG, was used as the percentage of peptide cross-linked dimer in the above formula. The percentage of cross-linkage of PG is generally considered important in reflecting the degree to which different linear chains of the glycan backbone are interconnected in forming a three-dimensional net of PG (6). Bi-linked dimer was included as peptide cross-linked dimer in the above formula even though it probably does not represent cross-linkage of different glycan strands.

Since penicillin reduced the amount of trimer detected from 6.0 to 2.5% of the total PG fragments, it was assumed that the difference was due to the penicillin-sensitive bonds of peptide cross-linked trimers. Because data concerning the amount of glycan trimer which may have been present was not obtained, the upper limit of 6% was used as percentage of peptide cross-linked trimer.

Using these values the upper limit for percentage of cross-linkage of protoplast PG was about 8%. Obviously, protoplasts synthesize very little cross-linked PG compared to walls of exponential-phase cells in which the peptide cross-linking is about 55 to 60% (16; Dezelee and Shockman, in press).

**Synthesis of soluble PG fragments by protoplasts during early and late stages of growth.** When protoplasts were observed by phase-contrast microscopy immediately after inoculation into growth medium, essentially no streptococcal-like forms were observed. The majority of the protoplasts appeared as almost perfect spheres, but about 15% of the population were irregular spheres. It was thought that these irregularities in the shapes of some protoplasts might be due to the presence of very small amounts of cell wall which had not been completely solubilized during lysozyme digestion. After 60 min of regrowth less than 1% of the protoplasts were irregular in shape (presumably a result of continued action of lysozyme and native autolysin in the remaining wall). To determine if the peptide cross-linked material found in PG fractions was a result of transpeptidation of newly made wall to undigested old wall, the following experiment was performed. Protoplasts were prepared in the usual manner and inoculated into medium containing L-\(^[^3]^H\)lysine (0.5 \(\mu\)Ci/ml). After 72 min of regrowth, L-\(^[^3]^C\)lysine (0.2 \(\mu\)Ci/ml) was added and culture was incubated for an additional 78 min. The supernatant was then hydrolyzed with lysozyme (Boehringer, 1 mg/ml) and run on the Sephadex G-50 and G-25 columns. This experiment permitted evaluation of PG synthesis.
during early and late stages of regrowth. For example, if the rate of dimer synthesis relative to monomer synthesis was greater during the first half of the growth period than the second half, the ratio of [\(^{1}\text{H}\)]lysine-containing dimer to [\(^{1}\text{H}\)]lysine-containing monomer would be greater than the ratio of [\(^{14}\text{C}\)]lysine-containing dimer to [\(^{14}\text{C}\)]lysine-containing monomer. Data shown in Table 3, however, are not compatible with this possibility. The relative amounts of monomer, dimer, and trimer found in [\(^{1}\text{H}\)]lysine-containing PG were almost identical with those found in [\(^{14}\text{C}\)]lysine-containing PG. Furthermore, when the components of isolated dimer were analyzed on the basis of their \(\beta\)-elimination products, the relative amounts of peptide dimer, glycan dimer, and bi-linked dimer were essentially the same for both labels. This suggests that synthesis of peptide cross-linked material was not limited to the initial stages of protoplast growth.

**DISCUSSION**

Data in this and the preceding report (12) indicate that wall-free protoplasts synthesize soluble PG fragments, consisting largely of uncross-linked disaccharide peptide monomer units, in amounts and at rates similar to those calculated for the synthesis of insoluble, relatively highly cross-linked PG by intact streptococci (Table 1, footnote b).

These observations suggest that these fragile bodies are fully capable of carrying out the entire series of reactions (6) leading to the formation of completed disaccharide-peptide units. In fact, as demonstrated in the following paper (13), protoplasts are capable of assembling these units into soluble glycan chains which were also found in the growth medium.

In addition to quantitative differences in the degree of cross-linking of the protoplast PG product in comparison with PG found in walls of intact streptococci, protoplasts synthesized two forms of bisdisaccharide-decapeptide dimers which were not detected in wall preparations isolated from intact cells. All three forms of dimer retained the 2:1 molar ratio of [\(^{14}\text{C}\)]acetate:[\(^{1}\text{H}\)]lysine characteristic of disaccharide-peptide fragments isolated from both streptococcal walls (Dezelee and Shockman, in press) and protoplast supernatant (12) and indicative that these products contain both the disaccharide and peptide moieties of PG (Dezelee and Shockman, in press). One component (glycan dimer, Fig. 2) apparently consists of two disaccharide-peptide monomer units coupled via a glycosidic linkage (i.e., a tetrasaccharide).

<table>
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<th>Component</th>
<th>Continuously labeled PG ([\text{(^{1}\text{H})}])-lysine (%)</th>
<th>PG labeled during late stages of growth ([\text{(^{14}\text{C})}])-lysine (%)</th>
<th>([\text{(^{1}\text{H})}])-lysine in dimer (%)</th>
<th>([\text{(^{14}\text{C})}])-lysine in dimer (%)</th>
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<td>Analysis of total PG</td>
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<td>Monomer</td>
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<td>Dimer</td>
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*Protoplasts were grown continuously in \([\text{\(^{1}\text{H}\)}]\)lysine (0.5 \(\mu\text{Ci/ml}\)). \([\text{\(^{14}\text{C}\)}]\)lysine (0.2 \(\mu\text{Ci/ml}\)) was added after 72 min of regrowth and the culture was incubated an additional 78 min. The culture supernatant was harvested, treated with lysozyme, and fractionated on connected columns of Sephadex G-50 and G-25. Values for continuously labeled PG and PG labeled during late stages of growth are based on incorporation of \([\text{\(^{1}\text{H}\)}]\)lysine and \([\text{\(^{14}\text{C}\)}]\)lysine, respectively. Components of isolated dimer were identified and quantitated on the basis of their products after treatment with 0.05 N NaOH.

This isomer is resistant to digestion by both hen egg-white lysozyme and the Chalaropsis B muramidase. Although the reason for the resistance of the protoplast product to hydrolysis is not known with certainty, it seems possible that a difference in amino sugar residues may play a role. In other species resistance to hen egg-white lysozyme has been attributed to the absence of \(N\)-acetyl groups on the glucosamine residue (8) or to the presence of \(O\)-acetyl groups (1).

The second unusual form of dimer was resistant to hydrolysis by mild alkali (the \(\beta\)-elimination reaction) and was presumed to be bi-linked dimer (Fig. 2); that is, disaccharide-peptide monomer units linked through both glycosidic and peptide bonds. However, the resistance of the material in peak A, Fig. 1, to alkali also could be attributed to the absence of a reducing group on a free muramic acid residue. Since nonreducing 6-anhydro-\(N\)-acetylmuramic acid residues have been found in the PG of E. coli and Salmonella typhi (17), this possibility can-
not be ruled out. The identity of this alkali-resistant component of the protoplast dimer is currently under investigation. The inability of a high concentration of penicillin to completely prevent the synthesis of dimer (and trimer) fraction(s) (Table 1) was explained by the presence of components other than typical peptide cross-linked dimer.

Previous studies in this laboratory (9) indicated that autolasts and lysozyme-induced protoplasts of *S. faecalis* did not contain detectable levels of residual, insoluble wall. If the insertion of new precursors into preexisting wall occurs via transpeptidation (10, 11, 19) or if transpeptidation occurs after incorporation of oligomeric precursors into the wall via transglycosylation (10, 11), then the PG fragments synthesized by these wall-free protoplasts should be exclusively uncross-linked. However, small amounts of peptide cross-linked dimer both typical and atypical (presumed bi-linked) were found (Table 2). The synthesis of the same relative amounts of peptide cross-linked fragments during both early and late stages of protoplast growth (Table 3) indirectly suggests that the presence of a small amount of residual insoluble wall contamination on the surface of protoplasts at the beginning of protoplast growth is not an important factor in determining the number of peptide cross-links formed. It remains possible that cross-linking of PG fragments occurred between units which were still in the membrane-bound state. Transpeptidation to form peptide dimer (Fig. 2) would then involve separate membrane-associated glycan strands of PG which maintain a spatial relationship with the membrane-bound cross-linking enzyme similar to that which occurs during transpeptidation in intact cells. In contrast, the formation of bi-linked dimer (Fig. 2) would involve transpeptidation of the peptide side-chains of adjacent disaccharide-peptide units and this does not require a special geometric relationship between two substrates. It should be noted that linear PG in which adjacent monomer units are linked via peptide bridges would not interconnect separate glycan strands and therefore would not contribute to the rigid three-dimensional architecture of PG.

The presence of tetrasaccharide in the form of glycan and bi-linked dimers in lysozyme digests of protoplast supernatants suggests that protoplasts may actually synthesize soluble glycan chains. In the experiments described in this and the preceding paper (12), attempts to limit muramidase activity were not made, so that direct evidence regarding the ability of protoplasts to make glycan chains was not obtained. The following paper (13) reports results germane to this point.

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LITERATURE CITED


