Aminoglycoside-Resistant Mutation of *Pseudomonas aeruginosa* Defective in Cytochrome c\textsubscript{552} and Nitrate Reductase

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A gentamicin-resistant mutant of *Pseudomonas aeruginosa* PAO503 was selected after ethyl methane sulfonate mutagenesis. The strain, *P. aeruginosa* PAO2401 had increased resistance to all aminoglycosides tested but exhibited no change for other antibiotics. The mutation designated agIA (aminoglycoside resistance) was cotransducible with the 8-min iluB,C marker on the *P. aeruginosa* chromosome. It showed a marked reduction in cytochrome c\textsubscript{552} and nitrate reductase (Nar) and a change in terminal oxidase activity. Cytochrome c\textsubscript{552} is a component of the *P. aeruginosa* Nar. No changes in succinate and reduced nicotinamide adenine dinucleotide dehydrogenases, ubiquinone content, Mg\textsuperscript{2+}/Ca\textsuperscript{2+} membrane adenosine triphosphatase, and energy coupling of electron transport to adenosine 5'-triphosphate synthesis were detected. Transport of gentamicin and dihydrostreptomycin was impaired in PAO2401, but transport of proline, arginine, glutamine, glucose or the polyamine spermidine was not reduced. Ribosomes of PAO2401, and PAO503 bound dihydrostreptomycin equally well, and cell extracts did not inactivate gentamicin or dihydrostreptomycin. Strain PAO2401 is resistant to gentamicin and dihydrostreptomycin because of impaired transport of these compounds. The transport studies indicate a selective coupling of dihydrostreptomycin and gentamicin transport with terminal electron transport. This conclusion was supported by results from another mutant (PAO417-T2) with increased Nar activity, enhanced dihydrostreptomycin and gentamicin transport and a reduction in resistance to these drugs. These results are discussed in relation to a refined model for aminoglycoside transport and briefly relative to plasmid-mediated aminoglycoside resistance.

MATERIALS AND METHODS

**Bacterial strains.** Bacterial strains, R-factors, and sex factors are given in Table 1.

**Media.** Media used were citrate minimal medium (CMM) (19) supplied with required amino acids, nutrient broth (NB) (BBL Microbiology Systems), Trypticase soy broth (TSB) (BBL Microbiology Systems), and tryptic soy agar (TSA) (Difco). Anaerobic growth was in an anaerobic jar with a GasPak (BBL Microbiology Systems).

**Mutant isolation.** Ethyl methane sulfonate (0.1 ml) was added to 10 ml of an exponential-phase culture of *P. aeruginosa* PAO503 in CCM. This was incubated at 37°C for 60 min. The preparation was centrifuged, resuspended to a density of an absorbance of 0.1 at 600 nm in CMM and incubated for 2 h at 37°C. Portions of undiluted and diluted culture were plated onto CMM agar with 12.8 \(\mu\)g of Gm per ml. Unmutagenized cells give no colonies at 3.2 \(\mu\)g of Gm per ml with an identical inoculum.

**Antibiotic susceptibility testing.** Minimal inhib-
Table 1. P. aeruginosa strains, R-factors, and sex factors

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype</th>
<th>Strain collection, B.W. Holloway, Monash University</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO503</td>
<td>met-9011</td>
<td>This paper. Gm-resistant mutant of PAO503. Spontaneous Gm-susceptible revertant of PAO2401.</td>
</tr>
<tr>
<td>PAO2401</td>
<td>met-9011 agI A</td>
<td>B.W. Holloway Transductant of PAO641 selected for ilv independence.</td>
</tr>
<tr>
<td>PAO2404</td>
<td>met-9011</td>
<td>B.W. Holloway</td>
</tr>
<tr>
<td>PAO641</td>
<td>car-9, trpF, ilvB C</td>
<td>PAO503 with R plasmid pLB130 (orR130)</td>
</tr>
<tr>
<td>PAO2408</td>
<td>car-9 trpF A, agI A</td>
<td>PAO503 with R plasmid pLB151 (or R151).</td>
</tr>
<tr>
<td>PAO643</td>
<td>car-9 pro-64 trpB67 na1 A9</td>
<td>PAO2401 with R plasmid pLB130</td>
</tr>
<tr>
<td>PAO503(pLB130)</td>
<td>met-9011, Str', Su', Gm'</td>
<td>PAO2401 with R plasmid pLB151</td>
</tr>
<tr>
<td>PAO503(pLB151)</td>
<td>met-9011, Str', Su', Gm', Tm', Ch'</td>
<td>PAO2401 with R plasmid pLB130</td>
</tr>
<tr>
<td>PAO2401(pLB130)</td>
<td>met-9011, agI A, Str', Su', Gm', Tm', Ch'</td>
<td>PAO2401 with R plasmid pLB151</td>
</tr>
<tr>
<td>PAO25</td>
<td>argF leu-10 chl-2</td>
<td>B.W. Holloway Spontaneous Sm-resistant derivative of PAO227. MIC of Sm, &gt;1,000 μg/ml, no change in other aminoglycoside resistance.</td>
</tr>
<tr>
<td>PAO227 Str'</td>
<td>ilvB, C his-II lys-12, met-28 trpC, D pro-73 leu-10 strA</td>
<td>PAO25 containing R68.45 and having chromosomal mobilization ability (Cma).</td>
</tr>
<tr>
<td>PAO25 (R68.45)</td>
<td>arg-F leu-10, Rc', Km', Ch'</td>
<td></td>
</tr>
</tbody>
</table>

Iteratory concentrations (MICs) were determined on TSA with a multiple inoculator delivering 1 μl/inoculum. The inoculum was about 10^7 colonies of an exponential-phase culture grown in TSB. Endpoints determined after 18 h of incubation at 37°C were defined as less than five colony growths. Disk testing was carried out by inoculating TSA by the procedure of Bauer et al. (2). Disks were 7 mm in diameter and contained per disk: carbenicillin, 100 μg; ticarcillin, 75 μg; polymyxin B, 30 μg; chloramphenicol, 100 μg; rifampin, 100 μg; nalidixic acid, 100 μg; thiosulfate, 1 mg; Gm, 10 μg; tobramycin, 10 μg; amikacin, 30 μg; kanamycin, 200 μg; Sm, 100 μg; neomycin, 30 μg; spectinomycin, 500 μg; and pipercillin, 10 μg.

Conjugation and transduction. Conjugation and transduction were performed as previously described (19). Mapping times are based on the recent map of the P. aeruginosa PAO chromosome (13).

Ribosomal binding of DHS and assays for aminoglycoside inactivation. Ribosomal binding of dihydrostreptomycin (DHS) and assays for aminoglycoside inactivation have been described elsewhere (4).

Uptake of DHS, amino acids, and glucose. Uptake of DHS and Gm was determined as previously described (5, 6, 9) with NB and CMM media. Uptake of glutamine, proline, serine, and arginine was performed on exponential-growth-phase cells grown in CMM. Cells were washed twice with CMM (minus citrate) and resuspended at 37°C to 2 × 10⁶ cells per ml in 4 ml of CMM with chloramphenicol (300 μg/ml) and incubated for 2 min. The 14C-amino acid spermidine or glucose was added, and portions (0.5 ml) were filtered on nitrocellulose filters (0.2 μm, Millipore Corp.) and washed with 5 ml of 1% NaCl. Filters were dried and counted in Omniflor (New England Nuclear). Spermidine uptake was performed both with and without the addition of chloramphenicol. Specific activities and initial concentrations were: proline, 283 mCi/mmol, 0.91 μM; glutamine, 42 mCi/mmol, 2.95 μM; spermidine, 85 mCi/mmol, 2.95 μM; arginine, 340 mCi/mmol, 0.75 μM. Glucose uptake was measured with NB in place of CMM because glutarate represses glucose transport systems in P. aeruginosa. Otherwise, the procedure was identical except that chloramphenicol was omitted. The specific activity of [14C]glucose was 291 mCi/mmol, and the initial concentration was 1.71 μM.

Electron transport and energy coupling. Assays were performed as described elsewhere for succinate dehydrogenase (1), reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (17), ubiquinone content (20), starved whole-cell adenosine triphosphate (ATPase) synthesis (23), cytochrome (4), nitrate reductase activity (22), and Mgs²⁺/Ca²⁺-activated adenosine triphosphatase (ATPase) (21). Oxidative phosphorylation was determined by the following method. The reaction mixture consisted of air-saturated 50 mM tris(hydroxymethyl)ammonomethane-hydrochloride (pH 7.4), 5 mM MgCl₂, 0.15 mM adenosine diphosphate, 0.75 mM adenosine monophosphate, 1.0 mM inorganic phosphate, 0.25 mM ethylenediaminetetraacetic acid (EDTA), and 0.8 mg of membranes prepared as described by Cox et al. (8) in a final volume of 2.5 ml in an oxygen electrode (YS1, model 53) chamber at 37°C. The assay was started with the addition of substrate (10 mM succinate or 1 mM NADH) and continued until anaerobiosis was obtained. For ATP determination, samples of 160 μl of the mixture were transferred to 40 μl of cold 30% perchloric acid. Samples were centrifuged in a Beckman microfuge to remove the protein precipitate. ATP was determined by the luciferase assay (16) on the supernatant of samples taken at zero time and at anaerobiosis. Control preparations were run simultaneously with no added substrate. P:O ratios were expressed as the ratio of nanomoles of ATP formed per minute per milligram of protein to the nanogram-atomic of O per minute per milligram of protein.

RESULTS

Mutant isolation and general characteristics. Colonies resistant to four times the con-
centration of Gm needed to completely inhibit strain PA0503 were obtained at a frequency of about 5 x 10⁻⁴/viable cell plated. Three phenotypes were clearly defined from 250 purified clones by examining the level of Gm resistance, anaerobic growth with nitrate, and aerobic growth at 43°C compared with 37°C in minimal medium without citrate but with either glucose (0.2%) or succinate (0.2%). A variety of other properties examined did not differentiate between phenotypes. Strain PA02401 was selected for further study based on initial results obtained from mapping and transport studies. Resistance to Gm, Sm, tobramycin, amikacin (Table 2), kanamycin, and neomycin was elevated for PA02401 when tested by MIC and disk methods. No change in susceptibility of PA02401 to carbenicillin (Table 2), ticarcillin, piperacillin, tetracycline, polymyxin B, chloramphenicol, rifampicin, nalidixic acid, sulfonamides, and spectinomycin compared with PA0503 was detected by either method.

Strain PA02401 did not grow anaerobically in TSB with 0.4% KNO₃, whereas PA0503 grew well under these conditions. Doubling times at 37°C in TSB and CMM with methionine, respectively, were 42 and 51 min for PA0503 and 127 and 165 min for PA02401. Growth of PA02401 in succinate (0.2%) CMM (less citrate) with methionine at 42°C was very poor, whereas PA0503 grew under these conditions. Spontaneous revertants were readily selected under the last conditions at a frequency of 10⁻⁴/viable cell plated. Five revertants were examined and had parental antibiotic susceptibility, growth rates, and anaerobic growth patterns. One of these, PA02404, was chosen for comparison with PA02401 and PA0503 in selected studies. Spontaneous reversion was a problem with studies performed with PA02401. At the termination of all experiments, cultures were checked for reversion. Only those experiments were used where reversion was undetectable.

Mapping of aminoglycoside resistance. Plasmid R68.45 was introduced into PA02401 from PA025 (R68.45) by selection for methionine auxotrophy and carbenicillin (500 µg/ml) resistance. PA02401 (R68.45) was conjugated with PA0227 Str⁻, and recombinants were selected on 500 µg of Sm per ml and minimal medium supplemented with five of six amino acids (see Table 3) required by PA0227 Str². One hundred recombinants for each selected marker were scored for coinheritance of Gm resistance (20 µg/ml). A second cross of PA02401 (R68.45) x PA0643 was performed selecting for the markers given in Table 3. These crosses indicated that the aminoglycoside resistance locus was between pro-64 at 4 min and car-9 at 9 min and that there was very high linkage to iltB,C at 8 min. Transduction with F116L using PA0641 was carried out. Aminoglycoside resistance was 50% cotransducible with iltB,C, demonstrating the mutation is in the 8-min region. The Gm-resistant transductants of PA0641 designated PA02408 had identical properties to PA02401 with respect to aminoglycoside resistance, inability to grow anaerobically, and reduced growth rate.

Ribosomal binding of Sm and inactivation of Sm and Gm. Ribosomes prepared from PA0503, PA02401, and PA02404 bound about 0.85 molecules of DHS per ribosome. Cell-free extracts of these strains did not inactivate Gm or Sm. It is unlikely that a single ribosomal mutation would result in the broad aminoglycoside resistance shown by PA02401 or that enzymic inactivation of the aminoglycosides would be acquired by mutation. However, both of these changes has been shown to alter DHS and Gm uptake, and they should be excluded before measuring uptake.

Uptake of DHS and Gm. Strain PA02401 has significantly reduced uptake of DHS (10 µg/ml) in NB (Fig. 1) and Gm (5 µg/ml) in NB (Fig. 2) compared with PA02404 and PA0503. A similar reduction of Gm (50 µg/ml) and DHS (50 µg/ml) uptake in CMM for PA02401 compared with PA0503 and PA02404 was also found.

### Table 2. MICs of aminoglycoside antibiotics and carbenicillin for R⁻ and R⁺ P. aeruginosa PA0503 and PA02401

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gm</th>
<th>Tobramycin</th>
<th>Sm</th>
<th>Amikacin</th>
<th>Carbenicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0503</td>
<td>5</td>
<td>1</td>
<td>60</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>PA02401</td>
<td>75</td>
<td>15</td>
<td>240</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>PA0503(pLB130)</td>
<td>100</td>
<td>1</td>
<td>600</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>PA02401(pLB130)</td>
<td>200</td>
<td>1</td>
<td>900</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>PA0503(pLB151)</td>
<td>100</td>
<td>15</td>
<td>N.D.</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>PA02401(pLB151)</td>
<td>&gt;750</td>
<td>150</td>
<td>N.D.</td>
<td>100</td>
<td>400</td>
</tr>
</tbody>
</table>

* MICs performed with TSA (Difco) by multiple inoculator method using 10⁵ cells per inoculated spot. N.D., Not determined.
The preceding results demonstrate that PAO2401 accumulates Gm and DHS less effectively than PAO503 and PAO2404. The difference in transport cannot be ascribed to differences in cell wall, ribosomal affinity for DHS, or inactivation of DHS or Gm.

Transport of other compounds. To determine whether the transport deficiencies for Gm and DHS were indicative of a general transport defect, accumulation of several other compounds was measured. These included proline (proton motive force-dependent transport in Escherichia coli); glutamine (ATP-dependent transport in E. coli); the positively charged amino acid arginine; a polypeptide, spermidine (a streptomycin-induced polyamine transport system has been postulated) (14); and glucose. The results portrayed in Fig. 3 to 5 show that under conditions where Gm and DHS transport is reduced in PAO2401, no significant reduction in transport rates is seen for any of the above compounds. Thus, the transport defect in PAO2401 is not one resulting in generalized transport deficiency.

Glutamine transport rates were slightly greater in PAO2401 than PAO503. The transport of glucose in minimal medium with no additional energy source was less in PAO2401 than that in PAO503 (data not shown). With an additional energy source as in NB (Fig. 5), no difference was seen. It is probable that PAO2401 has a reduced level of endogenous energy reserves.

Components of electron transport and energy coupling. The inability of PAO2401 to grow anaerobically in TSB with 0.4% KNO₃.

To exclude changes in the outer membrane and peptidoglycan as a cause for aminoglycoside resistance in 2401, Gm uptake was measured in spheroplasts of PAO503 and PAO2401. The results as shown in Fig. 2 demonstrate that spheroplasts of PAO2401 retain the reduced transport capability for Gm compared with spheroplasts of PAO503 seen in whole cells. Spheroplasts were prepared as described (9) with 0.25 mM EDTA.

The graphs show accumulation of DHS and Gm at 37°C in nutrient broth. Accumulation of DHS at 5 μg of DHS per ml by whole cells of P. aeruginosa PAO503 (---) and PAO2401 (-----) and accumulation of Gm at 2 μg/ml in nutrient broth with 20% sucrose.

### Table 3. Conjugational mapping

<table>
<thead>
<tr>
<th>Selected markers</th>
<th>Map position (min)</th>
<th>Coinheritance of resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross PAO2401(R68.45) x PAO227 Str'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tllB,C*</td>
<td>8</td>
<td>90</td>
</tr>
<tr>
<td>his-11*</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>lys-12*</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>trpC,D*</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>pro-73*</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>leu-10*</td>
<td>60</td>
<td>3</td>
</tr>
</tbody>
</table>

* From the FP-2 origin in P. aeruginosa PAO.
* One hundred recombinants were analyzed for coinheritance of Gm resistance by plating on amino acid-supplemented minimal medium with 20 μg of Gm (as sulfate) per ml.

**Fig. 1.** Accumulation of DHS at 37°C with time at 10 μg of DHS per ml in nutrient broth. P. aeruginosa PAO503 (---), PAO2401 (-----), PAO2404 (---), PAO417 (△ △ △), and PAO417-T2 (△ △ △).

**Fig. 2.** Accumulation of Gm at 37°C with time in nutrient broth. Accumulation at 5 μg of Gm per ml by whole cells of P. aeruginosa PAO503 (●●●●●●) and PAO2401 (△ △ △ △) in nutrient broth with 20% sucrose.
suggested this mutant was defective in electron transport. A series of investigations were carried out to assess most of the parameters of electron transport and energy coupling. The results provided in Table 4 show that no significant differences were detected between PAO503 and PAO2401 in assays for succinic and NADH dehydrogenases, ubiquinone content, and Mg²⁺/Ca²⁺-dependent membrane ATPase. Both strains coupled energy from electron transport to ATP synthesis as shown by measurement of starved whole-cell ATP synthesis and determination of P:O ratios with isolated cytoplasmic membranes (Table 5).

Cytochrome spectra of air-oxidized versus dithionite-reduced cells (Fig. 6) showed that the cytochrome c₅₅ band at 552 nm and the β band at 523 nm were markedly diminished in PAO2401. These findings illustrate that the absorption maximum at 552 nm in PAO2401 is less than 5% of that in PAO503. Thus, cytochrome c₅₅ is absent or markedly diminished in PAO2401.

Oxygen consumption by isolated membrane of PAO2401 is reduced in rate relative to the parental or revertant strains. In addition, con-

![Graph](image1)

**Fig. 3.** Uptake of (A) spermidine and (B) glutamine by whole cells of *P. aeruginosa* PAO503 (○-○○) and PAO2401 (□-□) in minimal medium containing citrate.

![Graph](image2)

**Fig. 4.** Uptake of (A) arginine and (B) proline by whole cells of *P. aeruginosa* PAO503 (□-□) and PAO2401 (○-○) in minimal medium containing citrate. Uptake of proline in (B) by *P. aeruginosa* PAO417 (▲-▲-▲) and PAO417-T2 (△-△-△) in minimal medium containing citrate.

![Graph](image3)

**Fig. 5.** Uptake of glucose by whole cells of *P. aeruginosa* PAO503 (□-□-□) and PAO2401 (■-■) in nutrient broth.
Table 4. Assays of membrane functions

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ubiquinones (nmol/mg [dry wt])</th>
<th>Mg&quot;⁺/Ca⁺ ATPase (nmol of inorganic phosphate per mg of protein per 5 min)</th>
<th>Succinate dehydrogenase (nmol of DCPIP reduced per min/mg of protein)*</th>
<th>NADH dehydrogenase (nmol of DCPIP reduced per min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-EDTA⁺</td>
<td>+EDTA⁺</td>
<td></td>
</tr>
<tr>
<td>PA0503</td>
<td>2.47 x 10⁻⁴</td>
<td>1.50</td>
<td>&lt;0.05</td>
<td>2.05</td>
</tr>
<tr>
<td>PA02401</td>
<td>2.74 x 10⁻⁴</td>
<td>1.85</td>
<td>&lt;0.05</td>
<td>1.82</td>
</tr>
<tr>
<td>PA02404</td>
<td>2.6 x 10⁻⁴</td>
<td>1.75</td>
<td>0.05</td>
<td>1.85</td>
</tr>
</tbody>
</table>

* EDTA concentration, 10 mM; DCPIP, dichlorophenolindolphenol.

Table 5. ATP synthesis from starved whole cells and P:O ratios of membrane fractions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Starved whole cells (nmol of ATP per mg of protein)</th>
<th>Membrane fractions*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0⁺</td>
<td>3⁺</td>
</tr>
<tr>
<td>PA0503</td>
<td>0.90</td>
<td>4.4</td>
</tr>
<tr>
<td>PA02401</td>
<td>0.88</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Substrate used was sodium succinate (10 mM).
+ Time in seconds.

Table 6. Stimulation of O₂ consumption and susceptibility of membranes to inhibition by 0.1 mM KCN

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ratio of O₂ consumed in presence of substrate: no substrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADH</td>
</tr>
<tr>
<td>503</td>
<td>22.5</td>
</tr>
<tr>
<td>2401</td>
<td>4.5</td>
</tr>
<tr>
<td>2404</td>
<td>22</td>
</tr>
</tbody>
</table>

* Concentrations: NADH, 1 mM; succinate, 10 mM. Buffer: 50 mM potassium phosphate (pH 7.6), 0.3 mg of protein per 3 ml of assay. Oxygen consumption measured with oxygen electrode.

Assumption is stimulated much less by NADH and succinate (Table 6) in PA02401 than PA0503. The stimulated consumption of oxygen by PA02401 in the presence of NADH or succinate is much more resistant to inhibition by 0.1 mM KCN than that of PA0503. These results indicate that different terminal cytochrome oxidases are in use in the two strains. Strain PA02401 uses primarily an oxidase relatively insensitive to KCN, whereas PA0503 probably uses this oxidase plus an oxidase more sensitive to inhibition by KCN.

Table 7 illustrates nitrate reductase (Nar) activities found in strains PA0503, PA02401, and PA02404. PA02401 has about 1/40 the activity of the other strains grown anaerobically with K nitrate, and K nitrite. Molybdate did not restore Nar activity. The marked reduction of Nar in PA02401 explains the strains inability to grow anaerobically in nitrate. PA02401 grows slowly in TSB-K nitrite (0.1%) and has nitrite reductase activity. PA0503, PA02401, PA02404, and PA02408 all grow on K nitrate as the only nitrogen source and, thus, assimilate nitrate. The assimilatory nitrate reductase is functional in PA02401.

MICs of aminoglycosides for strains with and without R plasmids. It has been proposed and supported by experimental data that R plasmid-specific enzymic modification results in aminoglycoside resistance by competition between the rates of enzymic modification and transport of aminoglycosides (9). This proposal predicts that a strain like PA02401 with defective Sm or Gm transport should have increased...
MICs for these drugs in the presence of modifying enzymes compared to PAO503. The results in Table 2 show this prediction is met. pLB130 specifies phosphorylation of Sm and acetylation of Gm. pLB151 specified adenylylation of Sm, Gm, and tolbramycin and $\beta$-lactamase-mediated hydrolysis of carbenicillin. MICs for the appropriate aminoglycosides are elevated in R* PAO2401 relative to R+ PAO503. No increase in resistance level to carbenicillin was detected.

Aminoglycoside susceptibility in a strain with increased Nar activity. P. aeruginosa PAO417-T2 was isolated as insensitive to aeruginocin 41 (AR41) and found to have at least fourfold-increased susceptibility to the aminoglycoside antibiotics streptomycin, kanamycin, Gm, tobramycin, sisomicin, lividomycin, and amikacin.

This mutant has not been accurately mapped or shown to involve a single mutation. However, it is interesting to note that it has about an eight- to ninefold increase in Nar activity (Table 7). The mutant shows no change in succinate and NADH dehydrogenase and $Mg^{2+}$/$Ca^{2+}$ membrane ATPase. The mutant PAO417-T2 has $3.2 \times 10^{-4}$ nmol of ubiquinone per mg of protein about 50% higher than the parent PAO417 ($2.2 \times 10^{-4}$ nmol/mg of protein). Parent and mutant show coupling between electron transport and ATP synthesis by both starved whole-cell ATP synthesis and P-O ratios (0.15).

PAO417-T2 accumulates more DHS at a concentration of 5 $\mu$g/ml than does PAO417. Similarly PAO417-T2 shows enhanced uptake of proline (Fig. 4B).

PAO417-T2 produces a brown pigment not detected in the parent. The pigment is probably identical to that described by Mann in wild-type P. aeruginosa (18). It obscures cytochrome absorption spectra so that evaluation of cytochromes could not be carried out. It is probable that the pigment can act as an electron carrier in electron transport.

**Table 7. Nitratereductase activity in strains of P. aeruginosa**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrate reductase (nmol of NO$_2$ per min per mg of protein)</th>
<th>Aerobic + K nitrate*</th>
<th>Anaerobic + K nitrate</th>
<th>Anaerobic + K nitrate + K nitrite*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO503</td>
<td>0.060</td>
<td>0.110</td>
<td>0.122</td>
<td></td>
</tr>
<tr>
<td>PAO2401</td>
<td>0.002 (No growth)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO2404</td>
<td>0.047</td>
<td>0.120</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>PAO417</td>
<td>0.042</td>
<td>0.080</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO417-T2</td>
<td>0.361</td>
<td>0.402</td>
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* K nitrate at 0.4%, K nitrite at 0.1% where added.

**DISCUSSION**

The mutation in PAO2401 most likely involves a single gene in view of the high reversion frequency and transducibility of the phenotype. It is probable that a component common to cytochrome c$\text{O}_{2}$ and the dissimilatory nitrate reductase (Nar) is either not formed or formed in a markedly reduced amount in PAO2401. Fewson and Nicholas have previously shown that the nitrate reductase complex from P. aeruginosa contains cytochrome c (11) which is consistent with this interpretation. The reduced oxygen consumption and relative resistance to KCN of oxygen consumption with succinate or NADH indicates a change in the nature of the predominant cytochrome oxidase used by PAO2401. It is likely that PAO2401 is principally using the nitrite-$O_2$-oxidoreductase generally detected in anaerobically or glucose-grown P. aeruginosa. This complex contains cytochrome d and a distinct cytochrome c. In general, cytochrome oxidases containing cytochrome d are relatively more resistant to inhibition by KCN (15).

Mutant PAO2401 illustrates the relationship between terminal electron transport and aminoglycoside transport. The defect in cytochrome c$\text{O}_{2}$ and Nar is not associated with a generalized transport deficiency. This suggests that there is a specific relationship between terminal electron transport and aminoglycoside accumulation. The last conclusion is firmly supported by studies examining aminoglycoside susceptibility with different electron acceptors for growth of E. coli. These investigations have shown that susceptibility to Gm, Sm, amikacin, kanamycin, and tobramycin is greatest with $O_2$ as the terminal electron acceptor. A significant decrease in susceptibility occurs with nitrate. A further marked decrease in susceptibility results when fumarate is the terminal acceptor or under fermentative conditions. Sm-dependent (Str$^d$) strains only grow well in oxygen. Anaerobic growth with nitrate of Str$^d$ strains is poor and growth does not occur with fumarate or under fermentative conditions. (H. M. Van Den Elzen and L. E. Bryan, Progr. Abstr. Internsci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no 284, 1978).

The decrease in Sm and Gm transport for PAO2401 and the increase in resistance and the reverse relationship for PAO417-T2 confirm the relationship between transport of and susceptibility to Sm and Gm.

The association between Sm and Gm transport and terminal electron transport, particularly cytochrome oxidase activity, suggests a specific coupling between these events. These find-
ings are consistent with a previous proposal (7) that Sm and Gm cross the cytoplasmic mem-
brane bound to a membrane component in-
volved in membrane energization from electron
transport. We are not aware of a conventional
transport system specifically coupled to terminal
electron transport. This view is supported by the
observations that proline, arginine, glutamine,
spermidine, and glucose transport is similar in
PA0503 and PAO2401.

Sm, Gm, and probably other aminoglycosides
are most likely transported into cells as strong
cations at physiological pH values. Little other
structural requirement seems taken into account
(7). Divalent cations in concentrations as low as
0.02 mM are effective antagonists of Sm and Gm
transport. The antagonism by divalent cations is
not specific and is consistent with an effect on
a single transport system. At higher concentra-
tions monovalent cations antagonize trans-
port, as does arginine (7).

Holtej has shown that the acceleration of Sm
transport which occurs after Sm binding to ri-
obosomes (EDP-II in our terminology [6]) is as-
associated with an acceleration of transport of
some polyamines. This has been proposed as
due to an induced polyamine transport system.
However, polyamines do not induce this trans-
port system (14). An alternative explanation for
EDP-II is that there is a general acceleration of
transport of strongly cationic compounds result-
ing from an increase in the cross-membrane
electrical potential (see below). It is likely that
such compounds enter cells driven primarily by
the membrane potential (ΔΨ) (interior negative)
component of the cellular proton motive force.
The latter explanation is favored by the obser-
vation that acceleration of transport of other
aminoglycosides and arginine occurs at the same
time (L. E. Bryan and S. Kwan, unpublished
data). In addition the rate of EDP-II is strongly
antagonized by Mg2+, Ca2+, Co2+, and Mn2+. The
evidence supplied from the study of PAO2401
shows that there is no change in the rate of
spermidine transport, which argues against the
specific use of a polyamine transport system.

Based on these studies and additional studies
by our laboratory as well as that of others (14),
the model previously proposed for aminoglyco-
side transport (7) can be refined as follows. Sm
and Gm exist as strong cations at physiological
pH. As such they bind to anionic sites on the
cell surface (energy-independent binding [6]).
These include negatively charged polar heads of
cytoplasmic membrane phospholipids (9). After
binding to polar phospholipids, Sm and Gm
associate with membrane “transporters” on the
basis of their positive charge but otherwise non-
specifically and are driven across the cytoplas-
mic membrane by the membrane potential (in-
terior negative) (energy-dependent transport).
The transporters are most effectively linked with
terminal electron transport particularly cyto-
chrome oxidase and nitrate reductase. The
ature of the transporters cannot be absolutely
defined. They are very unlikely to be a single,
specific transport carrier. The major probabili-
ties are that transporters are a component of the
cytoplasmic membrane used by terminal elec-
tron transport to energize the membrane (and
perhaps to translocate protons). There is evi-
dence suggesting these are respiratory quinones
(7) (L. E. Bryan and S. Kwan, unpublished
data). Another set of candidates which could act
as the Sm and Gm transporters are transport
carriers for a variety of cationic compounds,
including polyamines. The specificity and affini-
ty of the transporters for aminoglycosides are
known to be poor.

The driving force for Sm and Gm transport,
based on their cationic character, is probably
ΔΨ (12). It is a well established observation that
the activity of Sm and Gm is highly dependent
on pH. This is understood in terms of the above
proposal. Cellular proton motive force is consid-
ered a function of both a proton gradient (ΔpH)
and electrical potential (ΔΨ) across the cytoplas-
mic membrane. The ΔpH is reduced when extra-
cellular pH is elevated and the major component
of the driving force is ΔΨ. In terms of aminogly-
coside transport, the increase in ΔΨ provides a
greater driving force for Sm and Gm entry. The
reverse situation occurs under acidic conditions.
As shown by previous investigations as well as
here, with PA0417-T2, an increase in the rate of
Sm and Gm entry is associated with enhanced
Sm and Gm susceptibility. We have repeatedly
oberved that the rate of Gm and Sm transport
declines with falling pH and increases with in-
creasing alkalinity.

The response of Sm and Gm transport to ΔΨ
is also the probable reason for EDP-II or accel-
erated transport which results from ribosomal
binding of transported Sm (6). Dubin et al. ob-
served that a marked loss of K+ is initiated
almost coincidentally with the increased rate of
Sm transport (10). A selective K+ loss would
increase ΔΨ and result in more rapid Sm trans-
port as well as that of other strong cations as
polyamines, divalent cations, and arginine. The
reason for the K+ loss is not understood. How-
ever, our investigations have clearly shown that
Sm and Gm transport can be accelerated by
mutations affecting membrane energization (7),
including the 417-T2 mutant described here. It
seems likely that 417-T2 has an elevated proton
motive force as both proline and aminoglycoside
transport are much enhanced. We have at-
tempted to measure $\Delta A$ during treatment of cells with Sm but no clear result has been obtained due to the technical difficulties of combined measurement of Sm activity and $\Delta A$. We have, however, measured cellular ATP concentration and have shown repeatedly a 50% increase in cellular ATP concentration coincident with EDP-II. We have also shown that the transport of arginine a strong cation is accelerated with the onset of EDP-II (L. Bryan and S. Kwan, unpublished data), although transport of other amino acids including lysine is decreased at this time.

In summary Sm and Gm transport requires two major components. (i) Anionic transporters charged most effectively by cytochrome oxidase which exist in the cytoplasmic membrane and act to bind Sm and Gm on the basis of their positive charge, but otherwise nonspecifically. Transporters are most likely reduced quinones, or other as yet undefined proteins involved in membrane energization from electron transport. Also possible but less likely would be carriers for various cationic compounds.

(ii) An electrical potential ($\Delta \psi$) with the cell interior being negative and that the transport rate of Gm and Sm is governed by the magnitude of $\Delta \psi$.

Mutant PAO2401 would be transport defective for Sm and Gm because of reduced cytochrome oxidase activity and a reduced rate of reduction of the transporter.

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LITERATURE CITED