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Extent of Peptidoglycan O Acetylation in the Tribe *Proteeae*

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The degree of peptidoglycan O acetylation in 18 strains of the different genera of the tribe *Proteeae* (*Proteus*, *Providencia*, and *Morganella*) has been determined by high-performance liquid chromatography-based organic acid analysis of mild-base-released acetic acid and quantitation of peptidoglycan concentrations by simultaneous amino sugar-amino acid analysis using high-performance anion-exchange chromatography with pulsed amperometric detection. The *N*,*O*-diacetylmuramyl content of all isolated and purified peptidoglycans was greater than 29% and ranged up to 57% relative to total muramic acid concentration. Each of the O-acetylated peptidoglycans was found to be resistant to solubilization by hen egg white lysozyme.

The O acetylation of peptidoglycan occurs at the C-6 hydroxyl group of *N*-acetylmuramyl residues, producing the corresponding *N*,*O*-diacetylmuramyl derivatives within the eubacterial cell wall heteropolymer. A total of 11 species of eubacteria are currently known to possess O-acetylated peptidoglycan (reviewed in reference 6); these species include some important human pathogens, both gram positive (e.g., *Staphylococcus aureus* [11, 27]) and gram negative (e.g., *Neisseria gonorrhoeae* [2] and *Proteus mirabilis* [7, 20]). The complete extent of peptidoglycan O acetylation among the eubacteria, however, remains largely unknown. Little is also known with respect to the physiological role of the modification as it pertains to the various microorganisms, but it does confer resistance to the hydrolytic action of many muramidases (and hence conceivably autolysins), including hen egg white and human lysozymes (2, 4, 7, 18, 21, 23, 26). Several studies have shown that the extent of resistance to muramidases is directly related to the degree of the modification (7, 23, 26), which ranges between 20 and 70% (6). Thus, the peptidoglycan from 14 strains of *Proteus mirabilis*, characterized by 20 to 53% O acetylation, was shown to be resistant to hydrolysis by hen egg white lysozyme, while chemically de-O-acetylated material was completely solubilized and degraded to low-molecular-weight fragments (7). The consequences of the decreased susceptibility of O-acetylated peptidoglycan to solubilization are considerable in view of the detrimental effects exerted by circulating peptidoglycan fragments in a mammalian host, including the induction of rheumatoid arthritis (reviewed in references 6 and 25).

Here, a study of all species of the tribe *Proteeae* was initiated to discern the prevalence of peptidoglycan O acetylation among this important group of human pathogens. By using high-performance liquid chromatography (HPLC)-based analysis procedures for the quantitation of both acetic acid and peptidoglycan (muramic acid), all species of the genera *Proteus*, *Providencia*, and *Morganella* are shown to possess the modification.

Bacterial strains and preparation of peptidoglycan. All of the bacterial strains used in this study were obtained from the American Type Culture Collection, Rockville, Md., with the exception of *Morganella morganii* UGM 326 and UGM 92, *Providencia stuartii* UGM 603, and *Providencia rettgeri* UGM 565, which were from the University of Guelph Department of Microbiology culture collection. *Escherichia coli* CHS4 and *Pseudomonas aeruginosa* PAO1 were kindly provided by J. Wood and J. Lam, Department of Microbiology, University of Guelph, respectively. All bacteria were maintained on nutrient agar slants at 4°C.

Cultures (50 ml) of the various microorganisms were grown in nutrient broth supplemented with 30 mM glucose at 37°C on a rotary shaker (200 rpm). After 3 to 5 h of incubation, the cells (optical density at 578 nm of 0.60 to 0.70) were harvested by centrifugation (Sorvall RC-2B; DuPont Sorvall) at 8,000 × *g* and 4°C for 15 min, washed twice with 10 mM sodium phosphate buffer (pH 6.5) and then resuspended in 5 ml of water (pH 5.5 to 6.0). Insoluble peptidoglycan was extracted from whole cells by the boiling 4% sodium dodecyl sulfate (SDS) procedure as modified by Hoyle and Beveridge (16). The SDS-insoluble peptidoglycan was collected by ultracentrifugation [Beckman L5-50; Beckman Instruments (Canada) Ltd., Mississauga, Ontario, Canada] at 160,000 × *g* for 60 min at 20°C. The pellet was washed twice by centrifugation with acidified water and resuspended in 600 μl of water.

Determination of peptidoglycan O acetylation. Aliquots (200 μl) of peptidoglycan suspensions were incubated at ambient temperature for at least 3 h with an equal volume of either 160 mM NaOH or 160 mM sodium phosphate buffer (pH 6.5). The peptidoglycan was collected by centrifugation at 33,000 × *g* for 10 min in a microcentrifuge (Biofuge 13; Heraeus Instruments, Osterode, Germany). The supernatant was filtered through a Millipore HA 0.45-μm-pore-size membrane [Millipore (Canada) Ltd., Mississauga, Ontario, Canada], and quantitation of released acetate was accomplished by HPLC employing an HPX-87H organic acid column [Bio-Rad Laboratories (Canada) Inc., Mississauga, Ontario, Canada] as previously described (7). Quantitation of peptidoglycan (simultaneous analysis of amino sugars and amino acids) was performed on acid hydrolysates by HPLC-based anion-exchange chromatography employing pulsed-amperometric detection (5), and the levels of muramic acid were used for quantitative purposes since this amino sugar is unique to peptidoglycan. Samples of peptidoglycan (40 to 80 μg) were hydrolyzed in 4 M HCl at 104°C for 12 h in vacuo.

A typical HPLC chromatogram obtained during the analysis for the ester-linked acetate from preparations of the *Proteeae* peptidoglycan (*O*-acetate released by mild saponification) is presented in Fig. 1. Identical samples treated with 80 mM sodium phosphate buffer (pH 6.8) were prepared as controls, and no released acetate was detected. In addition,

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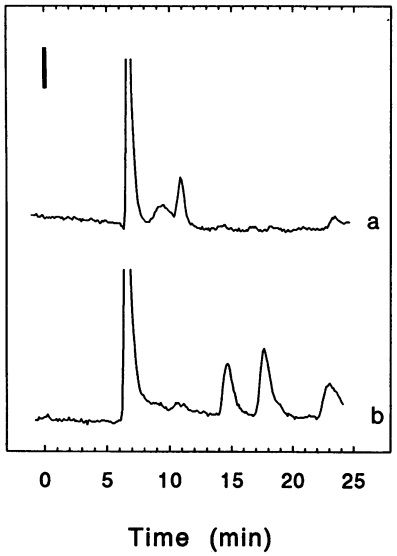

FIG. 1. Determination of peptidoglycan O acetylation by HPLC-based organic acid analysis. Samples of *Proteus vulgaris* ATCC 13315 peptidoglycan were treated with 80 mM sodium phosphate buffer (pH 6.6) (A) or 80 mM NaOH (B) for 3 h at 25°C. The filtered supernatants were injected onto a Bio-Rad HPX-87H HPLC column and eluted with 20 mM H_2SO_4 at 0.6 ml min^{-1} and 45°C. The bar represents 5.0 × 10^{-4} absorbance units at 215 nm. Acetic acid is eluted at 14.8 min.

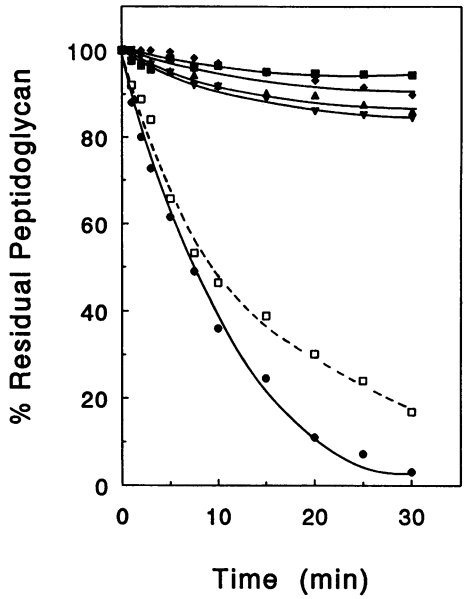

FIG. 2. Extent of solubilization of O-acetylated and non-O-acetylated peptidoglycan by hen egg white lysozyme. Representative samples of peptidoglycan (approximately 0.4 mg ml^{-1}) in 100 mM sodium phosphate buffer (pH 6.6) were incubated at 25°C with 10 U of lysozyme, and the decrease in A_{660} monitored with time is expressed as a percentage of an appropriate control that lacked muramidase addition. Shown are the peptidoglycans isolated from *P. aeruginosa* or *E. coli* (●), *Providencia stuartii* (▼), *Providencia rettgeri* (◆), *M. morganii* (▲), and *Proteus penneri*, native (■) and de-O acetylated (80 mM NaOH, 25°C, 3 h) (□).

no acetate was observed in the negative-control preparations of either *E. coli* or *P. aeruginosa* peptidoglycan that had been treated with either NaOH or buffer. The extents of peptidoglycan O acetylation as determined by the HPLC assay for the various *Proteeae* species are listed in Table 1. All of the 18 strains tested had levels of O-acetylated peptidoglycan greater than 28% (molar ratio of acetate to muramic acid). *Providencia stuartii* ATCC 35031 possessed the highest level of O acetylation at 57.3%, while on average, strains of *Proteus vulgaris* were the least modified (29 to 35%). Hitherto, none of these species of *Proteeae* except *Proteus mirabilis* and *Proteus vulgaris* (6) were known to produce O-acetylated peptidoglycan. The type strain of *Proteus mirabilis* (ATCC 29906), which was not previously characterized for the modification, was found to produce O-acetylated peptidoglycan at levels similar to those of the other 15 characterized strains (viz., 20 to 53%) (7).

TABLE 1. Extent of O-acetylated peptidoglycan among the *Proteeae*

Species	Strain	% *O*-Acetyl[a]
Proteus mirabilis	ATCC 29906 (type)	41.5
P. myxofaciens	ATCC 19692 (type)	52.8
P. penneri	ATCC 33519 (type)	36.0
P. vulgaris	ATCC 13315 (neotype)	31.3
	ATCC 33420	34.9
	ATCC 8427	29.4
	ATCC 6380 (OX19)	32.3
Morganella morganii	ATCC 25830 (neotype)	45.7
	UGM 326	43.0
	UGM 92	49.6
Providencia alcalifaciens	ATCC 9886 (type)	42.4
P. heinbachae	ATCC 35613 (type)	33.6
P. rettgeri	ATCC 29944 (type)	36.6
	UGM 565	42.1
P. rustigianii	ATCC 33673 (type)	40.9
P. stuartii	ATCC 29914 (type)	53.6
	ATCC 33672	39.7
	ATCC 35031	57.3
	UGM 603	39.4

[a] Values represent means of at least two independent determinations and are relative to muramic acid concentrations.

Susceptibility of *Proteeae* peptidoglycan to lysozyme. The turbidometric assay of Hash (14) was used to assess the susceptibility of the peptidoglycan preparations to hen egg white lysozyme (a muramidase) as previously described (7). Briefly, evenly suspended (sonicated) aliquots (200 μl) of insoluble peptidoglycan were added to 500 μl of 100 mM sodium-potassium phosphate buffer (pH 6.6) in a 1-cm-path-length glass cuvette (1-ml capacity) to give the desired concentration of substrate. Hydrolytic reactions were initiated with the addition of 10 μl of hen egg white lysozyme (1,000 U ml^{-1}), and the decrease in turbidity at 660 nm was monitored with time.

Representative time course curves for the lysozyme-catalyzed hydrolysis of SDS-insoluble peptidoglycan isolated from *P. aeruginosa*, *E. coli*, and species of the tribe *Proteeae* are presented in Fig. 2. The muramidase completely solubilized both the *P. aeruginosa* and *E. coli* peptidoglycans within 30 min of enzyme introduction. In contrast, none of the naturally occurring peptidoglycans isolated from the various species of the tribe *Proteeae* were susceptible to the enzymatic hydrolysis, even upon prolonged incubation. Complete lysozyme-catalyzed solubilization of these latter peptidoglycan samples required the prior removal of *O*-acetyl moieties by mild base hydrolysis.

Discussion and conclusions. The peptidoglycan from all of the species of the tribe *Proteeae* was shown to be modified by *O*-acetyl groups. Accurate quantitation of peptidoglycan O acetylation was achieved by employing HPLC procedures for both acetic acid and amino sugar analysis. All of the strains examined possessed peptidoglycan that was both O acetylated and resistant to lysozyme hydrolysis. The degree of O acetylation ranged between 28 and 57%, which is similar to that observed in other bacteria (6). Approximately 34 to 52% of the muramyl residues of *N. gonorrhoeae* are known to be N,O diacetylated (26), with a preponderance of strains possessing above 40%. The muramyl residues of *S. aureus* peptidoglycan are about 60% O acetylated (11), but this value appears to increase to approximately 82% when cells are cultured in the presence of the bacteriostatic drug chloramphenicol (18, 24). This latter observation is consistent with the O acetylation of peptidoglycan as being a maturation process (6) and the fact that the extent of O acetylation is observed to increase during the stationary phase (18).

The physiological role of peptidoglycan O acetylation remains unknown, but it is speculated to control the action of autolysins. Indeed, many muramidases, including human and egg white lysozymes (2, 4, 7, 18, 21, 23, 26), do not act on the modified peptidoglycan, while others, such as the enzymes from *Chalaropsis* sp. (15) and *Streptomyces globisporus* (mutanolysin) (13), are not affected by the acetyl substitutions. Thus, O acetylation may block the action of a specific group of autolysins while not affecting others. In addition, there has been at least one report of an autolysin with selectivity for O-acetylated peptidoglycan (1).

The three genera of the tribe *Proteeae* constitute a medically important group of bacteria in the family *Enterobacteriaceae*. A number of species represent important human pathogens and together are responsible for approximately 10% of all nosocomial infections in North America (3). The presence of O-acetylated peptidoglycan in a number of pathogenic bacteria, including all of the members of the tribe *Proteeae*, may signify an important correlation between the modification and pathogenicity. Furthermore, the optimal expression of many of the pathobiological activities of peptidoglycan (reviewed in references 6 and 25), including the induction of rheumatoid arthritis (8–10, 12, 17, 19, 22), requires the persistence and accumulation of large peptidoglycan fragments in the host. That O acetylation has been shown to prevent its hydrolysis by mammalian lysozymes underscores the importance for further studies relating to this modification of peptidoglycan.

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