Myxospore Formation in *Myxococcus xanthus*: Chemical Changes in the Cell Wall During Cellular Morphogenesis

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Vegetative cells of *Myxococcus xanthus* (strain FB) were induced to form myxospores by the glycerol induction technique. Several structural changes took place in the peptidoglycan during myxospore formation. The percent of the peptidoglycan comprised of monomer (disaccharide peptide) decreased from about 20% to approximately 7%. The proportion of the total diaminopimelic acid possessing a free amino group decreased about 11%. A carbohydrate containing only glucose was found to be bound, possibly covalently, to the vegetative cell and myxospore peptidoglycan. The amount of carbohydrate relative to peptidoglycan decreased by two-thirds during myxospore formation. None of the above changes in the peptidoglycan were observed in a mutant (strain GNI) of *M. xanthus* which was unable to convert to myxospores when incubated in the glycerol induction medium, or in the parental wild type (FB) when it was incubated in induction medium lacking the myxospore inducer, glycerol.

In 1964, Dworkin and Gibson (2) demonstrated that when glycerol was added to a growing culture of *Myxococcus xanthus*, the population of long, thin vegetative cells rapidly and synchronously converted to spherical myxospores. Since myxospore formation requires that the shape of the cell wall change from a cylinder to a sphere, it is likely that specific alterations in the chemical nature of the cell wall occur during myxospore formation, although these may be only transient changes (10).

In an attempt to detect chemical changes in the cell wall during myxospore formation, White et al. (10) examined cell walls obtained from both vegetative cells and myxospores and concluded that: (i) the overall chemical composition of the peptidoglycan was similar in both cell types and resembled that found for *Escherichia coli*; (ii) both vegetative cells and myxospores contained approximately the same amount of peptidoglycan; (iii) the peptidoglycan did not appear to constitute a continuous sheath around the vegetative cell, but may have in the myxospore; (iv) the sizes of the fragments solubilized by muramidase ac-

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tivity were considerably larger in the myxospores; (v) galactosamine and glycine rapidly accumulated in the cell wall when the cells were converting from ovoids to spheres and were presumed to reflect the formation of capsular material.

In this paper we report that some of the larger fragments solubilized by lysozyme from vegetative cells or myxospores are bound to a carbohydrate consisting chiefly, if not entirely, of glucose. The amount of bound glucose decreases and the cross-linking of the peptidoglycan through diaminopimelate (Dpm) increases during myxospore formation. These chemical modifications were not observed in peptidoglycan from cells incubated in induction medium lacking glycerol or in a mutant of strain FB which was unable to convert to myxospores when incubated in glycerol induction medium.

MATERIALS AND METHODS

Organisms. *M. xanthus* strain FB (2) and a spontaneous mutant (GNI), which did not convert to myxospores, were used in these studies. The mutant was selected by continuous subculture of FB in growth medium supplemented with 0.5 M glycerol (Orlowski et al., in press).

Cultivation. Both strain FB and GNI were grown in 2% Casitone (Difco), 1.0 × 10⁻⁴ M potassium
phosphate (pH 7.5), and $8 \times 10^{-4}$ M MgSO$_4$, as described previously (2). Cells in mid-log phase of growth were used for myxospore production and cell wall preparation.

**Myxospore induction.** Myxospores were induced by the technique of Dworkin and Gibson (2), modified for heavy cultures by Sadler and Dworkin (7). The glycerol induction medium consisted of 1% Castone (Difco), $8 \times 10^{-4}$ M MgSO$_4$, and 0.5 M glycerol.

**Preparation of cell wall fractions.** Cell walls were prepared by a procedure modified from White et al. (10). Cells were harvested from the growth medium in a refrigerated centrifuge at 20,600 $\times$ g for 10 min, washed once with cold distilled water, and suspended in a small volume of cold glycerol induction medium. The suspension of cells was added to a larger volume of prewarmed glycerol induction medium (30 C) and vigorously aerated in a graduated cylinder (1, 7). For the preparation of walls from vegetative cells, hot sodium lauryl sulfate (90 C) was immediately added to the graduated cylinder, to a final concentration of 10%, and the lysed cells were heated for 20 min at 90 C. For the preparation of walls from myxospores, the detergent was not added until the cells had converted to spheres, which were not yet optically refractile in the phase microscope (about 70 min). Control preparations of FB incubated in the absence of glycerol, or of GNI incubated in the presence of glycerol, were allowed to incubate for 70 min before the detergent was added. The control cells remained as long rods during the 70-min incubation. The detergent-treated walls were collected by centrifugation at 100,000 $\times$ g for 2 hr at room temperature. The walls were then washed twice with room-temperature distilled water and treated with deoxyribonuclease until the viscosity decreased. The deoxyribonuclease-treated preparation was centrifuged at 100,000 $\times$ g for 4 C for 1 hr and washed once with cold distilled water. The washed walls were incubated with 0.7 mg of trypsin per ml (two times crystallized, Sigma Chemical Co.) in 0.05 M potassium phosphate buffer, pH 7.5, at 37 C for 4 hr. Trypsin-treated walls were centrifuged at room temperature for 1 hr at 100,000 $\times$ g, and the pellet was heated in 1% sodium lauryl sulfate for 15 min at 90 C. If the cell walls were not extracted with hot detergent after trypsin treatment, large amounts of carbohydrate not covalently bound to the peptidoglycan were solubilized by lysozyme treatment. The noncovalently bound carbohydrate was eluted in several peaks in the Sephadex columns, including the void volume, and was readily separated from the peptidoglycan during paper electrophoresis. The cell walls were then washed twice with distilled water at room temperature, twice with cold tris(hydroxymethyl)aminomethane (Tris) buffer, 0.05 M, pH 8.4 at 25 C, and twice with cold distilled water. Each centrifugation was at 100,000 $\times$ g for 1 hr. The resulting cell wall preparations were then treated with lysozyme, as described below.

**Lysozyme treatment.** The cell wall preparations (about 5 to 10 $\mu$moles of reducing power) were incubated with 200 $\mu$g of lysozyme per ml (grade A, Calbiochem) in 0.1 M ammonium acetate, pH 7.5, at 37 C for 24 hr. It was previously shown that such treatment solubilized over 90% of the peptidoglycan from vegetative cells or dark spheres (10; unpublished observations). The lysozyme-treated suspension was centrifuged at 43,500 $\times$ g for 1 hr at 4 C. The supernatant fluid was extracted with chloroform three times to extract lysozyme (6) and evaporated to dryness on a rotary flash evaporator.

**Column chromatography.** Peptidoglycan fractions were separated by gel filtration through Sephadex columns (Pharmacia, Inc., Piscataway, N.J.), as described by White et al. (10), after a method devised by D. J. Tipper (personal communication). The columns were equilibrated and eluted with 0.01 M lithium chloride in 0.02% sodium azide. Fractions (5 ml) were collected at a rate of approximately 12 ml per hr. Elution with water, as described by White et al. (10), did not separate all the peptidoglycan subunits (see Fig. 2). Pooled fractions were desalted by filtering through a column of Bio-Gel P2, using water as an eluant.

**Paper chromatography, electrophoresis, and thin-layer chromatography.** Neutral sugars were separated by descending paper chromatography on Whatman no. 1 paper using a solvent system composed of normal butanol-pyridine-water (6:4:3). The chromatograms were allowed to run for 24 hr, and the sugars were detected by alkaline silver nitrate (3). Samples were subjected to electrophoresis with a high voltage electrophoretor, (Gilford model D), at 2,000 v for 1 hr on Whatman no. 1 paper in pyridine-glacial acetic acid-water (1:1:0.289) at pH 3.7. Peptidoglycan components were detected with 0.5% ninhydrin (Sigma) in butanol. Diaminopimelic acid (Dpa) was separated from the other peptidoglycan components in acid hydrolysates by ascending chromatography on Silica Gel G-coated glass plates with a solvent system composed of normal butanol-glacial acetic acid-water (3:1:1).

**Chemical assays.** Reducing-power was determined by the Park and Johnson method as described by Ghyuysen et al. (4). N-acetyl amino sugars were estimated with the Morgan-Elson reaction as described by Ghyuysen et al. (4) using a 30-min heating period. N-acetylglucosamine was used as the standard in both assays. The glucose-containing carbohydrate was measured with the phenol-sulfuric acid method (5) and enzymatically with glucose oxidase (Glucostat reagent of Worthington Biochemical Corp.). The optimal hydrolysis conditions for release of the glucose from the peptidoglycan fractions were 1 N HCl at 105 C for 2 hr in sealed ampoules. Hydrolyzed samples were neutralized with an equal volume of 1 N sodium hydroxide before analysis with Glucostat. Fluorodinitrobenzene derivatives of Dpa (mono-dinitrophenyl [DNP]-Dpa) were made and estimated after thin-layer chromatography, as described by Ghyuysen et al. (4). The standard was bis-DNP-Dpa, which was assumed to produce 1.7 times the color of mono-DNP-Dpa (4). The proportion of the Dpa containing a free amino group was defined as the molar ratio of the amount of mono-DNP-Dpa to the total amount of Dpa contained in the acid hydrolysates. Each determination was re-
RESULTS

Gel filtration of lysozyme digests. A comparison of the sizes of fragments solubilized by complete lysozyme digestion of the peptidoglycan should reflect the extent of peptide cross-linking within the peptidoglycan, or the binding of non-peptidoglycan components to the peptidoglycan, or both. Accordingly, peptidoglycan from vegetative cells and from the sphere stage of myxospore formation were digested with lysozyme and chromatographed on Sephadex columns. Five peaks of material which gave a positive reducing power assay were obtained, but the first three were incompletely separated (Fig. 1). The first four peaks were Morgan-Elson positive. The fifth peak from the myxospore preparation contained some Morgan-Elson-positive material, and had been reported by White et al. (10) to contain glucosamine but not muramic acid or any amino acids. The molar ratio of reducing power to Morgan-Elson-positive material in all the peptidoglycan peaks was approximately 2 (Table 1), indicating that the lysozyme digestion was essentially complete (4). Based on published information (10) and on filtration of lysozyme digests of E. coli peptidoglycan (data not presented), monomer-sized fragments would be expected to elute in a position corresponding to peak 4, whereas dimer-sized fragments would be expected to elute between peaks 3 and 4. Most of the Dpm in peak 4 possessed a free amino group, supporting the conclusion that it was monomer (Table 1). White et al. (10) previously reported the absence of monomer-sized fragments in lysozyme digests of peptidoglycan from M. xanthus. The failure to detect monomer-sized fragments probably resulted from the use of water as an eluant rather than a medium of high ionic strength. Figure 2 is an elution profile of lysozyme-solubilized peptidoglycan from the vegetative cells of M. xanthus, eluted with water instead of 0.01 M lithium chloride, in 0.02% sodium azide. Only a small peak in the monomer range was detected. Similar chromatography of E. coli peptidoglycan failed to separate the monomer

![Figure 1. Gel filtration profiles of peptidoglycan fragments solubilized by lysozyme from dark sphere stage of myxospore formation (A) and vegetative cells (B). Numbers at top indicate boundaries used to delimit peaks 1 through 4 for further chemical characterization. Reducing power, ○: Morgan-Elson, Δ: phenol-sulfuric, ▲. V_e was 195 ml; glcNAc, N-acetylgucosamine.]
fraction. Peaks were pooled and desalted for further chemical characterization, as indicated by the numbers at the top of Fig. 1.

**Shift during myxospore formation in size of peptidoglycan subunits.** When the peaks of material eluted from the Sephadex columns were pooled and assayed for Dpm, the percentage of the peptidoglycan of monomer size in the vegetative cells decreased from about 20% to approximately 7% in the myxospores (Table 1). At the same time, there was an increase in the amount of peptidoglycan of the trimer-tetramer size.

**Degree of cross-linking by Dpm.** If the fragments of Morgan-Elson-positive material eluted from the Sephadex columns were composed of peptidoglycan cross-linked only by Dpm, one would expect that the fraction of the total Dpm which possessed a free amino group would be 100, 50, 33, and 25% for material eluted in the monomer, dimer, trimer, and tetramer regions, respectively. Based on the elution volumes of *E. coli* peptidoglycan and on published data (10), we estimate that peak 4 contained monomer (molecular weight 1,200), peaks 3 and 2 contained mixtures of compounds trimer and tetramer size, and peak 1 contained material larger than tetramer. Table 1 indicates that the Dpm eluted in peaks 2 through 4 possessed approximately the predicted values of free amino groups, although a little low. However, the Dpm eluted in the first peak from samples of vegetative peptidoglycan possessed a free amino group content that was too high to be consistent with the elution volume. To a lesser degree, this was also true of material eluted in peak 1 from the myxospore peptidoglycan. Similar data were reported by White et al. (10). The lysozyme digests were therefore examined for non-peptidoglycan material which may have been bound to the peptidoglycan fragments in the first peak, increasing their apparent size as judged by the elution volumes.

**Presence of carbohydrate containing glucose in the lysozyme digests.** When the fractions from the Sephadex columns were assayed for carbohydrate by the phenol-sulfuric acid assay, considerable carbohydrate was present in peak 1 (Fig. 1). The material was pooled and hydrolyzed. Paper chromatography revealed that the only non-peptidoglycan sugar present was glucose. When the hydrolysates were assayed for glucose by the Glucostat reagent, it was found that essentially all of the material which was phenol-sulfuric acid positive could be accounted for as glucose. The amount of glucose relative to Dpm was approximately two-thirds lower in the myxospores (Table 2).

**Binding of the carbohydrate to the peptidoglycan.** When the material in peak 1 from either the vegetative or myxospore peptidoglycan was pooled and subjected to paper elec-

![Figure 2](http://jb.asm.org/Downloaded from)

**Fig. 2.** Gel filtration profile of peptidoglycan fragments solubilized by lysozyme from vegetative cells of *M. xanthus* strain FB. Columns were equilibrated and eluted with distilled water. Reducing power, ●. V₀ was 250 ml.
TABLE 2. Glucostat and phenol-sulfuric acid assays of carbohydrate in peak 1a

<table>
<thead>
<tr>
<th>Cells</th>
<th>Phenol-sulfuric (nmoles)</th>
<th>Glucostat (nmoles)</th>
<th>Glucose/DAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative</td>
<td>3,460</td>
<td>3,680</td>
<td>2.6</td>
</tr>
<tr>
<td>Myxospore</td>
<td>1,469</td>
<td>1,500</td>
<td>0.67</td>
</tr>
</tbody>
</table>

a Peak 1 shown in Fig. 1 was pooled and assayed for phenol-sulfuric acid-positive material using a glucose standard. A portion of the sample was acid hydrolyzed and assayed for glucose with the Glucostat reagent, and a third portion was acid hydrolyzed and assayed for Dpm.

trophoresis, only one component was detected. The spot was both silver nitrate and ninhydrin positive, and migrated a short distance toward the positive pole. This material was eluted and partially hydrolyzed in 1 N HCl for 2 hr at 105 C. Upon electrophoresis, the partial acid hydrolysate yielded four components which were both ninhydrin and silver nitrate positive, and a fifth spot which was silver nitrate positive and migrated in the same position as the glucose standard. Three of the ninhydrin-positive spots corresponded to the positions of known peptidoglycan standards. The fourth spot traveled in the same position as glutamic acid, but since the material was silver nitrate positive, it was presumed to contain carbohydrate. This material was eluted and hydrolyzed completely in 6 N HCl at 105 C for 8 hr. The products of the latter hydrolysis were separated by paper chromatography and yielded materials which traveled in the same position as muramic acid, glutamic acid, alanine, and glucose. Traces of lysine were also observed.

Control samples from cells not converting to myxospores. Since myxospores were induced in a highly concentrated cell suspension (approximately 10⁶ cells/ml) containing one-half of the amount of Casitone and much less phosphate than was present in the growth medium, it was possible that the changes observed in the peptidoglycan reflected the conditions of cell incubation rather than myxospore formation. Therefore, two control preparations of peptidoglycan were made: (i) from cells incubated under the same conditions as an inducing culture but in the absence of glycerol, and (ii) from a mutant (GNI) incubated under identical conditions as the wild type but which did not form myxospores under induction conditions. Cell walls were prepared, and the peptidoglycan was solubilized with lysozyme. The peptidoglycan fragments were separated by gel filtration and the peaks were pooled. There was no shift in the fragment sizes, no change in the proportion of Dpm with a free amino group, nor were there any changes in the relative amounts of glucose (Tables 3, 4)

DISCUSSION

Whereas there is approximately 70% of the Dpm with a free amino group in E. coli (8), there is less than 50% in M. xanthus vegetative cells, and only 25 to 30% in the myxospore (10; see above). The small percentage of Dpm with a free amino group in M. xanthus is consistent with the discovery that most of the fragments of peptidoglycan released by lysozyme are larger than dimer size.

The percentage of monomer-sized fragments decreased from approximately 20 to 7% during myxospore formation. Since the total amount of peptidoglycan is approximately constant during myxospore formation (10), there was a shift from the monomer size to the larger sizes. This was accompanied by a decrease of approximately 11% in the proportion of Dpm possessing a free amino group. It appears, therefore, that part of the process of restructuring the cell wall during myxospore formation involves increased cross-linking between the peptides of the peptidoglycan or between peptidoglycan peptides and other wall components.

There is a carbohydrate composed only of glucose which may covalently bind to a portion of the peptidoglycan in both vegetative cells and myxospores. The conclusion that the carbohydrate may be covalently bound to the peptidoglycan is tentative and rests upon the finding that both carbohydrate and peptidoglycan travel coincidentally during paper electrophoresis, even after a partial acid hydrolysis of material eluted from the chromatogram. After partial acid hydrolysis, a component was isolated that contained muramic acid, alanine, glutamic acid, and glucose. The absence of glucosamine and Dpm was confirmed with an amino acid analyzer (data not presented). There were also small amounts of lysine detected. This material must be enriched for lysine, since hydrolyzed samples of unfractionated peptidoglycan yield only barely detectable quantities of lysine after chromatography (unpublished observations). One cannot be certain how much glutamic acid was associated with the glucose-containing compound, since free glutamic acid migrated in the same position as the partial acid hydrolysate during electrophoresis. The amount of glucose relative
to Dpm decreased by two-thirds during myxospore formation. One possible role for the carbohydrate would be as part of the non-peptidoglycan links postulated by White et al. (10) to hold the peptidoglycan together in the vegetative cell wall, but suggested to be absent from the myxospore wall. The increased cross-linking through Dpm in the myxospore would strengthen the wall as the postulated non-peptidoglycan links were removed.

The fragment of peptidoglycan which was analyzed after partial acid hydrolysis and found to contain glucose did not contain detectable Dpm. There is, therefore, no evidence that the carbohydrate described above can account for the fact that peptidoglycan with low cross-linking (high proportion of free amino groups on the Dpm) was eluted in the void volumes of the Sephadex columns described by us and White et al. (10). It is possible that the Dpm-containing peptidoglycan (which represents most of the peptidoglycan in peak 1) is also bound to non-peptidoglycan material but was not released during the mild acid hydrolysis employed.

None of the chemical changes within the peptidoglycan was observed when cells were incubated in myxospore induction medium lacking glycerol or when a mutant strain was used which was unable to convert to myxospores in glycerol induction medium. Although this does not prove a relationship between the alterations in chemical structure of the peptidoglycan and the change in shape of the cell during myxospore formation, it lends support to such a hypothesis. Detailed studies of the structure of the peptidoglycan and associated wall layers during myxospore formation should provide insight into the molecular basis for those processes which lead to changes in the shape of the bacterial cell wall.

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**LITERATURE CITED**


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