

## Characterization of Gentamicin 2'-N-Acetyltransferase from *Providencia stuartii*: Its Use of Peptidoglycan Metabolites for Acetylation of Both Aminoglycosides and Peptidoglycan

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The relationship between the acetylation of peptidoglycan and that of aminoglycosides in *Providencia stuartii* has been investigated both in vivo and in vitro. Adaptation of the assay for peptidoglycan *N*→*O*-acetyltransferase permitted an investigation of the use of peptidoglycan as a source of acetate for the *N* acetylation of aminoglycosides by gentamicin *N*-acetyltransferase [EC 2.3.1.59; AAC(2')]. The peptidoglycan from cells of *P. stuartii* PR50 was prelabelled with <sup>3</sup>H by growth in the presence of *N*-[acetyl-<sup>3</sup>H]glucosamine. Under these conditions, [<sup>3</sup>H]acetate was confirmed to be transferred to the C-6 position of peptidoglycan-bound *N*-acetylmuramyl residues. Isolated cells were subsequently incubated in the presence of various concentrations of gentamicin and tobramycin (0 to 5× MIC). Analysis of various cellular fractions from isolated cells and spent culture medium by the aminoglycoside-binding phosphocellulose paper assay revealed increasing levels of radioactivity associated with the filters used for whole-cell sonicates of cells treated with gentamicin up to 2× MIC. Beyond this concentration, a decrease in radioactivity was observed, consistent with the onset of cell lysis. Similar results were obtained with tobramycin, but the increasing trend was less obvious. The transfer of radiolabel to either aminoglycoside was not observed with *P. stuartii* PR100, a strain that is devoid of AAC(2')-Ia. A high-performance anion-exchange chromatography-based method was established to further characterize the AAC(2')-Ia-catalyzed acetylation of aminoglycosides. The high-performance liquid chromatography (HPLC)-based method resolved a tobramycin preparation into two peaks, both of which were collected and confirmed by <sup>1</sup>H nuclear magnetic resonance to be the antibiotic. Authentic standards of 2'-*N*-acetyltobramycin were prepared and were well separated from the parent antibiotic when subjected to the HPLC analysis. By applying this technique, the transfer of radiolabelled acetate from the cell wall polymer peptidoglycan to tobramycin was confirmed. In addition, isolated and purified AAC(2')-Ia was shown to catalyze in vitro the transfer of acetate from acetyl-coenzyme A, soluble fragments of peptidoglycan, and *N*-acetylglucosamine to tobramycin. These data further support the proposal that AAC(2')-Ia from *P. stuartii* may have a physiological role in its secondary metabolism and that its activity on aminoglycosides is simply fortuitous.

Bacterial resistance to aminoglycosides is usually conferred by one or more of three different classes of modifying enzymes. The free hydroxyl groups of various aminoglycosides can be either phosphorylated by *O*-phosphotransferases or adenylated by *O*-adenyltransferases, while the free amino groups serve as the substrate for *N*-acetyltransferases (5). The latter enzymes comprise four distinct classes of *N*-acetyltransferases, designated AAC(1), AAC(2'), AAC(3), and AAC(6') based upon the site of acetylation of the streptomycin core of the aminoglycoside antibiotics. Each enzyme of the respective four classes is both species and substrate specific (for a recent review, see the work of Shaw et al. [36]). Most genes encoding these enzymes are plasmid borne, but one of the two aminoglycoside resistance factors in *Providencia stuartii*, a gentamicin 2'-*N*-acetyltransferase (EC 2.3.1.59), is chromosomally encoded (7, 27, 28, 47). This enzyme appears to be ubiquitous in *P. stuartii* regardless of the aminoglycoside resistance phenotype (28, 34). The *aac*(2')-Ia gene is normally expressed at low levels, but high-level expression confers resistance to the clinically important

aminoglycosides, gentamicin, tobramycin, sisomicin, netilmicin, and 6'-*N*-ethylnetilmicin (34).

Expression of the *aac*(2')-Ia gene is controlled by at least two *trans*-acting negative regulators (33, 34). The atypical nature of the genetic regulation of AAC(2')-Ia production prompted investigations into a potential physiological function of the enzyme. Mutant strains that either over- or underexpress *aac*(2')-Ia have been shown to have altered cell morphologies (30, 33, 34) in addition to differences in extents of peptidoglycan *O* acetylation (30). Thus, previous investigations have shown that AAC(2')-Ia contributes to, but is not solely responsible for, the *O* acetylation of peptidoglycan in *P. stuartii* (30).

The *O* acetylation of peptidoglycan occurs in a number of important human pathogens, including *Staphylococcus aureus*, *Neisseria gonorrhoeae*, and each genus of the tribe *Proteeae* (*Providencia*, *Proteus*, and *Morganella*) (8, 10). This modification to the C-6 hydroxyl groups of *N*-acetylmuramyl (MurNAc) residues confers resistance to hydrolysis by muramidases, including human milk and hen egg white lysozymes (12, 35). The extent of peptidoglycan *O* acetylation varies naturally between approximately 20 and 70% (8, 10), and muramidase resistance has been shown to be proportional to the level of this modification (12, 35). Little is known about the pathway for the *O* acetylation of peptidoglycan, but substantial evidence indicates that it follows the formation (transpeptidation) of peptide cross-links in peptidoglycan (for a review, see reference 10).

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On the basis of radiotracer studies, an *N*→*O*-acetyltransferase has been proposed to catalyze the transfer of acetyl groups from the N-2 position of either *N*-acetylglucosaminyl (GlcNAc) or MurNAc residues to the C-6 position of the latter during the turnover of peptidoglycan (Fig. 1, reaction 1) (13, 14).

In the present study, the role of *O*-acetylated peptidoglycan and its metabolic subunits serving as a reservoir of acetyl moieties for the AAC(2')-Ia-catalyzed acetylation of aminoglycosides has been investigated. The radioassay for the peptidoglycan *N*→*O*-acetyltransferase was employed to follow the transfer of radiolabelled acetate *in vivo*. In addition, a method for aminoglycoside analysis based on high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was employed to monitor the transfer *in vitro* of acetate from different sources to tobramycin by a purified preparation of AAC(2')-Ia.

## MATERIALS AND METHODS

**Chemicals.** EDTA, morpholinepropanesulfonic acid (MOPS), mutanolysin, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co., St. Louis, Mo. Tryptone, yeast extract, and Bacto Agar were obtained from Difco Laboratories, Detroit, Mich. Gentamicin sulfate, tobramycin, and *N*-[acetyl-<sup>3</sup>H]acetyl-D-glucosamine were obtained from ICN Biomedicals, Montreal, Quebec, Canada, while *N*-[1-<sup>3</sup>H]acetyl-D-glucosamine and *N*-[1,6-<sup>3</sup>H]acetyl-D-glucosamine were purchased from Amersham International, Amersham, United Kingdom, and American Radiolabeled Chemicals, Inc., St. Louis, Mo., respectively. Liquiscint was obtained from National Diagnostics, Atlanta, Ga. Affi-Gel 10 was purchased from Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario, Canada. All other chemicals and reagents were obtained from Fisher Scientific, Toronto, Ontario, Canada, and were of reagent grade or better. Deionized, charcoal-filtered water was supplied by an on-line Milli-Q purifying system (Millipore Corp., Bedford, Mass.).

**Bacterial strains.** *P. stuartii* PR50, PR51, and PR100 (34) were maintained on Luria-Bertani (LB) agar plates at 4°C with passage onto fresh plates every 3 weeks. Stock cultures were stored at -70°C in the presence of 50% glycerol and recovered as required.

**Radiolabelling of O-acetylated peptidoglycan.** LB medium (200 ml) supplemented with dextrose (100 mM final concentration) and *N*-acetyl-D-glucosamine (100 mM final concentration) was inoculated and incubated at 37°C, until growth reached an optical density at 600 nm (OD<sub>600</sub>) of 0.2 to 0.3. At this stage (early exponential phase), 30 μCi of the radiolabelled compound of interest ([acetyl-<sup>3</sup>H]GlcNAc [4.5 Ci · mmol<sup>-1</sup>] or [1-<sup>3</sup>H]GlcNAc [5.6 Ci · mmol<sup>-1</sup>]) was added, and growth was allowed to continue until an OD<sub>600</sub> of 0.6 to 0.8 was obtained (late exponential phase).

**Isolation of O-acetylated peptidoglycan.** Cells were resuspended in 50 ml of 50 mM sodium phosphate buffer, pH 6.5, at 4°C. Insoluble peptidoglycan was isolated from cells by employing the boiling SDS procedure described by Dupont and Clarke (12). SDS-insoluble peptidoglycan was recovered by ultracentrifugation at 160,000 × *g* for 50 min at 25°C. The insoluble material was washed twice with 50 mM sodium phosphate buffer, pH 6.5, and recovered via ultracentrifugation with an Airfuge ultracentrifuge [Beckman Instruments (Canada) Inc., Mississauga, Ontario, Canada] at 160,000 × *g* for 15 min at room temperature. The pellet was then used immediately as required.

**Solubilization of radiolabelled O-acetylated peptidoglycan.** To test the efficiency of the above radiolabelling procedure (counting efficiency is enhanced by prior solubilization) or to prepare *O*-acetylated peptidoglycan as an acetate source in the *in vitro* assay, peptidoglycan was isolated (see above) and solubilized by incubation in 200 μl of 500 U of mutanolysin ml<sup>-1</sup> in 100 mM sodium phosphate buffer, pH 6.8, for 4 h. Aliquots of these digestion reactions were used in the *in vitro* experimental section or mixed with 5 ml of Liquiscint scintillation cocktail and counted with a liquid scintillation analyzer (Tri-Carb 2000; Canberra-Packard Ltd., Mississauga, Ontario, Canada) to determine the extent of label associated with *O*-acetylated peptidoglycan.

**Release and quantitation of O-acetyl groups from peptidoglycan.** Quantitation of radiolabelled and nonradiolabelled *O*-linked acetate was achieved by mild base hydrolysis of SDS-insoluble peptidoglycan as previously described (12). Briefly, SDS-insoluble peptidoglycan was incubated in the presence of 450 μl of 50 mM NaOH (final concentration) for 2 h at room temperature. The mild base-treated peptidoglycan was sedimented by centrifugation with an Airfuge ultracentrifuge at 160,000 × *g* for 15 min at room temperature. The supernatant was collected and analyzed (or stored at 4°C for later analysis) for acetate by injection onto a 7.8- by 300-mm HPX-87H organic acid high-performance liquid chromatography (HPLC) column [Bio-Rad Laboratories (Canada) Inc.] at 35°C with 10 mM sulfuric acid serving as eluent at a flow rate of 0.6 ml · min<sup>-1</sup>. Acetate was detected by monitoring the *A*<sub>210</sub> of the column effluent. In radiolabel

studies, fractions eluting at 15.9 min (acetate) were collected and counted for levels of radioactivity by liquid scintillation counting.

**Assay of aminoglycoside acetylation *in vivo*.** Radiolabelled peptidoglycan from *P. stuartii* PR50 was obtained as previously described (cells grown in LB broth containing 30 μCi of either [acetyl-<sup>3</sup>H]GlcNAc or [1-<sup>3</sup>H]GlcNAc). Cells containing radiolabelled peptidoglycan, once harvested, were resuspended in 6 ml of 50 mM sodium phosphate buffer, pH 6.5, at 4°C and divided into four or six aliquots (as required for the experiment). The cell suspensions were immediately harvested at 3,200 × *g* (Biofuge A) at 4°C for 20 min. The cells were resuspended in the same buffer and incubated with varying concentrations of aminoglycoside (gentamicin or tobramycin) ranging from 0 to 300 μg · ml<sup>-1</sup> for 2 h at 37°C. Following the incubation, cells were recovered by centrifugation (14,900 × *g*, 20 min, 4°C), and the supernatant was stored at -20°C until required, while the cells were resuspended in 1 ml of the phosphate buffer and used as required.

A modified phosphocellulose paper binding assay, originally developed by Benveniste and Davies (4), was used to measure the transfer of radiolabel from a suitable cofactor to the antibiotic (aminoglycoside). Briefly, volumes of the desired fraction(s) were filtered through the aminoglycoside-binding phosphocellulose P81 paper (Whatman Laboratories Ltd., Maidstone, England) and dried. The filters were then washed with 10 ml of dH<sub>2</sub>O, dried, and counted for radioactivity by placing the filter paper into 5 ml of scintillation cocktail.

**Isolation and purification of AAC(2')-Ia.** A 2-liter culture of *P. stuartii* PR51, a strain that overproduces AAC(2')-Ia (34), was grown to late exponential phase (OD<sub>600</sub> of 0.6 to 0.8) in LB broth at 37°C. The cells were harvested by centrifugation (8,000 × *g*, 4°C, 15 min), and the cell paste was resuspended in 30 mM Tris-HCl, pH 8.0, containing 20% sucrose and 1 mM EDTA and subjected to osmotic shock (29). The resulting supernatant containing the released crude enzyme preparation was stored at -20°C until required.

AAC(2')-Ia was partially purified by affinity chromatography on gentamicin-Affi-Gel 10 by the procedure of Williams and Northrop (46) with modification. Briefly, the crude enzyme preparation (20 ml) was applied to the affinity matrix in 10 mM Tris-HCl, pH 7.8, containing 150 mM EDTA. The column was washed extensively with the same buffer until the *A*<sub>280</sub> of the eluate fell below 0.01, and then the bound enzyme(s) was eluted with the application of 1 M NaCl in 10 mM Tris-HCl, pH 4.5. Fractions (1.5 ml) containing protein were dialyzed against water at 4°C (two times, 2 liters, 16 h) and concentrated twofold by osmosis with polyethylene glycol 8000 (Carbowax). Analysis of this enzyme preparation by SDS-polyacrylamide gel electrophoresis revealed a major band with an apparent *M*<sub>r</sub> of 23,700. This is close to the *M*<sub>r</sub> of 20,073 as predicted from the open reading frame of *aac*(2')-Ia (34). Enzyme preparations were used immediately or stored at -20°C until required.

**HPLC-based assay for AAC activity.** The acetylation of tobramycin by AAC(2')-Ia was assayed by HPAEC-PAD by a modified method of Statler (42). Briefly, the sample(s) was resolved by the HPAEC separation under alkaline conditions, which provided an analytical method with sensitivity equal to or better than that of current HPLC methods that employ postcolumn derivatization (42). The HPAEC-PAD was performed with a Dionex series 4500 HPLC system equipped with a pulsed electrochemical detector, an advanced computer interface with a 486 computer, and a Bio-Rad model AS-100 HPLC autosampler. Samples were injected onto a CarboPac PA1 analytical column, previously equilibrated with 7.5 mM NaOH at 1 ml · min<sup>-1</sup>. A linear gradient to 50 mM NaOH over 10 min was immediately applied, and after standing at 50 mM NaOH for 2 min, the column was washed with 1 M Na-acetate in 150 mM NaOH for 8 min before reequilibration in starting buffer. The PAD detector settings were 0.13 V (E<sub>1</sub>), 0.6 V (E<sub>2</sub>), and -0.6 V (E<sub>3</sub>) with 500-, 100-, and 50-ms applied durations, respectively. Integration was performed over 500 ms beginning at 300 ms, and the response time was set at 200 ms. The postcolumn reagent, 300 mM NaOH, at 1 ml · min<sup>-1</sup> was used to raise the pH and ionic strength to levels required for sensitive detection. Aminoglycosides and potentially modified aminoglycosides eluting from the column were collected and counted for levels of radioactivity. In some cases, postcolumn ninhydrin detection was performed with a Beckman model 232 postcolumn reactor [Beckman Instruments (Canada) Ltd.] and Trione (ninhydrin) reagent (Pickering Laboratories, Mountain View, Calif.). The resulting eluents were monitored by visible *A*<sub>570</sub> with a Beckman model 166 UV/Vis detector.

**Compositional analysis of tobramycin.** The compositional analysis of tobramycin was performed by an HPAEC-PAD procedure, as previously described (9), following hydrolysis in 4 M HCl at 95°C for 18 h, *in vacuo*.

**NMR spectroscopy.** <sup>1</sup>H nuclear magnetic resonance (NMR) spectra were recorded with a Varian Unity AM-400 spectrometer at 400 MHz and are expressed in parts per million relative to 3-(trimethylsilyl)propionic-2,2,3,2-d<sub>4</sub> acid as an internal standard. Isolated samples were taken up in and dried from D<sub>2</sub>O at least twice prior to analysis in D<sub>2</sub>O.

**Preparation of 2'-N-acetyltobramycin by AAC(2')-Ia.** A 750-μl sample of purified AAC(2')-Ia in 10 mM Tris-HCl, pH 5.5, containing 15 mM EDTA was mixed with 2.5 mg (3.2 μmol) of tobramycin (added in four aliquots over 60 min) and [acetyl-<sup>3</sup>H]acetyl-coenzyme A (CoA) (0.23 Ci · mol<sup>-1</sup>) in a total volume of 950 μl. After incubation for 60 min at 37°C, the mixture was stored at -20°C until required for HPAEC analysis.

The acetylation of tobramycin by AAC(2')-Ia with other potential sources of acetate donor was investigated. Radiolabelled and mutanolysin-digested, *O*-acetylated peptidoglycan was prepared as described above, and 70- to 95-μg

TABLE 1. Assay for peptidoglycan *N*→*O*-acetyltransferase activity in *P. stuartii*

<i>P. stuartii</i> strain	Amt of radiolabelled material released from isolated peptidoglycan after incubation with <sup>a</sup> :					
	Buffer <sup>b</sup>		Base <sup>c</sup>			
	cpm	% of total	Total		As acetate <sup>d</sup>	
	cpm	% of total	cpm	% of total	cpm	% of total
PR50 (wild type)	88	1.8	1,990	39	880	17
PR100 [ <i>aac</i> (2')-Ia mutant]	10	0.08	3,510	28	640	5.1

<sup>a</sup> *n* = 2.<sup>b</sup> 50 mM sodium phosphate, pH 6.5.<sup>c</sup> 50 mM sodium hydroxide.<sup>d</sup> Isolated by HPLC.

samples were added to 100  $\mu$ l of the AAC(2')-Ia enzyme preparation in 500  $\mu$ l of 50 mM sodium phosphate buffer, pH 6.8. Tobramycin (2.5 mg) was added in four equal aliquots over 60 min and incubated at 37°C with shaking for a further hour. It was then stored at -20°C until required for HPAEC analysis. In other experiments, the radiolabelled peptidoglycan was replaced with 10  $\mu$ Ci of either [*acetyl*-<sup>3</sup>H]GlcNAc (1.03 Ci  $\cdot$  mol<sup>-1</sup>) or [1,6-<sup>3</sup>H]GlcNAc (60 Ci  $\cdot$  mol<sup>-1</sup>). For each of the above experiments, control reactions in which reaction mixtures were prepared and treated as described above except for the exclusion of the enzyme preparation were performed.

## RESULTS

**O acetylation of *P. stuartii* PR50 and PR100.** The peptidoglycan of *P. stuartii* PR50 and PR100 cells was labelled with [<sup>3</sup>H]acetate by culturing the two strains in LB broth containing [*acetyl*-<sup>3</sup>H]GlcNAc. The peptidoglycan was isolated from harvested cells, and the ester-linked (*viz.*, O-linked) acetate was

released by mild base hydrolysis. Approximately 39% of the total radioactivity associated with the peptidoglycan isolated from cells of strain PR50 was recovered in the supernatants of these mild base hydrolysates (Table 1). This radioactive supernatant was subjected to organic acid analysis by HPLC, and the peak eluting as acetic acid was collected. Scintillation counting of the recovered acetate showed it to be radioactive, and the levels accounted for almost half of the radioactivity associated with the base hydrolysate, or 17% of the total radioactivity associated with the original peptidoglycan sample. The remainder of the radioactivity in the supernatant of the mild base hydrolysate is likely associated with the *N*-acetyl moieties of the amino sugars of small peptidoglycan fragments that are solubilized by the base treatment, as observed with *Proteus mirabilis* (13). Buffer treatment (negative control) of the ra-

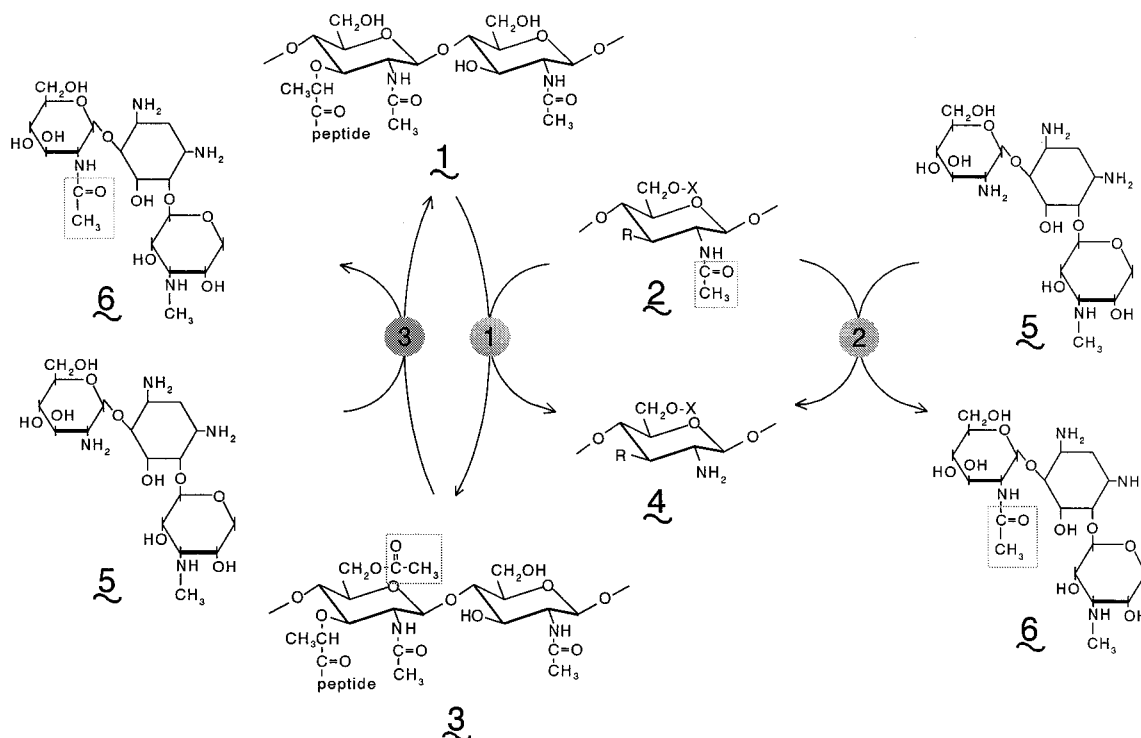


FIG. 1. Proposed pathway for the acetylation of both peptidoglycan and aminoglycosides by AAC(2')-Ia. (Reaction 1) The O acetylation of peptidoglycan (1) has previously been shown to involve the transfer of acetate from the *N*-acetylmuramyl or *N*-acetylglucosaminyl residues of peptidoglycan (2), which may or may not be previously O acetylated (X = H or acetate), to the C-6 position of muramyl residues (3), resulting in the formation of the free amine (4), which subsequently appears in the medium. R = lactyl ether peptide. (Reactions 2 and 3) AAC(2')-Ia is also postulated to catalyze this reaction or, in the presence of aminoglycosides (5, gentamicin), transfer the N-linked (2) (reaction 2) and/or O-linked (3) (reaction 3) acetate to the N-2 position of the antibiotic (6).



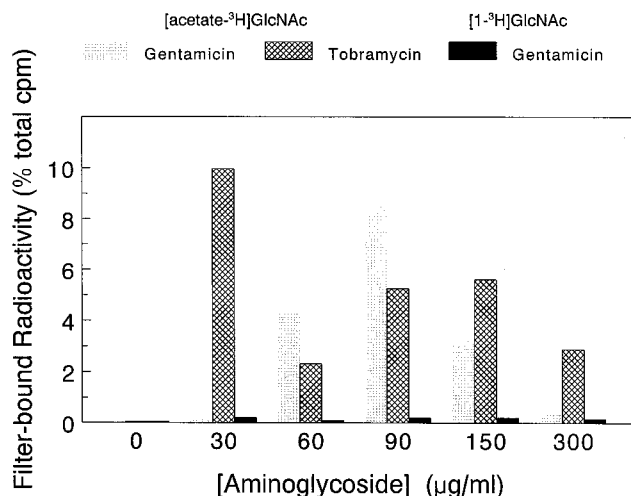


FIG. 2. Filter paper assay of aminoglycoside acetylation using radiolabelled peptidoglycan as the acetate donor. Cells of *P. stuartii* PR50 containing peptidoglycan prelabelled with either [*acetate*-<sup>3</sup>H]GlcNAc or [<sup>1</sup>-<sup>3</sup>H]GlcNAc were incubated with varying concentrations of aminoglycoside for 2 h at 37°C and then were recovered by centrifugation. The cells were ruptured by sonication, insoluble debris was removed by centrifugation, and volumes of the supernatant were filtered through the aminoglycoside-binding phosphocellulose P81 paper. The filters were washed with 10 ml of dH<sub>2</sub>O, dried, and subjected to scintillation counting. The results are presented as a percentage of the total radioactivity associated with the entire cellular fraction.

radioactive peptidoglycan released less than 2% of the total radioactivity into the supernatant, and no acetate was detected in these samples. Similar results were obtained with *P. stuartii* PR100, a mutant with a frameshift mutation in *aac*(2')-Ia, but the overall levels of peptidoglycan O acetylation were lower (Table 1). These data suggest that the process of peptidoglycan O acetylation in *P. stuartii* is the same as with *P. mirabilis*, involving the putative peptidoglycan N→O-acetyltransferase in transferring acetate from the N-2 position of either GlcNAc or MurNAc to the C-6 position of the latter moiety within the peptidoglycan sacculus (Fig. 1, reaction 1).

**Peptidoglycan as a source of acetate for aminoglycoside acetylation.** AAC(2')-Ia has previously been shown to contribute to the O acetylation of peptidoglycan in *P. stuartii* (30, 31). In light of the data presented above, we wanted to investigate whether this enzyme could utilize the acetate associated with peptidoglycan for the acetylation of aminoglycoside antibiotics in vivo. To determine if the acetate from O-acetylated peptidoglycan or one of its components, GlcNAc, could serve as the acetate source for aminoglycoside modification, *P. stuartii* PR50 cells with specifically radiolabelled peptidoglycan were incubated with varying concentrations of either gentamicin or tobramycin. The aminoglycosides were subsequently recovered from various cellular fractions and assayed for radioactive acetate by two independent methods.

**Phosphocellulose binding assay.** The aminoglycosides present in the supernatants of sonicated *P. stuartii* cells prelabelled in their peptidoglycan with [*acetyl*-<sup>3</sup>H]GlcNAc were recovered by filtration through the cation-exchange P81 phosphocellulose filter paper. The total counts for all radiolabel associated with the phosphocellulose paper relative to the total radiolabel associated with the samples of the intact cells are presented in Fig. 2. These data indicated that, as the concentration of gentamicin incubated with the cells increases, so does the radioactivity associated with the cation-exchange filter papers. This trend was followed to concentrations of approxi-

TABLE 2. Phosphocellulose P81 filter paper assay for acetylation of tobramycin using [*acetyl*-<sup>3</sup>H]GlcNAc as acetate source<sup>a</sup>

<i>P. stuartii</i> strain	Radioactivity (cpm) associated with:			
	Total sample <sup>b</sup>	Phosphocellulose filters		
		Total bound <sup>c</sup>	NaOH wash <sup>d</sup>	
		Released	Bound	
PR50	103,200	7,570	4,230	1,280
PR100	76,900	145	0	13

<sup>a</sup> Cells, prepared from cultures incubated in the presence of [*acetyl*-<sup>3</sup>H]GlcNAc, were incubated with 50 µg of tobramycin ml<sup>-1</sup>.

<sup>b</sup> Supernatant of cells incubated with tobramycin after isolation, washing, and sonication.

<sup>c</sup> Associated radioactivity after filtration of supernatant of sonicated cells.

<sup>d</sup> Filters were incubated in 5 mM NaOH for 60 min at ambient temperature.

mately 90 µg · ml<sup>-1</sup>. However, beyond this concentration, the levels of radioactivity associated with the filters decreased. For tobramycin, this trend was not observed, but label was still found to be associated with the ion-exchange filters. In an attempt to control for the possibility that the labelled acetate was transferred following the catabolism of the supplemented peptidoglycan precursors, experiments were repeated but with non-acetate-labelled GlcNAc. Thus, [<sup>1</sup>-<sup>3</sup>H]GlcNAc was incorporated into the peptidoglycan of cells as described in Materials and Methods. Results with [<sup>1</sup>-<sup>3</sup>H]GlcNAc for these radiotracer studies indicated that no radiolabel was found associated with the filter papers (Fig. 2).

To confirm the specific role of AAC(2')-Ia in the transfer of radiolabel from peptidoglycan to aminoglycoside, experiments similar to those described above were repeated with tobramycin with both the wild-type PR50 strain and an isogenic mutant strain, PR100 [frameshift mutation in *aac*(2')-Ia]. Radioactivity remained associated only with the phosphocellulose filters treated with tobramycin-containing fractions isolated from *P. stuartii* PR50, i.e., negligible radiolabel was associated with the filter papers when PR100 was used (Table 2). With the wild-type strain PR50, approximately 60% of the bound radioactivity could be recovered from the cation-exchange filters by elution with 5 mM NaOH (Table 2).

**HPAEC assay.** The HPAEC-PAD method was adopted as a means to more directly assay and characterize the acetylation of aminoglycosides. As shown in Fig. 3A, HPAEC effected a clear separation of GlcNAc from two other distinct peaks. Only these latter peaks were detected with the ninhydrin-based postcolumn detector (*A*<sub>570</sub>), indicating the presence of free amines. Confirmation that the ninhydrin-positive peaks of Fig. 3A resulted from the resolution of a tobramycin complex, presumably originating from the nebramycin source, was obtained by both <sup>1</sup>H NMR spectroscopy and compositional analysis. Thus, the <sup>1</sup>H NMR spectra obtained with the material collected in the two ninhydrin-positive peaks were identical to those of authentic tobramycin (Fig. 4), with the exception of the C-1' and C-1' proton resonances at δ 5.08 and δ 5.17, respectively, which were masked by the large water peak. Compositional analysis by HPAEC-PAD of tobramycin following acid hydrolysis revealed major components, one of which was identified as glucosamine (data not shown). Analogous chromatographic profiles were obtained with the acid hydrolysates of the material collected in the putative tobramycin fractions of Fig. 3A.

Prelabelled cells were incubated in the presence of tobramycin and then sonicated to release cytoplasmic material. Supernatants from these preparations were injected onto the

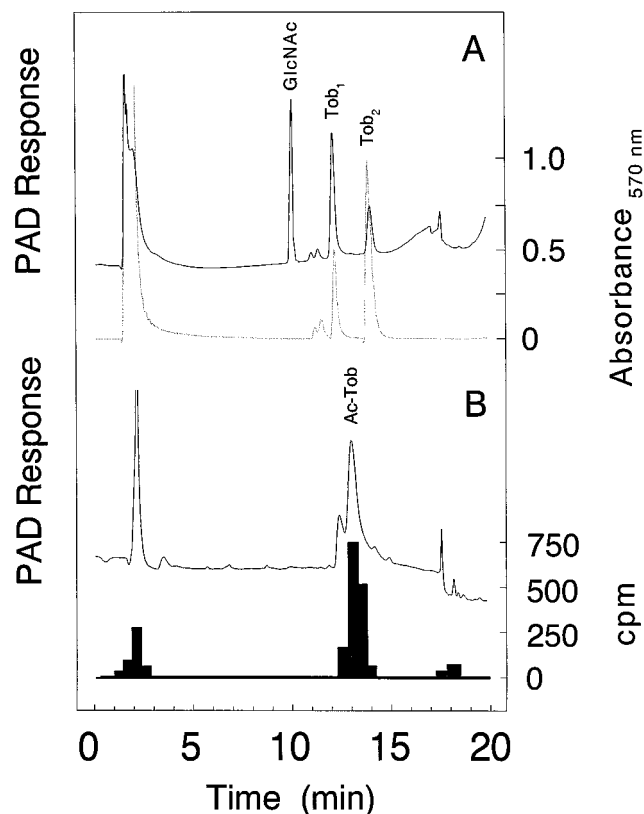


FIG. 3. HPAEC of tobramycin. Samples were injected in 100  $\mu$ l onto a CarboPac PA1 column, previously equilibrated with 5 mM NaOH. A linear gradient to 50 mM NaOH at 1 ml  $\cdot$  min $^{-1}$  over 10 min was immediately applied, and the eluent was monitored by both pulsed-amperometric and ninhydrin detection. (A) Analysis of a mixture of tobramycin (Tob<sub>1</sub> and Tob<sub>2</sub>; 5  $\mu$ g) and GlcNAc (50  $\mu$ g). (B) HPAEC analysis of *in vivo* tobramycin acetylation. Cells with peptidoglycan prelabelled with [*acetyl*- $^3$ H]GlcNAc were incubated with tobramycin for 60 min. The cells were recovered by centrifugation and sonicated, and the supernatants of recentrifugation were applied to phosphocellulose paper. The bound cationic species were eluted with 5 mM NaOH and injected onto the CarboPac PA1 column. The peak eluting at 13.5 min was tentatively assigned as *N*-acetyltobramycin (Ac-Tob).

HPAEC column, and eluting peaks were collected for analysis by scintillation counting. Preliminary experiments were not conclusive, although radiolabelled peaks were detected in the chromatogram (data not shown).

The HPAEC-PAD-based experiments described above were modified to include a preisolation step with the aim of removing any residual radiotracer compound (*viz.*, [*acetyl*- $^3$ H]GlcNAc) and, possibly, peptidoglycan turnover products that retained the radioactivity. Thus, prior to injection onto the HPAEC column, cell sonicates were first filtered through the cation-exchange phosphocellulose P81 paper. The HPAEC profile of the filter paper eluent (Figure 3B) revealed the presence of tobramycin (retention times, 12.5 and 14.5 min) and a large radioactive peak with a retention time of 13.5 min. The radioactive peak was tentatively identified as the *N*-acetylated derivative of tobramycin.

**Purification and assay of AAC(2')-Ia *in vitro*.** AAC(2')-Ia was isolated from the periplasm of an overproducing strain of *P. stuartii*, PR51, and it was partially purified by affinity chromatography on gentamicin-Affi-Gel 10. The enzyme was recovered as the major protein with an apparent molecular mass of 23.7 kDa, as judged by SDS-polyacrylamide gel electro-

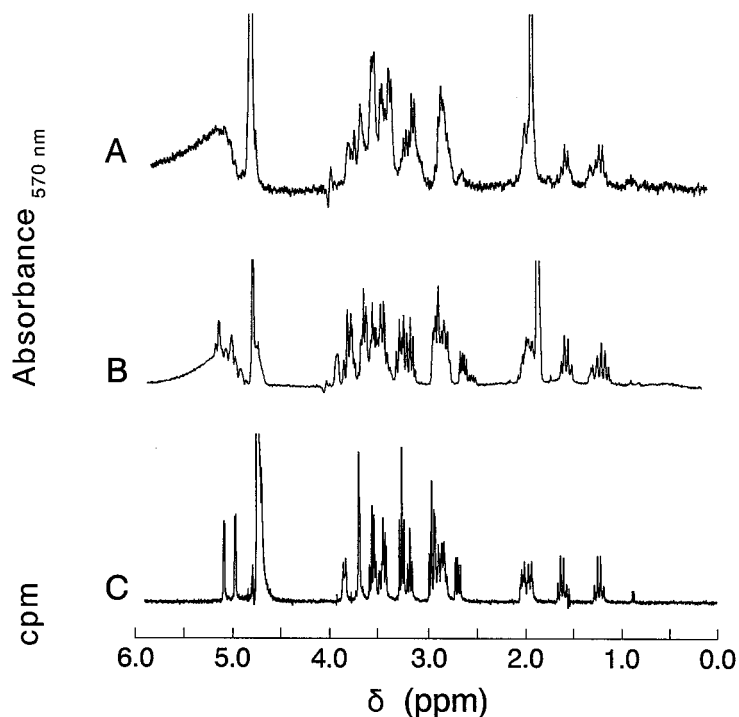


FIG. 4.  $^1$ H NMR spectrometric analysis of tobramycin. Spectra were obtained in D<sub>2</sub>O. (A) Authentic tobramycin (4 mg); (B and C) material recovered by HPAEC comprising peaks labelled Tob<sub>1</sub> and Tob<sub>2</sub>, respectively, from Fig. 3.

phoresis, which is close to the  $M_r$  of 20,073 as predicted from the open reading frame of *aac(2')-Ia* (34). Preliminary characterization of enzymatic activity involved incubation of the enzyme with tobramycin and its previously known cosubstrate, acetyl-CoA. Thus, enzyme preparations in 10 mM Tris-HCl, pH 5.5, containing 15 mM EDTA were incubated for a total of 2 h in the presence of both tobramycin and [*acetyl*- $^3$ H]acetyl-CoA. Aliquots were withdrawn during the incubation and subjected to HPAEC analysis, and the chromatograms obtained were compared to those obtained for untreated tobramycin. As shown in Fig. 5, two new peaks result with retention times approximately 0.7 min after the two tobramycin peaks. These two new peaks, which were found to increase in size with the concomitant decrease of the tobramycin peaks as the reaction proceeded over time, are consistent with the expected chromatographic behavior on an anion-exchange resin of cationic aminoglycosides with a modified (acetylated) amino functionality (*i.e.*, one less positive charge). Confirmation that the two new peaks at 13.5 and 15.2 min were the *N*-acetylated derivatives of tobramycin was obtained by scintillation counting of the eluents; both new peaks of Fig. 5B were found to contain  $^3$ H.

Given the apparent dual role of AAC(2')-Ia in acetylating both aminoglycosides and peptidoglycan (30, 31), studies were undertaken to identify other possible sources of acetate donor for the enzyme. The *in vivo* experiments described above suggested that peptidoglycan and its precursor GlcNAc may serve as sources of acetate in place of acetyl-CoA for the acetylation of aminoglycosides. Hence, the protocol for the production of 2'-*N*-acetyltobramycin was repeated as above, but the radiolabelled acetyl-CoA was substituted with [*acetyl*- $^3$ H]GlcNAc. To serve as a negative control for the transfer of radioactive acetate, [1,6- $^3$ H]GlcNAc was also tested. Preliminary examination

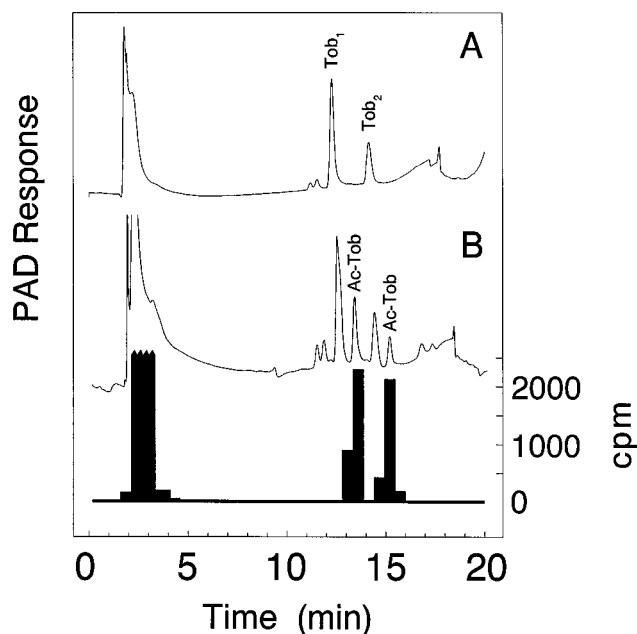


FIG. 5. HPAEC of 2'-N-[acetyl-<sup>3</sup>H]acetyltobramycin prepared by isolated AAC(2')-Ia. The acetylated tobramycin was prepared by incubation of the antibiotic with [acetyl-<sup>3</sup>H]acetyl-CoA and purified AAC(2')-Ia. The chromatographic conditions were the same as described in the legend to Fig. 3.

of the reaction products by HPAEC revealed considerable interference in the chromatographic profile which precluded the resolution of tobramycin and its derivative(s). This problem was circumvented by the extraction of the aminoglycoside from the reaction mixtures with cation-exchange phosphocellulose P81 paper prior to HPAEC. The treated filter papers were washed and then incubated with 5 mM NaOH to recover bound material for its injection onto the HPAEC column. Analysis of reaction mixtures incubated with [acetyl-<sup>3</sup>H]GlcNAc revealed the presence of a radiolabelled peak with a retention time of 13 min (Fig. 6A), which corresponded to *N*-acetyltobramycin. However, when the non-acetate-labelled GlcNAc was used ([1,6-<sup>3</sup>H]GlcNAc), the *N*-acetyltobramycin produced was not radiolabelled (Fig. 6B). As a further control for the assay, reaction mixtures incubated in the absence of either the radiolabelled GlcNAc or the enzyme preparation failed to produce *N*-acetyltobramycin (data not shown).

To test the possibility that O-acetylated peptidoglycan could, like GlcNAc, substitute for acetyl-CoA as a source of acetate, radiolabelled and mutanolysin-solubilized material in which the radiolabel was associated with acetyl moieties was prepared, as described in Materials and Methods. HPAEC analysis of reaction mixtures incubated for 1 h with this peptidoglycan preparation together with tobramycin and purified AAC(2')-Ia again indicated the formation of tritium-labelled 2'-*N*-acetyltobramycin (Fig. 6C). As above, control reaction mixtures devoid of either the peptidoglycan or the enzyme preparations did not contain the *N*-acetyltobramycin product (data not shown).

## DISCUSSION

The routine assay adopted for aminoglycoside modification involves the binding of the antibiotic to the cation-exchange phosphocellulose filter paper after incubation in the presence of the appropriate radiolabelled cosubstrate. For *N*-acetyl-

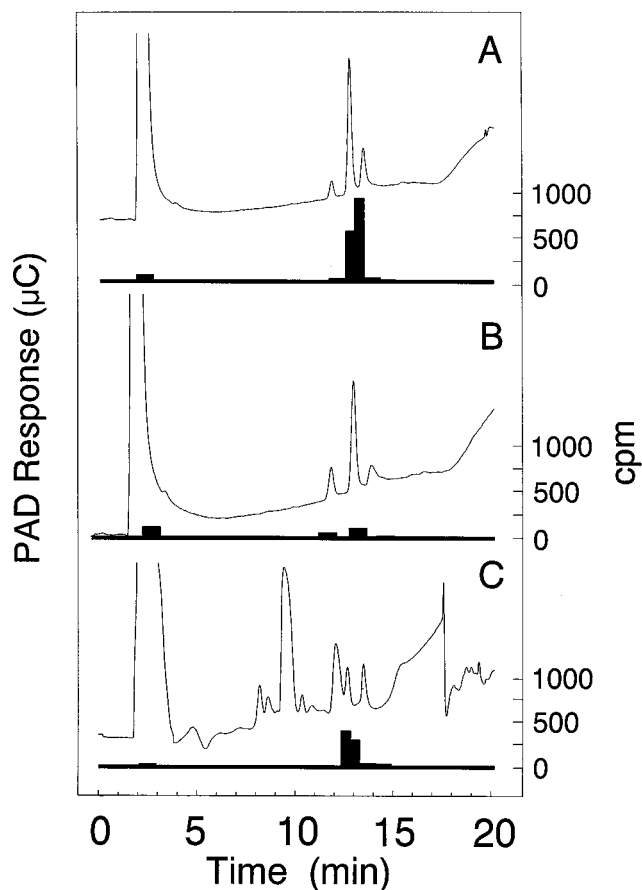


FIG. 6. In vitro acetylation of tobramycin by purified *P. stuartii* AAC(2')-Ia. The enzyme and tobramycin were incubated in the presence of [acetyl-<sup>3</sup>H]GlcNAc (A), [1,6-<sup>3</sup>H]GlcNAc (B), and mutanolysin-digested O-acetylated peptidoglycan containing [<sup>3</sup>H]acetate moieties (C). After centrifugation, reaction mixture supernatants were subjected to HPAEC analysis (conditions described in the legend to Fig. 3) followed by scintillation counting of collected fractions.

transferases, the employed cosubstrate is typically [acetyl-<sup>14</sup>C]CoA, but its substitution with either [acetyl-<sup>3</sup>H]GlcNAc or radiolabelled peptidoglycan fragments (specifically labelled at the N-2 and/or O-6 position of both *N*-acetylglucosaminyl and *N*-acetylmuramyl residues) led to the recovery of radioactive filters. This activity of AAC(2')-Ia from *P. stuartii* was observed both in vivo and in vitro. In the case of the in vivo reactions, the reaction was dependent upon the concentration of gentamicin up to 90 μg · ml<sup>-1</sup> (2× MIC). Above this level, the added gentamicin was thought to induce autolysis (23) and consequently to result in the disruption of the acetyl transfer pathway. Application of an HPAEC protocol for the analysis of aminoglycosides, previously developed by Statler (42), combined with <sup>1</sup>H NMR spectroscopy permitted the identification of the radiolabelled product as the acetylated antibiotic. These assays were conducted with gentamicin and tobramycin, and it is assumed that the other aminoglycosides recognized by the *P. stuartii* AAC(2')-Ia (i.e., sisomicin, paromycin, neomycin B, and kanamycin B and C [4, 36]) would also accept acetate from peptidoglycan or its components.

The apparent ability of AAC(2')-Ia to utilize different acetate sources that are structurally unrelated (viz., acetyl-CoA and peptidoglycan components) for the acetylation of aminoglycosides in addition to its activity as a peptidoglycan O-

acetyltransferase (30, 31) may seem very unusual. However, Udou and coworkers (43) have previously demonstrated that the AAC(2') from *Mycobacterium fortuitum* possesses affinity for compounds other than aminoglycosides. Among other compounds, several sugars relevant to this study (e.g., GlcNAc, MurNAc, galactosamine, and trehalose) were found to be inhibitory toward the acetylation of tobramycin in vitro.

Taken together, the observations discussed above support the pathway for the AAC(2')-Ia-catalyzed acetylation of both peptidoglycan and aminoglycosides as presented in Fig. 1. As previously determined for *P. mirabilis* (13, 14) and established in this study for *P. stuartii*, acetate from the *N*-acetylglucosaminyl and/or *N*-acetylmuramyl residues of peptidoglycan is transferred to the C-6 position of the muramyl residues by a membrane-associated acetyltransferase (reaction 1). With *P. stuartii*, certain aminoglycosides compete for this acetate as it may be transferred directly to their 2'-*N* position (reaction 2). At this time, the possibility that the ester-linked acetate may be transferred from the C-6 muramyl residues of pre-O-acetylated peptidoglycan to aminoglycoside (reaction 3) cannot be excluded. This latter point may be resolved by further radiotracer studies involving an in vitro peptidoglycan biosynthesis assay. Regardless, reaction 2 or 3 accounts for the observed acetylation of aminoglycoside with the concomitant decrease in peptidoglycan O acetylation (30, 31).

In general, very little is known about the enzymology of the aminoglycoside-modifying enzymes. Indeed, only one aminoglycoside, 2'-*N*-acetyltransferase, has been isolated and partially characterized (4). Unlike a number of other acetyltransferases, which comprise a family of integral membrane proteins (39), the site of action of aminoglycoside-modifying enzymes, intracellular or periplasmic, is still in question. The amikacin 3'-phosphotransferase of *Escherichia coli* has been suggested to be located in the cytoplasm (32), whereas both the gentamicin 3'-*N*-acetyltransferase (46) and a streptomycin-spectinomycin adenylyltransferase of *E. coli* (22) have been reported to be periplasmically located. This latter situation appears to be more reasonable since an efficient mechanism of resistance would involve the modification of an aminoglycoside before it enters the cytoplasm. Analysis of the amino-terminal sequences of a number of aminoglycoside-modifying enzymes has identified prominent signal sequences, particularly among the AAC(3) and AAC(6') enzymes (17, 36). In addition, the alginate acetyltransferase gene, *algF* (15, 37), encodes a leader sequence (37), implying that this enzyme, which O acetylates the mannuronate residues of alginate, also functions in the periplasm of *Pseudomonas aeruginosa*. That the *P. stuartii* AAC(2')-Ia was both isolated by osmotic shock and found to catalyze the transfer of acetate from either peptidoglycan or GlcNAc for both the N acetylation of aminoglycosides (this study) and the O acetylation of peptidoglycan (30) strongly suggests its localization within the periplasm. In support of this view, secondary structure predictions by the method of Klein et al. (24) indicate the AAC(2')-Ia to be a peripheral membrane protein. This algorithm was proven to be relatively reliable, as we used it to successfully predict that the *E. coli* penicillin-binding protein 5 is also a peripheral membrane protein. Thus, as with the penicillin-binding proteins (40), it is conceivable that the AAC(2')-Ia is associated with the outer leaflet of the cytoplasmic membrane.

The *aac(2')-Ia* gene has been reported to not encode a signal sequence (34), indicating that, if it is indeed exported, an alternative protein translocation mechanism to the general secretory pathway must be utilized. Closer examination of the C-terminal region of the deduced AAC(2')-Ia amino acid sequence (34) reveals the presence of a potential signal se-

quence. Algorithms developed by both Garnier et al. (18) and Gascuel and Golmard (19) predict the presence of an amphipathic  $\alpha$ -helix containing both a charged and an uncharged cluster of amino acids at the C-terminal region of the AAC(2')-Ia sequence. The characteristics of this proposed signal sequence closely resemble those observed for other exported proteins, as exemplified by the genetically related hemolysins of *E. coli* (20, 41) and the *Proteaeae* (*Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii*) (25). AAC(2')-Ia appears to lack only the hydroxylated amino acid cluster of the four sequence requirements (potential amphipathic  $\alpha$ -helix, charged cluster, uncharged region, hydroxylated cluster) identified for efficient translocation. However, site-directed mutagenesis has been used to show that C-terminal sequences devoid of this cluster of hydroxylated amino acids are still secreted, albeit to a lesser extent (approximately 30% efficiency) (41). A recent study by Chervaux and Holland (6) involving both random and directed mutagenesis of the *E. coli* hemolysin A gene indicated that the preservation of a conserved acidic (Glu-978) and an aromatic (Phe-989) residue in the C-terminal secretion signal was essential. With the *P. stuartii* AAC(2')-Ia, Glu-148 and Trp-156 of region I (helix II) may fulfill this role. To further support this hypothesis, hydrophobic cluster analysis (16, 26) of the C terminus of the deduced AAC(2')-Ia amino acid sequence also bears striking similarities to that of the *E. coli* hemolysin (data not shown).

Homology studies conducted by Shaw et al. (36) had indicated that the *P. stuartii* AAC(2')-Ia enzyme was unique. It was found to be unrelated to any other aminoglycoside-modifying enzyme, for which considerable amino acid sequence homologies are otherwise observed. AAC(2')-Ia was thus suggested to comprise its own class (36). Recently, the chromosomally encoded AAC(2')-Ib from *M. fortuitum* was added to this class of acetylating enzymes since it was found to be 38% identical and 63% similar to *P. stuartii* AAC(2')-Ia. A search of the available data banks had not found any other protein to which these AAC(2')s may be related (34). Further analysis conducted in the present study indicated that they do not appear to contain the imperfect tandem hexapeptide repeat motif ([Ile, Leu, Val]-Gly-X-[Gly, Asn, Asp]-[Val, Ala]) observed in a number of bacterial transferases, including the *E. coli* transferases, serine acetyltransferase, thiogalactoside acetyltransferase, and UDP-*N*-acetylglucosamine acetyltransferase (44).

Whereas peptidoglycan or its precursor GlcNAc appears to be a reservoir of acetate moieties for the AAC(2')-Ia-catalyzed modification of aminoglycosides, it is unlikely that it serves as the sole source, but what else is available? With in vitro assays, acetyl-CoA is utilized by AAC(2')-Ia, but this metabolite would not be available in the periplasm. If the primary site of action of the acetyltransferase is indeed the periplasm, it is conceivable that a transmembrane reaction analogous to that observed in the Golgi vesicles (21) and lysosomes (2) of rat liver exists. In these systems, acetyl-CoA in the cytoplasm provides the source of acetate to the respective membrane-bound acetyltransferases which subsequently translocate it to the interior of the Golgi apparatus and lysosomes to acetylate sialic acids and glucosamine, respectively. However, the AAC(2')-Ia of *P. stuartii* does not appear to be a transmembrane protein (34), indicating that, for this postulate, a separate protein complexed with AAC(2')-Ia would be required to perform the translocation of acetate. Only further biochemical investigation and analysis will bear out the true pathway for aminoglycoside acetylation.

The observations that the AAC(2')s of *P. stuartii* and *M. fortuitum* are chromosomally encoded (1, 7, 27, 28, 47), that the expression of AAC(2')-Ia is highly regulated (33, 34), and that



both enzymes are ubiquitous in the respective bacteria regardless of the aminoglycoside phenotype (1, 28, 34, 43) have led to speculation that these enzymes may have a physiological or metabolic function unrelated to aminoglycoside modification (30, 33, 34, 43) and that their activity on the aminoglycosides is simply fortuitous for the producing cell. In view of both the structural relatedness between peptidoglycan and the aminoglycosides and the morphological changes observed upon the altered expression of AAC(2')-Ia (30, 34), it was thought that this AAC may have a role in the biosynthesis of peptidoglycan. Recent investigations confirmed its participation in the O acetylation of peptidoglycan in *P. stuartii* (30, 31), but subsequent Southern hybridization experiments have indicated that a gene homologous to *aac(2')-Ia* is not universally present in the closely related *Proteaceae* (11), each species of which has been shown to produce O-acetylated peptidoglycan (8). This would suggest that the true physiological role, if any, of the enzyme may involve another aspect of cell wall metabolism. One other possibility could concern the acetylation of lipopolysaccharide. In support of this postulate, only the two species of the *Proteaceae*, *Providencia rettgeri* and *Proteus penneri*, which are currently known to O acetylate their lipopolysaccharides (3, 38, 45) were shown to encode DNA sequences with homology to *aac(2')-Ia* (11).

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