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EVIDENCE FOR PEPTIDOGLYCAN-ASSOCIATED PROTEIN(S) IN *Neisseria gonorrhoeae*

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Summary: Growth pH markedly influenced the composition of the cell envelope of *Neisseria gonorrhoeae*. The composition of the peptidoglycan from cells grown at pH 7.2 and 8.0 consisted primarily (91%) of muramic acid, glutamic acid, alanine, *meso*-diaminopimelic acid, and glucosamine in approximate molar ratios of 1:1:2:1:1. The peptidoglycan from cells grown at pH 6.0 contained an accessory protein(s) which accounted for 42% of the weight of the isolated complex.

The chemical composition of purified gonococcal peptidoglycan from cells grown at pH 7.2 and 37° C was initially reported by Hebeler and Young (1) and subsequently confirmed by Wolf-Watz et al. (2). Both groups of investigators found muramic acid, glutamic acid, alanine, *meso*-diaminopimelic acid and glucosamine in approximate molar ratios of 1:1:2:1:1, respectively. In this respect, the peptidoglycan of the gonococcus is typical of all the gram-negative bacteria, thus far examined (3, 4, 5) and probably belongs to group chemotype I (3, 5).

In cells grown at 37° C in medium buffered at pH 7.2, no accessory polymers were identified in association with the peptidoglycan component of *Neisseria gonorrhoeae* (1, 2). It should be noted that amino acid analyses of gonococcal peptidoglycans indicated that minor amounts of aspartic acid, glycine, and threonine were present (1); however, on a weight basis, these amino acids accounted for less than 1 percent of the dry weight of the peptidoglycan preparations. Similar results were reported by Wolf-Watz et al. (2).

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In this communication, we report that the pH of growth can modify the chemical composition of the peptidoglycan with respect to the presence of accessory polymers.

MATERIALS AND METHODS

Growth conditions and media. Strains of N. gonorrhoeae were grown in liquid gonococcal broth (6) buffered at pH 6.0, 7.2, or 8.0 with N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid at a concentration of 30 mM. After final adjustment of the pH with HCl or NaOH, the medium was sterilized by autoclaving and then supplemented with $NaHCO_3$ (420 mg/l), glucose (5 g/l) and IsoVitaleX enrichment (BBL) (1% v/v). All cultures were grown and harvested at mid-logarithmic phase as described previously (6).

Isolation of peptidoglycan. The peptidoglycan of N. gonorrhoeae was isolated as previously described (7). Essentially, harvested cells were boiled in 4 percent sodium dodecyl sulfate (SDS) for 3 hours and allowed to stand overnight at room temperature. The digest was then centrifuged at 60,000 x g for 45 min and the pellet (peptidoglycan) extensively washed by centrifugation with water.

Analytical methods. The amino acid and amino sugar analysis was performed on preparations of purified peptidoglycan hydrolyzed in 4 N HCl at 105° C for 14 hours on a Beckman model 119 amino acid analyzer as described previously (7). Samples of purified peptidoglycan treated with either trypsin (10 µg/ml) or lysozyme (10 µg/ml) were subjected to SDS-polyacrylamide gel electrophoresis (7.5% gels) and stained according to the procedure of Segrest and Jackson (8).

RESULTS

Table 1 shows the percent dry weight of the apparent peptidoglycan fraction as a function of growth pH. In the strains thus far examined, the apparent increase of the peptidoglycan fraction isolated from cells grown at pH 6.0 ranged from 2 to 8 times the amount isolated from cells grown at either pH 7.2 or 8.0.

Subsequent studies have demonstrated that the apparent increase in peptidoglycan was not due to an overproduction of peptidoglycan per se, but was mainly due to the presence of an associated protein(s). Repeated attempts to dissociate the protein(s) by additional extractions in boiling 4% SDS were unsuccessful. The peptidoglycan-protein complex also remained associated after SDS-polyacrylamide gel electrophoresis and was found at the top of the gel (within an R_f of 0.1). Suspensions of the peptidoglycan-protein complex from cells grown at pH 6.0 decreased in optical density following treatment

Table 1

Effect of Growth pH on the Apparent Peptidoglycan Content of Neisseria gonorrhoeae[a]

	Peptidoglycan (percent of dry weight)		
Strain	pH 8.0	pH 7.2	pH 6.0
RUG 40	0.9	1.0	8.0
F62	ND[b]	1.4	13.4
JW31	1.9	2.5	11.4
RUG 50	ND	1.1	4.5
CS7	ND	9.2	18.5

[a]Peptidoglycan was isolated as described in Materials and Methods.

[b]ND = not determined.

with either 10 µg/ml of lysozyme, trypsin, chymotrypsin, or pronase. Suspensions of the peptidoglycan-protein complex from cells grown at pH 7.2 or 8.0 decreased in optical density only when treated with lysozyme.

An amino acid analysis was performed on the peptidoglycan-protein complex isolated from strain RUG 40 grown at pH 8.0, 7.2, and 6.0 (Table 2). The complex isolated from cells grown at pH 8.0 or 7.2 did not contain significant amounts of amino acids beyond those found in the peptide portion of the peptidoglycan. The peptidoglycan-protein complex from cells grown at pH 8.0 and pH 7.2 consisted of 9.5% and 9.1% protein respectively (Table 3). The complex isolated from cells grown at pH 6.0 contained significant concentrations of all amino acids except cystine and methionine (Table 2). About 42%, by weight, of the complex isolated from these cells consisted of accessory protein(s) (Table 3). There was not a consistent increase in the molar ratio of the various amino acids of the protein(s). Further studies

Table 2

Amino Acid Composition of the Peptidoglycan-Protein Complex of Neisseria gonorrhoeae RUG 40[a]

Protein[b]	pH of Growth Media					
	pH 8.0		pH 7.2		pH 6.0	
	nmol	µg	nmol	µg	nmol	µg
Amino acids						
Aspartic acid	12.7	1.46	8.1	0.93	42.1	4.84
Threonine	6.3	0.64	2.9	0.29	17.9	1.81
Serine	10.4	0.90	8.3	0.72	22.3	1.94
Glutamic acid	ND	ND	ND	ND	19.0	2.45
Proline	ND	ND	ND	ND	17.6	1.71
Glycine	16.9	1.08	13.7	0.78	31.0	1.82
Alanine	ND	ND	ND	ND	27.1	1.92
Half-cystine	ND	ND	ND	ND	ND	ND
Valine	7.2	0.68	13.2	0.13	15.6	1.47
Methionine	ND	ND	ND	ND	ND	ND
Isoleucine	3.7	0.41	10.5	1.19	22.4	2.53
Leucine	13.2	1.49	4.9	0.55	27.7	3.13
Tyrosine	2.8	0.46	ND	ND	8.9	1.45
Phenylalanine	5.2	0.76	ND	ND	14.5	2.13
Lysine	6.0	0.77	27.7	3.55	108.4	13.87
Histidine	ND	ND	2.0	0.27	44.9	6.15
Arginine	ND	ND	ND	ND	122.4	10.09
Peptidoglycan[c]						
Muramic acid	105.0	24.46	92.0	21.44	106.4	24.70
Glucosamine	102.0	16.42	87.0	14.01	84.0	13.52
Alanine	165.0	11.70	161.0	11.43	166.0	11.82
Diaminopimelic acid	100.0	12.90	100.0	12.90	100.0	12.90
Glutamic acid	98.0	12.60	92.0	11.87	100.0	12.90

[a]The growth and isolation of the peptidoglycan-protein complex from N. gonorrhoeae strain RUG 40 was as described in Materials and Methods. Hydrolysis was performed in 4 N HCl, 105° C for 14 hrs. Correction for components degraded by HCl was obtained from a standard curve of samples hydrolyzed for various periods; the degradation for muramic acid and glucosamine was 36 and 26.5% respectively.

[b]Correction was made to account for the gain of H_2O during hydrolysis.

[c]Amino acid analysis was determined by standardizing diaminopimelic acid to 100 nmoles. The typical peptidoglycan components used in this determination were muramic acid, glucosamine, alanine, diaminopimelic acid, and glutamic acid in a 1:1:1.65:1:1 ratio respectively. Components found in the peptidoglycan were subtracted from the protein portion of the peptidoglycan-complex.

Table 3

Percent Distribution of Protein and Peptidoglycan in the Peptidoglycan-Protein Complex Isolated from N. gonorrhoeae RUG 40[a]

Growth pH	Fraction[b]	
	Peptidoglycan (%)	Protein (%)
8.0	90.5	9.5
7.2	90.9	9.1
6.0	57.8	42.2

[a]The growth and isolation of the peptidoglycan-protein complex from N. gonorrhoeae RUG 40 was as described in Materials and Methods.

[b]Percent of each fraction was determined by weight as calculated from data in Table 2.

are required to determine how many proteins are actually present and whether lipid is associated with the protein(s).

DISCUSSION

The chemical composition of the growth medium has a significant effect on the macromolecular content of the bacterial cell surface. As early as 1965, changes in the galactosamine content and some minor variations in amino acids, as a function of growth phase and the carbon source in the medium, were noted in the cell wall of Bacillus subtilis (9). More recently, it was determined that major shifts of polymers, such as the substitution of teichuronic acid for teichoic acid could be produced by limiting the phosphorus content of the growth medium (10). Similarly, it has been possible to vary the envelope of gram-negative organisms such as Aerobacter aerogenes or N. sicca by changes in the growth conditions (11, 12).

Although many organisms produce major shifts in the pH of poorly buffered medium during growth, little attention has been directed to an analysis of the effect of hydrogen ion concentration on the cell envelope. While N. gonorrhoeae can grow at pH values ranging from 5.8 to 8.0 (13), the present study represents the first attempt to analyze the consequences on the attendant metabolic alterations on the cell envelope. The data presented clearly establish that there is a major increase in the protein content of the peptidoglycan isolated from gonococci grown at pH 6.0. Furthermore, this effect does not appear to be strain specific. At present, it is not possible to ascertain whether this increment in the amino acid content reflects an increase in more than one protein and whether the protein(s) varies among strains of gonococci.

The exact physiologic significance of the protein(s) remains to be elucidated. Currently, we favor the hypothesis that this protein is covalently attached to the peptidoglycan in a fashion analogous to the lipoprotein of Escherichia coli (14). Our analyses have not permitted us to establish whether there is lipid associated with this protein or what residue in the peptidoglycan serves as the binding site for the protein. Presumably, this protein serves a role in anchoring the outer membrane to the peptidoglycan. The increase in protein could be related to either an increased rate of synthesis or a slower rate of degradation at pH 6.0. These possibilities are currently under investigation.

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