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A Simple Gel Electrophoretic Method for Analyzing the Muropeptide Composition of Bacterial Peptidoglycan

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The muropeptide composition of bacterial peptidoglycan is currently most efficiently determined by reverse-phase high-pressure liquid chromatography (HPLC). Though sensitive, the HPLC procedure is technically demanding and has been applied to a relatively small number of bacterial strains and species. We have found that fluorescence-assisted carbohydrate electrophoresis (FACE) is a simple, rapid method by which reducing muropeptides from multiple peptidoglycan samples can be visualized. Individual reducing muropeptides were covalently labeled with the fluorescent molecule 8-aminonaphthalene-1,3,6-trisulfonic acid, after which they were separated by electrophoresis through a 35% polyacrylamide gel and visualized by exposure to UV light. FACE detected the appropriate numbers of reducing muropeptides in the proper proportions for four bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Yersinia enterocolitica*. As little as 2 to 5 pmol per muropeptide was detected when the intensity of the fluorescent signal was measured with a charge-coupled device camera, at a level of sensitivity between 50 and 250 times higher than that of the classic HPLC technique. Thus, FACE may be used to identify interesting peptidoglycan samples prior to more-extensive analysis by HPLC, or FACE may eventually replace HPLC for some applications.

The rigid element of the cell wall in eubacteria is peptidoglycan, a macromolecule in which two components, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), alternate to form a carbohydrate polymer (the glycan chain). From NAM extends a side chain of five amino acids (e.g., L-Ala–D-Glu–*meso*-DAP–D-Ala–D-Ala), and two such peptides from adjacent glycan chains can be joined covalently (17, 29). Thus, peptidoglycan is a lattice of glycan chains connected by multiple peptide cross-links. Variation in the length, composition, and linkage of peptide side chains produces more than 100 different peptidoglycan types (25), and at least 40 to 50 individual compounds compose the peptidoglycan of *E. coli* (6, 7).

The preferred method of analyzing the chemical structure of peptidoglycan is to digest it with muramidase, which cleaves the glycan chain into individual NAG-NAM disaccharide subunits (muropeptides) (6, 7), after which the individual components are separated. Muropeptide composition was first measured by paper chromatography, which could distinguish disaccharide monomers and multimers (19, 22). However, the richness in the number and types of muropeptides was not appreciated until 1983, when *Escherichia coli* muropeptides were separated by reverse-phase high-pressure liquid chromatography (HPLC) (8). Five years later, the complete method was described, and many of the individual muropeptides were identified (6). This technology provides the basis for all current peptidoglycan analysis.

The HPLC procedure was a considerable improvement over previous methods for separating muropeptides. However, despite this advance, from 1983 to 1994 the technique was used to analyze peptidoglycans from only five different gram-negative bacteria (1, 3, 6, 7, 20, 28), three different gram-positive bacteria (2, 5, 26), and one eukaryotic organelle (21). In most cases these determinations were made only once. It was not until 1995 that nine additional gram-negative organisms were added to this list, all from a single laboratory (23, 24). For the most part, HPLC has been used to analyze *E. coli* peptidoglycans from a limited number of mutants and growth conditions. Thus, application of the technique has been restricted to a few laboratories and a few special circumstances. This situation exists because, though effective, the HPLC procedure is technically demanding, requiring meticulous control of several parameters (6). One of its practitioners has acknowledged this state of affairs by admitting that ". . .[the HPLC] methodology is still far from routine" (24).

A simpler alternative to HPLC would stimulate a broader investigation of peptidoglycan structure, enabling the analysis of numerous bacteria and bacterial mutants. For this purpose, we adopted a modified gel electrophoresis technique and have found it to be a fast, sensitive alternative to HPLC.

Preparation of peptidoglycan from bacteria. Bacteria were diluted from a fresh overnight culture and grown at 37°C in 400 ml of M9 medium (18) for 3 to 4 doublings to an A_{550} of ~0.5. Cells were cooled rapidly to 4°C and harvested by centrifugation at 10,000 × *g* for 15 min at 4°C, and the wet weight of the pellet was determined. Peptidoglycan was isolated essentially as described previously (6, 9, 11). Washed bacteria were resuspended to 0.2 g/ml in H_2O, the mixture was added dropwise with vigorous stirring to an equal volume of boiling 8% sodium dodecyl sulfate (SDS) and boiled for 30 min, and the lysate was cooled overnight to room temperature. Insoluble peptidoglycan was pelleted by ultracentrifugation at 100,000 × *g* for 60 min at room temperature, and the pellet was washed and repelleted at least four times in distilled water until the SDS concentration was below 1 mg/ml, as determined by the methylene blue assay (10). The pelleted peptidoglycan was resuspended in 5 ml of 10 mM Tris-HCl (pH 7.0)–10 mM NaCl and solubilized by sonication in closed microcentrifuge tubes for 1 to 2 min. Glycogen contamination was removed by adding imidazole to 0.32 M and α-amylase to 100 μg/ml and incubating the mixture for 2 h at 37°C. Pronase (pretreated by incubation at 60°C for 2 h to inactivate lysozyme) was added to 200

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μg/ml, and the sample was incubated at 60°C for 1.5 h to remove the bound lipoprotein. This mixture was added, with vigorous stirring, to an equal volume of boiling 8% SDS and boiled for 15 min, after which the peptidoglycan was pelleted, washed free of SDS as described above, resuspended in 0.02% NaN_3, and stored at −20°C.

Preparation and fluorescent labeling of muropeptides. Muropeptides were prepared by digesting a sample of peptidoglycan from 5×10^9 to 10×10^9 bacteria (1/10 the amount of the total peptidoglycan preparation) with 10 μg of *N*-acetylmuramidase SG (USB Specialty Biochemicals, Amersham Life Sciences, Cleveland, Ohio). Muropeptides released from intact peptidoglycan after digestion with muramidase have a terminal reducing sugar (except for those derived from the extreme end of each glycan chain). The reducing ends were labeled with the fluorescent molecule 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), as published previously (13, 27) and as described by the supplier (Glyko, Inc., Novato, Calif.).

Separation and visualization of labeled muropeptides by FACE. Fluorophore-assisted carbohydrate electrophoresis (FACE) was performed in a minigel apparatus under conditions previously described (13, 27). The gels and buffers used are those described by Laemmli (16) but without SDS and β-mercaptoethanol. Fluorescent-labeled muropeptides were loaded onto a 35% acrylamide gel, separated by electrophoresis for 90 min, and visualized by exposing the gel to a 365-nm-wavelength light. The bands were photographed with a Polaroid MP-4 camera fitted with a filter normally used for photographing DNA stained with ethidium bromide. Duplicate samples were sent to Glyko, Inc., where muropeptides were separated under the same conditions, and the gel was UV irradiated and photographed by a charge-coupled device (CCD) camera. Because ANTS labeling is virtually complete and adds one fluorophore per muropeptide, the molar quantity of each muropeptide was determined by comparing its fluorescence with a standardized ladder of maltose oligomers (12, 14). Fluorescence intensity and the relative and molar quantities of each band were determined with Glyko FACE analytical software.

FACE detects muropeptides with greater sensitivity than does HPLC. Peptidoglycan was prepared from six different strains of *E. coli* and from *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Yersinia enterocolitica*. Muropeptides were separated by electrophoresis and were compared with one another and with previously reported HPLC analyses. In all cases, the CCD camera detected more bands than did film photography or visual inspection of UV-irradiated gels (compare the results from photography [Fig. 1A] with those from the CCD camera [Fig. 1B]). In addition, data from the CCD camera were immediately available for quantitation of each compound by direct comparison with oligosaccharide standards on each gel.

Approximately 20 to 23 muropeptide bands were visible in peptidoglycan digests from *E. coli* (Table 1 and Fig. 1B, 2, and 3). In comparison, classic HPLC analysis detects 22 to 23 individual reducing muropeptides, accounting for ~95% of the total number of components in *E. coli* peptidoglycan (6, 7, 23). The remaining 5% are anhydromuramic acids, representing the modified ends of glycan chains (6, 7); compounds derived from these ends are not labeled with ANTS. By comparing the fluorescence intensity of each band to that of a standard, as few as 2 to 5 pmol of muropeptide per band was detected by CCD camera capture (Table 1). This number is near the current theoretical detection limit of 0.2 pmol per band, which may decrease with improved CCD cameras (14). For comparison, the detection limit per muropeptide by HPLC is 100 to 500 pmol (7), although radiolabeled muropeptides can be detected by HPLC with a sensitivity equivalent to that of FACE for unlabeled muropeptides.

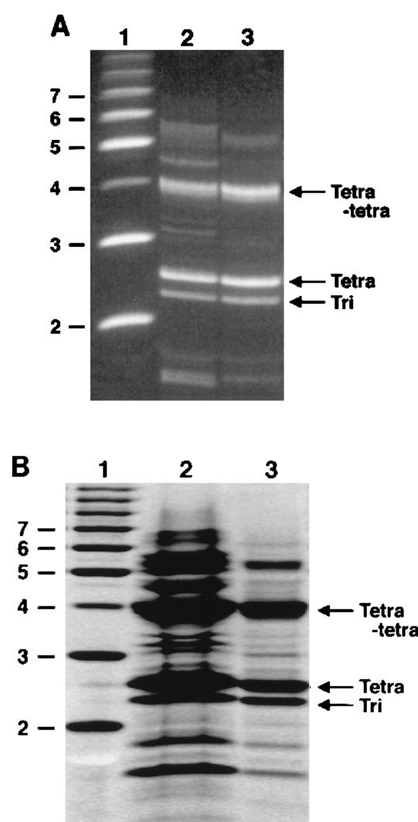

FIG. 1. Electrophoretic separation of fluorescent-labeled muropeptides from bacterial peptidoglycan, visualized by photography and CCD camera. Muropeptides were prepared from *E. coli* and *P. aeruginosa*, labeled with the fluorescent dye ANTS, and separated by electrophoresis through a 35% polyacrylamide gel as described in the text. (A) The gel was illuminated by light from a 365-nm-wavelength UV transilluminator, and fluorescent bands were visualized by Polaroid photography. (B) The gel was illuminated by a 360-nm-wavelength UV light, and fluorescent bands were detected by data capture with a CCD camera. Lanes: 1, maltose oligomer standards; 2, muropeptide sample from *E. coli* CSQ (W1485 *supE lacI*^{q}); 3, muropeptide sample from *P. aeruginosa* (lab strain). The numbers on the left indicate the number of maltose monomers in each oligomer; band no. 4 contains 25 pmol of oligomer and is the fluorescent standard against which the molar quantities of other bands were measured. Provisional identification of three muropeptides are indicated on the right: Tri, disaccharide-tripeptide monomer; Tetra, disaccharide-tetrapeptide monomer; Tetra-tetra, dimer of two tetra monomers cross-linked via the tetrapeptide side chains.

Preliminary identification of muropeptide bands. The amount of each muropeptide band was expressed as a percentage of the total peptidoglycan in each sample (Table 1, and data not shown), and these percentages were used for the preliminary identification of a few major muropeptides. The two most prominent muropeptide bands separated by FACE accounted for 33.5 and 25.7% of the total in *E. coli* peptidoglycan (Table 1). In accord with previous HPLC analyses (7), these bands were provisionally identified as the tetra compound (disaccharide with a 4-amino-acid side chain) and the tetra-tetra compound (two disaccharides cross-linked via two 4-amino-acid side chains), respectively (Fig. 1 and 2). The amounts of these two components as measured by FACE are equivalent to the amounts detected by HPLC in *E. coli* (35.9 and 27.3%) (7), *Bordetella pertussis* (31.8 and 22.3%) (28), and *Haemophilus influenzae* (34.8 and 28.4%) (1).

TABLE 1. Actual and relative amounts of muropeptides from *E. coli* and *P. aeruginosa*

No. of maltose monomers[a]	Quantity (pmol) of muropeptide band in[b]:			% Total luminance in[c]:	
	Oligosaccharide standard	*E. coli*[d]	*P. aeruginosa*	*E. coli*	*P. aeruginosa*
10	14.97				
9	21.39				
8	27.13	5.31		0.2	
7	55.07	24.24		0.8	
6	94.90	34.72		1.2	
			4.76		0.5
		190.65		6.5	
		28.10		1.0	
5	156.39	29.86	50.80	1.0	5.5
		22.42	5.96	0.8	0.6
		186.01		6.3	
4	25.00	753.51	368.95	25.7	39.7
		33.84	2.61	1.2	0.3
		2.95		0.1	
			2.96		0.3
		51.26		1.7	
			8.87		1.0
3	152.50				
		12.17	3.93	0.4	0.4
		980.81	344.58	33.5	37.1
		314.92	98.09	10.7	10.6
2	119.11	4.94	3.37	0.2	0.4
		70.42	11.85	2.4	1.3
		6.87	1.47	0.2	0.2
		167.11	20.18	5.7	2.2
		1.73		0.1	
		3.5		0.1	
		4.5		0.2	
1	39.43				

[a] The number of maltose monomers comprosing each oligosaccharide in the standard mixture visible in the left-most lane of each figure.
[b] The amount and approximate location of each muropeptide band relative to the oligosaccharide standards. Quantitation was performed by using the fluorescence intensity of oligosaccharide no. 4 (25 pmol) as a reference point.
[c] The fluorescence luminance of each muropeptide band divided by the total luminance of all bands in the sample.
[d] Muropeptide separations are those depicted in Fig. 1A.

FACE detects differences in muropeptide profiles among bacteria. The ability of the FACE technique to distinguish differences in the muropeptide composition among different peptidoglycans was tested in two ways. First, we compared samples from different species of bacteria. Recently, Quintela et al. (23) used HPLC to analyze the peptidoglycan composition of *E. coli* and nine other organisms. We prepared muropeptides from three of these: *P. aeruginosa*, *E. cloacae*, and *Y. enterocolitica* (Fig. 1B, lane 3, and Fig. 2, lanes 4 and 5). It was easy to visualize the differences in muropeptide compositions among these species. In each case, the number of reducing muropeptides visible in the FACE gel was equal to or greater than the number detected by HPLC. For example, HPLC analysis detected 4 major and ~11 minor reducing muropeptides from *P. aeruginosa* (23), and FACE analysis detected the same numbers (Table 1 and Fig. 1B, lane 3). Similar results were observed for peptidoglycan from *E. cloacae* (Fig. 2, lane 4), in which FACE detected 18 to 20 muropeptides compared with ~18 by HPLC (23), and from *Y. enterocolitica* (Fig. 2, lane 5), in which FACE detected 19 to 20 muropeptides compared with ~18 by HPLC (23). Therefore, for each bacterium, the FACE technique accurately characterized the total number of different muropeptides and the number of major muropeptides and gave equivalent measurements for the ratios of the tetra-tetra compounds to the tetra components.

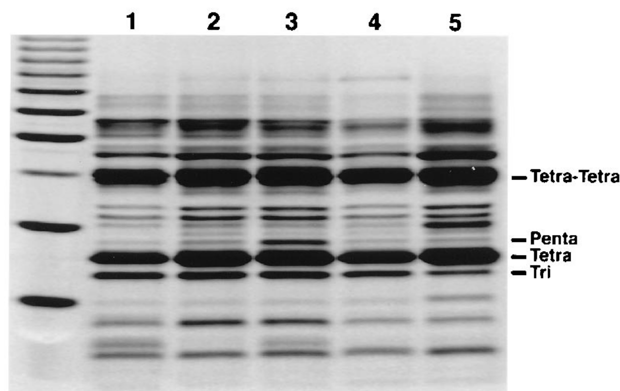

FIG. 2. Muropeptides profiles of *E. coli*, *Enterobacter cloacae*, and *Y. enterocolitica*. Muropeptides were prepared from the peptidoglycan of three strains of *E. coli* and from *E. cloacae* and *Y. enterocolitica*. Lanes: left lane (not numbered), maltose oligomer standards (band no. 4 is the third band from the bottom); 1, *E. coli* CSQ (W1485 *supE lacI*[q]); 2, *E. coli* ED3184 (4); 3, *E. coli* D456 (*E. coli* ED3184 ΔPBP 4 ΔPBP 5 ΔPBP 6) (4); 4, *E. cloacae* (lab strain); 5, *Y. enterocolitica* (lab strain). Provisional identification of four muropeptides are indicated on the right: Penta, disaccharide-pentapeptide monomer, and the other three are described in the legend to Fig. 1.

FACE detects differences in muropeptide profiles among *E. coli* mutants. The second test to access the ability of FACE to distinguish differences in peptidoglycan composition was the comparison of samples from different mutants of *E. coli*. We prepared peptidoglycan from three mutants of *E. coli* with deletions of PBPs 4, 5, and 6 (4); PBP 7 (our lab); or AmpC (our lab). The muropeptide compositions of these mutants were compared with those of their parents, by the FACE technique (Fig. 2 and 3). The muropeptide composition of the Δ4/Δ5/Δ6 mutant was different from that of its parent (Fig. 2, lanes 3 and 2, respectively). In particular, one band just larger

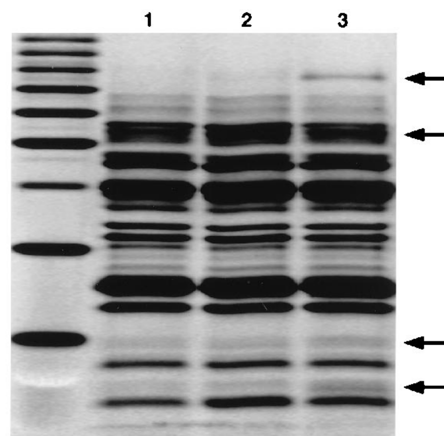

FIG. 3. Muropeptides profiles of *E. coli* and two isogenic mutants. Muropeptides were prepared and labeled with ANTS as described. Lanes: left lane (not numbered), maltose oligomer standards (band no. 4 is the third band from the bottom); 1, *E. coli* JC9387 (*E. coli* Genetic Stock Center, accession no. CGSC 6613); 2, an isogenic mutant of *E. coli* JC9387 from which the gene for PBP 7 was deleted (this lab); 3, an isogenic mutant of *E. coli* JC9387 from which the gene for AmpC was deleted (this lab). The arrows on the right indicate muropeptide bands that are more intense in the ΔAmpC mutant than in the parent (lane 3 versus lane 1, respectively).

than the tetra component (labeled "Penta" in Fig. 2) was increased 3.6-fold as determined by the fluorescence intensity. When peptidoglycan is synthesized by ether-permeabilized *E. coli* cells, the monomeric pentapeptide component (a single disaccharide with a 5-amino-acid side chain) is increased 3.4-fold in a PBP 4 mutant, 6.6-fold in a PBP 5 mutant, and 10-fold in a PBP 6 mutant (15). The results from FACE analysis are consistent with just such an increase in the pentapeptide component. The quantitative difference between the results reported here and those reported for *E. coli* cells harboring single mutations may be explained by the fact that in the earlier results the composition of newly synthesized peptidoglycan in ether-permeabilized cells was determined (15), whereas we measured the total peptidoglycan of undisturbed cells.

Peptidoglycan was also prepared from *E. coli* mutants lacking either PBP 7 or AmpC (Fig. 3). The PBP 7 mutant showed no alteration in muropeptide composition compared with the parental strain (Fig. 3, lane 2). However, minor alterations were apparent in the peptidoglycan of the *ampC* mutant. FACE analysis indicated that three or four muropeptides increased in the *ampC* mutant compared with its parent (Fig. 3, lanes 3 and 1, respectively). This is the first evidence that native *E. coli* AmpC protein plays a role in determining the peptidoglycan structure. We are not yet able to specify the nature of these alterations.

Advantages of FACE. Compared with the established HPLC technique, the FACE procedure separates and detects an equivalent number of reducing muropeptides at a level of sensitivity 50 to 250 times higher, permitting the detection of qualitative and quantitative differences in the muropeptide composition among bacterial strains and between mutants of a single species. While doing this, FACE analysis offers several advantages over reverse-phase HPLC. Chief among these is simplicity: muropeptide labeling is easy and polyacrylamide gel electrophoresis is readily accessible. In addition, FACE avoids the complexities associated with HPLC. For example, migration of muropeptides in the gel system is easily standardized against a ladder of maltose oligomers, whereas HPLC requires frequent standardization with a set of known, purified muropeptides (23). A second advantage is the rapidity with which samples can be separated and visualized and the results can be analyzed. Currently, a single peptidoglycan sample requires 2 to 3 h of HPLC separation (6, 7, 23, 24). In a minigel format, FACE requires 90 min and as many as 14 samples (7 in each of two gels) can be separated simultaneously, simplifying comparisons among them. In contrast, HPLC comparisons are complicated by the fact that elution profiles change with minor variations in conditions during a series of HPLC runs (6). Finally, the increased sensitivity of FACE means that less peptidoglycan is needed for each assay.

One notable weakness of the FACE technique is its inability to detect nonreducing 1,6-anhydromuropeptides, because the fluorescence-labeling procedure requires a reducing end. Although these anhydromuropeptides compose only ~5% of the total muropeptides derived from peptidoglycan, they are important because they define one end of each glycan chain. Thus, HPLC analysis remains the sole method for determining this aspect of peptidoglycan structure.

Summary. The muropeptide composition of bacterial peptidoglycan has been investigated in so few cases that we do not have enough data to draw conclusions about the biochemical roles of individual components. The simplicity and speed of FACE analysis will make it possible to survey the peptidoglycan composition of a much larger spectrum of the bacterial world than has heretofore been possible. The FACE procedure is already sufficiently powerful to serve as a screening device to identify interesting peptidoglycan samples prior to extensive HPLC analysis, and further work will establish whether FACE can replace or supersede HPLC for some applications. The ability to rapidly analyze muropeptides should lead to new insights regarding the relationships between peptidoglycan structure and bacterial physiology.

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