Evidence for N→O Acetyl Migration as the Mechanism for O Acetylation of Peptidoglycan in *Proteus mirabilis*

CLAUDE DUPONT† and ANTHONY J. CLARKE*

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 22 March 1991/Accepted 14 May 1991

O-acetylated peptidoglycan was purified from *Proteus mirabilis* grown in the presence of specifically radiolabelled glucosamine derivatives, and the migration of the radiolabel was monitored. Mild-base hydrolysis of the isolated peptidoglycan (to release ester-linked acetate) from cells grown in the presence of 40 μM [acetyl-3H]N-acetyl-D-glucosamine resulted in the release of [3H]acetate, as detected by high-pressure liquid chromatography. The inclusion of either acetate, pyruvate, or acetyl phosphate, each at 1 mM final concentration, did not result in a diminution of mild-base-released [3H]acetate levels. No such release of [3H]acetate was observed with peptidoglycan isolated from either *Escherichia coli* incubated with the same radiolabel or *P. mirabilis* grown with [1,6-3H]N-acetyl-D-glucosamine or [α-1-14C]glucosamine. These observations support a hypothesis that O acetylation occurs by N→O acetyl transfer within the sacculus. A decrease in [3H]acetate release by mild-base hydrolysis was observed with the peptidoglycan of *P. mirabilis* cultures incubated in the presence of antagonists of peptidoglycan biosynthesis, penicillin G and N-cycloserine. The absence of free-amino sugars in the peptidoglycan of *P. mirabilis* but the detection of glucosamine in spent culture broths implies that N→O transacylation is intimately associated with peptidoglycan turnover.

Following the lysis of invading microorganisms, mammalian tissues clear cellular debris, including peptidoglycan (PG), very rapidly through the action of the hydrolytic enzymes of either phagocytic cells or serum. However, in some instances, large-molecular-weight fragments of PG have been observed to persist and circulate in the host organism. These large PG fragments have been shown to induce diverse pathobiological and pathophysiological effects, many of which have been recently reviewed (36). Some of these include the induction of slow-wave sleep, complement activation, pyrogenicity, modulation of blastogenesis, and arthritogenicity. In vivo studies with the pathogens *Staphylococcus aureus* and *Neisseria gonorrhoeae* indicated that the phenomenon of PG persistence is directly attributable to the presence of numerous O-acetyl substituents on the glycan backbone (5, 15, 24, 34, 40).

O-acetylated PG has been observed in a number of bacteria, including many pathogenic species, both gram positive (e.g., *S. aureus* [17, 41]) and gram negative (e.g., *N. gonorrhoeae* [40] and *Proteus mirabilis* [14]). O acetylation of PG occurs at the C-6 hydroxyl group of N-acetylmuramyl residues, producing the corresponding 2,6-diacetylmuramyl derivative. This modification to PG inhibits the hydrolytic activity of lysozymes (reference 13 and references therein), presumably through steric hindrance since the C-6 hydroxyl moiety of the substrate directly participate in its binding to the active-site cleft of the enzyme (3, 23). The PG of some species of eubacteria has been reported to be O acetylated up to 70%, thereby conferring both intrinsic and complete resistance to lysozyme hydrolysis (30).

O-acetylated PG was first observed independently by two different groups 30 years ago (1, 7), and the biological significance of this modification was discerned soon after (6). Whereas its role in resistance to muramidases and the consequences of such have been well documented, very little is known concerning the biosynthetic process involved with the O acetylation of PG. There is evidence to suggest that O acetylation occurs after nascent PG strands have been both attached and cross-linked to the preexisting sacculus (10, 18, 26–28, 39). By pulse-chasing radiolabelled N-acetyl-D-glucosamine (GlcNAC) into the PG of *P. mirabilis*, Gmeiner and Kroll (18) revealed that only non-O-acetylated PG subunits are incorporated into the growing polymer. It has also been observed that the O acetylation of PG continues in a cell-free biosynthetic system of *P. mirabilis* PG (30). Lear and Perkins (26–28), using a similar experimental strategy, obtained analogous results with *N. gonorrhoeae*. Indeed, searches for lipid (bactoprenyl)-linked N-acetylglucosaminyl-N,O-diacylmuramyl pentapeptide precursors in either the cytoplasm or the cytoplasmic membrane proved futile (27, 39). These observations suggest that an acetyl transferase is present external to the cytoplasm. Comparison of the penicillin-binding protein (PBP) profiles of both penicillin-resistant and penicillin-sensitive strains of *N. gonorrhoeae* with the extent of PG O acetylation led Dougherty to implicate PBP 2 as having a role in O acetylation (9, 11). However, it is likely that the reduced levels of PG O acetylation in penicillin G-treated cells are consequential of incomplete cross-linking caused by the inhibition of transglycosidase or transpeptidase, possibly PBP 2. Nothing else is known about the putative acetyltransferase(s), and the source of acetate is unknown. Through in vivo labelling experiments with *P. mirabilis*, we provide evidence to suggest that acetate is transferred from the N-2 position of either N-acetylgulosaminyl or N-acetylmuramyl residues to the C-6 hydroxyl group of the latter.

* Corresponding author.
† Present address: Department of Chemistry, Carlsberg Laboratorium, Valby, Copenhagen DK 2500, Denmark.
MATERIALS AND METHODS

Bacterial strains. P. mirabilis 19 was kindly provided by J. Gmeiner, Technische Hochschule Darmstadt, Germany; a stock laboratory strain was obtained from H. Perkins, Liverpool, United Kingdom. These strains have been extensively characterized (4, 29, 31). Other P. mirabilis strains (ATCC 12453 and ATCC 7002) and P. vulgaris ATCC 13315 were purchased from the American Type Culture Collection, Rockville, Md. Escherichia coli K-12(CSH4) was provided by J. Wood, University of Guelph. All bacteria were maintained on nutrient agar slants at 4°C.

Growth conditions. For radiolabelling experiments, P. mirabilis strains were cultivated in aerated nutrient broth supplemented with 5.0 g of NaCl, 4.5 g of Na2HPO4, and 3.6 g of glucose per liter on a rotary shaker at 200 rpm and incubated on nutrient agar slants at 37°C. Growth was monitored at final concentrations of 2 to 5 mg·ml−1. Aliquots (200 μl) of these suspensions were incubated overnight at ambient temperature with an equal volume of either 40 mM NaOH or 40 mM sodium phosphate buffer (pH 6.5). The PG was collected by centrifugation at 160,000 × g for 15 min at 20°C in a Beckman Airfuge, and the pellet was washed once by centrifugation with 200 μl of 0.1% sodium azide. The supernatants were pooled and filtered through a Millipore HA 0.45-μm-pore-size membrane (Millipore Ltd., Mississauga, Ontario, Canada). Released acetate was detected and quantitated by high-pressure liquid chromatography (HPLC), using an Aminex HPX-87H (Bio-Rad) organic acid column (7.8 by 300 mm) as previously described (13). Fractions (0.5 ml) of the column effluent were collected and, together with a 100-μl aliquot of the original supernatant, were separately counted for levels of radioactivity by liquid scintillation counting. The PG recovered from the pellets of the Airfuge centrifugation was solubilized by treatment with 5 to 10 U of mutanolysin in 10 mM Tris·HCl (pH 6.80) containing 8 mM MgCl2 at 37°C with gentle agitation for 2 h prior to the quantitation of radioactivity remaining bound.

Radiolabelling in the presence of antibiotics. Cultures of P. mirabilis 19 were grown in minimum medium (100 ml), supplemented with 30 mM glucose and 40 μM GlnAc, for 2 h at 37°C (A578 of 0.15 to 0.2), and then 20 μCi of [acetyl-3H]GlcNAc was added. After a 2-h incubation in the presence of radiolabel, sterilized penicillin G, chloramphenicol, and D-cycloserine were introduced into the separate cultures at final concentrations of 10, 5.0, and 2.5 mg·ml−1, respectively. After a 2-h incubation, now in the presence of both antibiotic and radiolabel, the cultures were rapidly chilled in an ice bath and PG was isolated as described above. Aliquots (2 ml) of cultures were harvested both immediately before antibiotic introduction and at the end of the incubation period and mixed with an equal volume of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) solution. The samples were well mixed and kept at 0°C for at least 1 h. The TCA precipitates were filtered over a glass fiber membranes (GF/F; Whatman Ltd., Maidstone, United Kingdom) and washed with 10 ml of ice-cold 5% (wt/vol) TCA. The membranes were dried (60°C, 2 h), transferred to glass scintillation vials, and counted for radioactivity following the addition of 5 ml of scintillation cocktail.

Detection of free amines in peptidoglycan and turnover products. P. mirabilis 19 was cultured at 37°C with aeration in the minimal salts medium of Grabow and Smit (21) containing glucose as the carbon and energy source. Cultures (100 ml), incubated at 37°C until growth achieved an A578 of 0.80, were cooled to 2°C by swirling the flask in an ice-ethanol bath, and the cells were separated from the spent broth by centrifugation at 9,000 × g and 4°C. The culture supernatant was filtered through 0.45-μm-pore-size cellulose-acetate membranes (Millipore) to remove any residual cells and then lyophilized while the PG of the collected cells was extracted and prepared as described above. Samples of lyophilized purified PG (170 μg) were incubated in Milli-Q water, and the pH was adjusted to 10.4 with the addition of NaOH. These samples were then treated with 10 mM (final concentration) 5-dimethylaminopentane-1-sulfonate·Cl (dansyl·Cl)-acetone solution at 70°C for 1 h. The reaction solutions were lyophilized, and following acid hydrolysis in either 4 or 6 M HCl (100°C, 18 h), the dansylated samples were subjected to amino acid analysis by reverse-phase HPLC as described by Negro et
al. (33). Prior to dansylation, some samples of culture supernatant were suspended in 50 mM Tris·HCl buffer (pH 8.0) containing 0.2 μM pyridoxal phosphate and 50 mM α-ketoglutarate and treated with an aliquot (200 μl) of glutamate-pyruvate transaminase (0.5 mg·ml⁻¹). After 2 h of incubation at 37°C with shaking, the pH of the solution was raised to 10.2 to 10.6 with sodium hydroxide and dansylation was performed as described above.

Standards consisting of dansylated and didansylated diaminopimelic acid (DNS-DAP and (DNS)₂-DAP), dansylated muramic acid (DNS-Mur), and dansylated glucosamine (DNS-GlcN) were prepared as described above and purified by HPLC, using a Beckman Ultrasphere ODS column (5 μm; 4.6 by 150 mm) (33).

Analytical methods. PG concentrations were determined by amino acid analysis, using a Beckman Gold amino acid analyzer with postcolumn ninhydrin detection [Beckman Instruments (Canada)]. Samples of PG (0.15 mg) were hydrolyzed in vacuo with 4 M HCl at 110°C for 16 h. Acetate quantitation was also performed by HPLC on a Beckman system consisting of two model 110B pumps, a model 167 dual-channel rapid-scanning UV-visible detector, a model 406 analog interface, an IBM-XT computer controller with Beckman Gold chromatography software, and a Bio-Rad HPLC column heater as previously described (13). Radioactivity was measured with a Packard Tri-Carb 2000 liquid scintillation counter (Canberra-Packard Canada, Mississauga, Ontario, Canada) with Ecolume (ICN Biomedicals) or Liquisint (National Diagnostics, Manville, N.J.) as the scintillation cocktail. 2-Keto-3-deoxyoctonate acid was determined by the periodic acid-thiobarbituric acid method as described by Karkhanis et al. (25).

Enzymes and biochemicals. GlcNAc, acetyl phosphate, D-cycloserine, dansyl·Cl, dansylated alanine (DNS-Ala), DNS-Glu, D-glucosamine·HCl, mutanolysin, pyruvic acid, and SDS were purchased from Sigma Chemical Co., St. Louis, Mo. Boehringer Mannheim Canada, Laval, Quebec, Canada, supplied the pronase. Chloramphenicol was obtained from BDH Chemicals, Toronto, Ontario, Canada. Penicillin G was a product of Ayerst Laboratories, and nicotinic acid was obtained from Nutritional Biochemicals Corp. Ecolume, vitamin B₁₂, [¹⁴C]GlcN (specific activity, 45 mCi·mmol⁻¹; lot 3157143) and [acetate-³H]GlcNAc (specific activity, 10 Ci·mmol⁻¹; lots 3305141, 360912, and 4021162) were purchased from ICN Biomedicals Canada, Ltd., Montreal, Quebec, Canada. [¹⁶-³H]GlcNAc (specific activity, 34.2 Ci·mmol⁻¹; lot 2482-084) was provided by New England Nuclear Research Products, DuPont Canada, Dorval, Quebec, Canada.

RESULTS

Radionlabelling of peptidoglycan. Initial experiments investigating the mechanism of O acetylation pertained to the culture of both P. mirabilis Perkins and E. coli K-12(CSH4) in nutrient broth supplemented with 20 μM tritiated GlcNAc, with the radiolabel confined to specific sites in the carbohydrate. With [acetate-³H]GlcNAc, 14% ± 2.2% of the total added radioactivity was incorporated into the cells, and 27% ± 4.1% of this label (representing 3.7% of total added label) was confined to insoluble PG. Identical levels of radiolabel incorporation into P. mirabilis were documented by Martin and Gmeiner (30). LPS-free PG isolated and purified from such radiolabelled cells was subsequently incubated overnight with either 40 mM NaOH or 40 mM sodium phosphate buffer (pH 6.5). Following the removal of insoluble PG by ultracentrifugation, the supernatants were subjected to organic acid analysis by HPLC as previously described (13). Base treatment of PG isolated from P. mirabilis grown in the presence of [acetate-³H]GlcNAc led to the release of tritiated acetate (Fig. 1a). No such release of either radiolabel or acetate was observed from similar samples of PG treated with the phosphate buffer (Fig. 1b). Approximately 23% of the incorporated radioactivity was released as acetate from the insoluble PG after mild-base hydrolysis (Table 1), while no significant differences in the amounts of released radioactive acetate were observed between labeled PGs isolated from cultures of P. mirabilis Perkins grown in either nutrient broth or minimal salts medium.

Acetate was also detected from base-treated PG isolated from the same microorganism but incubated in medium containing either [¹⁻¹⁴C]GlcN or [¹⁶-³H]GlcNAc. However, the base-hydrolyzed acetate was not found to be radioactive in these cases; the radiolabel was confined to either C-1 or both C-1 and C-6, respectively, of the glucosamine (Table 1). Control experiments conducted with non-O-acetylated E. coli K-12(CSH4) indicated that this transfer was unique to P. mirabilis.
mirabilis. Neither acetate nor radiolabel was detected from base-treated PG isolated from E. coli cells that had been incubated with any of the radiolabelled precursors (Table 2).

Further studies with [acetate-3H]GlcNAc that involved a number of bacterial species and strains were carried out. The results of incubation of the various bacterial strains in nutrient broth supplemented with 40 μM [acetate-3H]GlcNAc are summarized in Table 2. In all cases, no acetate was detected when supernatants from buffer incubations were injected onto the HPLC column, and none of the radioactivity released was found to elute at the retention time of acetate. However, with the Proteus strains, significantly higher amounts of radioactivity were detected in the supernatants of mild-base incubations, and the levels varied with the particular strains. When these supernatants were subjected to HPLC analysis, acetate was detected in each case, and all radioactivity coeluted with authentic acetate. These data confirm that the release of radioactive acetate from specifically labelled and purified PG is correlated with the species of bacteria possessing O-acetylated PG.

Effects of antibiotics. The effects of various antibiotics on the extent of base-labile radioactive acetate released from P. mirabilis 19 cells incubated in the presence of [acetate-3H]GlcNAc are listed in Table 3. The addition of each of the antibiotics, at levels slightly lower than their respective MICs, to cultures preincubated with [acetate-3H]GlcNAc for 2 h drastically reduced the further incorporation of the radiolabelled precursor into the PG sacculus. The two antibiotics having a primary action on PG biosynthesis, penicillin G and D-cycloserine, also inhibited the transfer of labelled acetate from GlcNAc to a base-labile position. Penicillin G has previously been shown to block PG O acetylation in S. aureus (37), N. gonorrhoeae (4), and P. mirabilis (19). In contrast, the protein synthesis inhibitor chloramphenicol, while affecting the incorporation of [acetate-3H]GlcNAc into the PG sacculus, did not significantly alter the percentage of radioactive base-labile acetate in comparison with the control culture which lacked antibiotic addition. This finding is also consistent with observations made with S. aureus (24) and N. gonorrhoeae (35).

Source of the acetyl moiety for peptidoglycan O acetylation. The observations described above suggest that radioactive acetate derived from [acetate-3H]GlcNAc was specifically transferred in some manner to a base-labile position on PG. In an attempt to show that radioactive acetate is not released through the catabolism of [acetate-3H]GlcNAc and subsequently transferred to the C-6 of muramyl residues after prior activation, experiments using P. mirabilis 19 labeled with this radiolabel in the presence of other potential sources of acetate were carried out. Mild-base hydrolysis of PG isolated from P. mirabilis 19 grown in the presence of 40 μM [acetate-3H]GlcNAc resulted in the release of 23% ± 0.32% [3H]acetate. The inclusion of sodium acetate, sodium pyruvate, or acetyl phosphate, each at 1 mM final concentration, in the culture medium did not decrease the level of [acetate-3H]GlcNAc incorporation into the PG sacculus; moreover, no diminution of the level of radioactive acetate release (21% ± 2.9% for acetate; 25% ± 1.1% for pyruvate; 22% ± 1.7% for acetyl phosphate) was observed following the mild-base hydrolysis of the respective PG isolates.

Detection of free amines. If an N→O acetylation migration is occurring within the PG sacculus, then free amines of glucosaminyl or muramyl residues should be either present in PG itself or released as by-products of PG turnover. To test for the presence of free amines, the purified PG and concentrated spent broth of both P. mirabilis 19 and E. coli K-12(CHS4) cultures were dansylated by incubation with dansyl·Cl at 70°C for 1 h and then acid hydrolyzed in 4 M HCl at 100°C for 18 h. Representative results obtained by reverse-phase HPLC analysis of the dansylated and hydrolyzed adducts are shown in Fig. 2a. No DNS-Mur was evident in any of the samples tested, and DNS-GlcN was not detected in the PG preparations isolated from either P. mirabilis or E. coli. Evidence for the presence of DNS-GlcN in spent broth samples of P. mirabilis cultures was observed, but the DNS-GlcN eluted from the column as a shoulder to DNS-Ala due to the high concentration of the latter in the spent broth. To prevent this coelution of the two dansylated derivatives, most of the alanine present was converted to pyruvate by treatment of the spent broth samples with glutamate-pyruvate transaminase for 2 h at 37°C prior to dansylation. A clear separation of the two components was subsequently achieved by this transaminase pretreatment (Fig. 2b) and allowed for the quantitation of the DNS-GlcN contents. Treatment with the transaminase also seemed to substantially diminish the amount of DNS-DAP detected. It is conceivable that a peptide(s) was present in the commercial transaminase preparation which hydrolyzed the soluble peptidoglycan peptides and the resulting (DNS)2·DAP was subsequently lost under the large ammonia peak (Fig. 2b). Regardless, the levels of glucosamine released by P. mirabilis 19 detected by this dansylation procedure (Table 4).

---

**TABLE 1. Release of radiolabelled acetate from P. mirabilis Perkins peptidoglycan**

<table>
<thead>
<tr>
<th>Radiolabelled PG precursor</th>
<th>Radiolabelled acetate released after incubation with:</th>
<th>Buffer*</th>
<th>Base*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>[acetate-3H]GlcNAc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.54 (2.98)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>22.9 (8.25)</td>
</tr>
<tr>
<td>[acetate-3H]GlNAC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.12</td>
<td>1</td>
<td>22.7 (1.51)</td>
</tr>
<tr>
<td>[1-14C]GlcNAc</td>
<td>5.36</td>
<td>1</td>
<td>5.89 (0.225)</td>
</tr>
<tr>
<td>[1-14C]GlcNAC</td>
<td>13.9 (1.20)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td>14.2 (1.15)</td>
</tr>
</tbody>
</table>

---

**TABLE 2. Recovery of [3H]acetate from different sources of PG radiolabelled with [acetate-3H]GlcNAc**

<table>
<thead>
<tr>
<th>Source of [3H]PG</th>
<th>O acetylation (%)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>% of radiolabelled acetate released with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. mirabilis 19</td>
<td>52.8</td>
<td>Buffer*</td>
</tr>
<tr>
<td>P. mirabilis Perkins</td>
<td>40.2</td>
<td>3.54 (2.98)</td>
</tr>
<tr>
<td>P. mirabilis 7002</td>
<td>43.6</td>
<td>6.25</td>
</tr>
<tr>
<td>P. mirabilis 12453</td>
<td>24.1</td>
<td>2.52</td>
</tr>
<tr>
<td>P. vulgaris 13135</td>
<td>9.65</td>
<td>0.730 (0.0542)</td>
</tr>
<tr>
<td>E. coli K-12(CHS4)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.81 (2.69)</td>
<td>9.49 (3.27)</td>
</tr>
<tr>
<td>E. coli K-12(CHS4)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.542</td>
<td>0.751</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Isolated and purified PG (225 μg) was incubated with either buffer or base overnight at ambient temperature (23°C).

<sup>b</sup> 40 mM sodium phosphate buffer (pH 6.50).

<sup>c</sup> 40 mM (final concentration) sodium hydroxyde.

<sup>d</sup> Bacteria cultured in saline nutrient broth.

<sup>e</sup> Values in parentheses represent standard deviations of n samples.

<sup>f</sup> Bacteria cultured in the minimal salts medium of Grabow and Smit (21).

---

For the full text, please refer to the source: [Link](http://jb.asm.org/).
are comparable to the concentrations of diaminopimelic acid observed in samples of the spent broth not treated with transaminase, indicating that glucosamine is indeed an authentic product of PG turnover in this microorganism. No DNS-GlcN was observed in samples of either fresh medium or E. coli spent broth that had been treated in a similar way, suggesting a correlation between its release from a bacterial species and O acetylation.

**DISCUSSION**

We have presented evidence to suggest that O acetylation of PG arises via an N-O acetyl migration during PG turnover. Several lines of evidence strongly suggest that the radioactive acetate released from hydroxyl positions is transferred directly from the N-2 position of N-acetylglucosaminyl or N-acetyl muramyl residues and not from an intermediate precursor molecule derived through the catabolism of the supplement [acetyl-3H]GlcNAc. (i) Metabolic studies with members of the family Enterobacteriaceae have shown that in the presence of glucose, GlcNAc is transported into the cells by the phosphoenolpyruvate-dependent phosphotransferase system and directly incorporated into wall layers, as has been shown for LPS and PG (8). This has been shown to be the case for P. mirabilis (30) and was corroborated in this study by the finding that radioactive acetate is not released from base-treated PG prepared from cells incubated with either [1,6-3H]GlcNAc or [1,14C]GlcN. Moreover, the presence of acetate, acetyl phosphate, or pyruvate (other potential sources of transferred acetate) did not compete with [acetyl-3H]GlcNAc in the O-acetylation process. (ii) The inhibition of [3H]acetate transfer by antagonists of PG biosynthesis suggests the prerequisite incorporation of labelled subunits into PG prior to transfer of labelled acetate. Under the experimental conditions used, cells were preincubated with [acetyl-3H]GlcNAc for 2 h prior to the addition of the various antibiotics. This would have provided enough time for a hypothetical catabolism of GlcNAc in the cytoplasm and transfer of [3H]acetate to the sacculus, but such did not apparently occur. Instead, the subsequent administration of penicillin G, which blocks PG transglycosylation and transpeptidation reactions, among others, or d-cycloserine, an inhibitor of both alanine racemase and d-alanyl-d-alanine synthetase, were found to block the transfer of [3H]acetate. The fact that these inhibitors of PG maturation not only prevent the incorporation or further processing of [acetyl-3H]GlcNAc into the preexisting PG sacculus but also limit [3H]acetate transfer implies that

**TABLE 3. Effects of antibiotics on the transfer of [3H]acetate in the peptidoglycan of P. mirabilis 19 labelled with [acetyl-3H]GlcNAc**

<table>
<thead>
<tr>
<th>Antibiotic*</th>
<th>[acetyl-3H]GlcNAc incorporated into*:</th>
<th>Base-released [3H]acetate from SDS-insoluble PG*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCA-insoluble cell wall material</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Before addition</td>
<td>After addition</td>
<td>Increase (%)</td>
</tr>
<tr>
<td>None</td>
<td>1,426</td>
<td>2,638</td>
<td>85</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1,361</td>
<td>1,548</td>
<td>13</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>1,430</td>
<td>1,630</td>
<td>14</td>
</tr>
<tr>
<td>d-Cycloserine</td>
<td>1,372</td>
<td>1,847</td>
<td>35</td>
</tr>
</tbody>
</table>

* Antibiotic concentrations were 10.0, 5.0, and 2.5 mg · ml-1 for penicillin G, chloramphenicol, and d-cycloserine, respectively.

* Cells were cultured in the presence of 40 µM [acetyl-3H]GlcNAc for 2 h before the addition of antibiotics. TCA-insoluble cell wall material and SDS-insoluble PG were prepared as described in Materials and Methods.

* 40 mM (final concentration) sodium hydroxide, overnight at ambient temperature (23). An average of 1.39% of the [3H]acetate was released from PG samples incubated in 40 mM sodium phosphate buffer (pH 6.5).

* Calculated as the percentage of base-released counts relative to those of SDS-insoluble PG isolated from cells following 2 h of incubation in the presence of antibiotics.

**FIG. 2. Detection of free amines in the spent culture medium of P. mirabilis.** Lyophilized preparations of spent culture medium were dansylated and hydrolyzed as described in Materials and Methods. Aliquots (25 µl) of the hydrolyzed preparations were injected onto a Beckman ODS Ultrasphere reverse-phase HPLC column (5 µm; 4.6 by 150 mm) previously equilibrated in 14% acetonitrile in 25 mM sodium phosphate–25 mM sodium acetate (pH 6.8). The column was eluted with a gradient of acetonitrile (14 to 22% over 5 min, 22 to 34% over 10 min, and finally 34 to 62% over 22 min), and the effluent was monitored at 254 nm. (a) Dansylated and hydrolyzed spent culture medium; (b) the same sample of spent culture medium but treated with glutamate-pyruvate transaminase for 2 h at 37°C prior to dansylation and hydrolysis. Retention times for DNS-Glu, DNS-GlcN, DNS-Ala, DNS-DAP, DNS-NH2, and (DNS)2-DAP are 30.1, 36.7, 38.1, 51.2, 58.1, and 58.6 min, respectively.
TABLE 4. Amino acid and amino sugar composition of *P. mirabilis* spent culture medium

<table>
<thead>
<tr>
<th>Sample Treatment</th>
<th>DNS-Ala</th>
<th>DNS-Glu</th>
<th>DNS-GlcN</th>
<th>DNS-DAP</th>
<th>(DNS)-DAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unhydrolyzed</td>
<td>13.9</td>
<td>ND</td>
<td>2.93</td>
<td>0.222</td>
<td></td>
</tr>
<tr>
<td>Hydrolyzed 4 M HCl</td>
<td>14.33 (1.75)</td>
<td>0.0960 (0.031)</td>
<td>2.59 (0.245)</td>
<td>2.78 (0.0695)</td>
<td>0.231 (0.0178)</td>
</tr>
<tr>
<td>Hydrolyzed 6 M HCl</td>
<td>13.27 (1.25)</td>
<td>ND</td>
<td>3.75</td>
<td>2.32 (0.156)</td>
<td>0.92 (0.0304)</td>
</tr>
</tbody>
</table>

*Lyophilized spent culture supernatant was dansylated prior to hydrolysis in HCl at 110°C for 16 h. The dansylated amines were separated and quantitated by reverse-phase HPLC (see Materials and Methods). ND, Not determined. Values in parentheses are standard deviations.*


