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Aminoglycoside-Resistant Mutants of *Pseudomonas aeruginosa* Deficient in Cytochrome *d*, Nitrite Reductase, and Aerobic Transport

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Two gentamicin-resistant mutants of *Pseudomonas aeruginosa* PAO 503 were selected after ethyl methane sulfonate mutagenesis. Mutant PAO 2403 had significantly increased resistance to aminoglycoside but not to other antibiotics. Mutant PAO 2402 showed a similar spectrum of resistance but of lower magnitude. Both mutants showed no detectable cytochrome *d* and had a high frequency of reversion to a fully wild-type phenotype. PAO 2403 had a marked decrease and PAO 2402 had a moderate decrease in nitrite reductase activity. Both mutants had reduced uptake of gentamicin and dihydrostreptomycin. Mutant PAO 2403 showed a general decrease in transport rate of cationic compounds, whereas mutant PAO 2402 had only deficient glucose transport. Both mutants showed enhanced rates of glutamine transport and no change in glutamic acid transport. Other components of electron transport and oxidative phosphorylation were normal. These mutants involve ferrocytochrome c_{551} oxidoreductase formed only on anaerobic growth but illustrate transport defects in aerobically grown cells.

The selection of bacterial mutants with increased resistance to aminoglycoside antibiotics, particularly streptomycin and neomycin, has yielded target- and transport-type mutants. Target mutants show a narrow spectrum of resistance. Abnormalities of ribosomal 30S subunit proteins have been characterized for streptomycin and kanamycin resistance, and those of ribosomal ribonucleic acid have been characterized for kasugamycin resistance. A mutant affecting protein L6 of the 50S ribosomal subunit has been detected for gentamicin but only in association with a mutation affecting gentamicin transport (A. Bock, P. Buckel, A. Buchberger, and H. G. Wittmann, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 17th, New York, N.Y., abstr. no. 105, 1977).

Mutations affecting aminoglycoside transport exhibit a broad-spectrum increase in resistance to most, if not all, aminoglycosides, and those detected to date fall into two major classes. The first class affects aerobic electron transport and includes mutants with changes in cytochromes, respiratory quinones, and *cya* and *crp* mutations. The last exert their action apparently by causing reduction of the synthesis of components of the electron transport chain. The second class is uncoupling and membrane adenosine triphosphatase (ATPase) mutants, all of which apparently have some degree of increased proton leakage of the cytoplasmic membrane. Conversely, mutants with increased susceptibility have also been characterized and show increases in electron transport components or are deficient in ATPase without proton leakage (3).

Most of the preceding mutants have been obtained in *Escherichia coli*, a facultative organism. We have examined *Pseudomonas aeruginosa* broad-spectrum aminoglycoside-resistant mutants for two major reasons. *P. aeruginosa* grows anaerobically by nitrate respiration rather than by fermentation, and clinically significant broad-spectrum aminoglycoside resistance is common in isolates of this organism from patients receiving aminoglycoside therapy. We previously reported a mutant, defective in cytochrome c_{552} and nitrate reductase, which had impaired transport of streptomycin and gentamicin, but not of several other compounds, including polyamines (4). In this paper we report two additional mutants defective in cytochrome *d* and nitrite reductase, both of which have impaired aminoglycoside transport but different transport defects for other compounds.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and R plasmids are given in Table 1.

Media, mutant isolation, and antibiotic susceptibility testing. Media used were citrate minimal

TABLE 1. *P. aeruginosa strains and R plasmids*

Strain	Genotype/phenotype[a]	Comments
PAO 503	*met-9011*	Strain collection, B. W. Holloway, Monash University, Melbourne
PAO 2403	*met-9011 aglC19*	This paper; gentamicin-resistant ethyl methane sulfonate derivative of PAO 503
PAO 2402	*met-9011 aglB18*	This paper; low-level gentamicin-resistant ethyl methane sulfonate derivative of PAO 503
PAO 503(pLB130)	*met-9011*, Str^r, Su^r, Gm^r	PAO 503 with R plasmid pLB130
PAO 503(pLB151)	*met-9011*, Str^r, Su^r, Gm^r, Tm^r, Cb^r	PAO 503 with R plasmid pLB151
PAO 2403(pLB130)	*met-9011, aglC19*, Str^r, Su^r, Gm^r	PAO 2403 with R plasmid pLB130
PAO 2403(pLB151)	*met-9011, aglC19*, Str^r, Su^r, Gm^r, Tm^r, Cb^r	PAO 2403 with R plasmid pLB151
PAO 2402(pLB130)	*met-9011, aglB18*, Str^r, Su^r, Gm^r	PAO 2402 with R plasmid pLB130
PAO 2402(pLB151)	*met-9011, aglB18*, Str^r, Su^r, Gm^r, Tm^r, Cb^r	PAO 2402 with R plasmid pLB151

[a] Str, Streptomycin; Su, sulfonamide; Gm, gentamicin; Tm, tobramycin; Cb, carbenicillin; *agl*, aminoglycoside resistant.

medium (CMM) (4) (supplemented with methionine), nutrient broth (NB) (BBL Microbiology Systems), Trypticase soy broth (TSB) (BBL Microbiology Systems), and tryptic soy agar (TSA) (Difco Laboratories). Anaerobic growth was in an anaerobic jar with a GasPak (BBL Microbiology Systems). Mutant isolation with ethyl methane sulfonate was performed as previously described (10), selecting with 25 μg of gentamicin per ml. Antibiotic susceptibility testing was carried out as previously described by a multiple inoculator agar dilution system (4). Disk testing was performed by the procedure of Bauer et al. (2) with TSA.

Conjugation experiments. Conjugation and mapping experiments were performed as previously described (10).

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

Uptake of dihydrostreptomycin, gentamicin, amino acids, spermidine, glutamine, and glucose. Uptake of dihydrostreptomycin and gentamicin was determined as previously described (5–7) with NB, CMM, and aminoglycoside uptake media. Uptake of arginine, lysine, proline, spermidine, glutamine, glutamic acid, and glucose was performed as previously described (4). Specific activities and initial concentrations of ^{14}C-labeled compounds 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, 1.75 μM; glucose, 291 mCi/mmol, 1.71 μM; lysine, 343 mCi/mmol, 0.95 μM; glutamic acid, 267 mCi/mmol, 0.95 μM.

Electron transport and energy coupling. Assays were performed as previously described for succinate dehydrogenase, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase, ubiquinone content, starved whole-cell adenosine triphosphate (ATP) synthesis, cytochrome spectra, nitrate reductase activity, Mg^{2+}-Ca^{2+}-activated ATPase, oxidative phosphorylation, and oxygen consumption (4). Nitrite accumulation was determined by growing cells anaerobically at 37°C in TSB with 0.4% KNO_3 added. After overnight growth, cells were removed by centrifugation, and the supernatant was assayed for nitrite concentration (13). Supernatants were appropriately diluted where nitrite concentration was out of range of the assay. Nitrite reductase activity was assayed by determining the consumption of nitrite with time in the following assay mixture: 0.3 M potassium phosphate buffer (pH 7) (0.1 ml); 0.1 M sodium nitrite (0.1 ml); 0.7 mM methyl viologen (0.1 ml); $Na_2S_2O_4$-$NaHCO_3$ (1:1; 4 mg of each per ml) (0.1 ml); crude cell extract (0.1 ml). Cells were grown anaerobically in TSB with 0.4% KNO_3 overnight, then centrifuged at 3,000 × *g* for 20 min, washed once with 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) and suspended in phosphate buffer as used in the assay. Cells were broken in a French pressure cell and dialyzed overnight at 4°C against 1,000 volumes of phosphate buffer to remove any nitrite that had accumulated during growth. The protein content of the cell extract was adjusted so that 0.3 to 0.5 mg per assay was used. Results for nitrite reductase activity were based on the initial linear consumption of nitrite and given as nanomoles of nitrite consumed per minute per milligram of protein.

RESULTS

Mutant isolation, growth, and resistance. Mutants with a fourfold or greater increase in resistance to gentamicin were isolated at a frequency of about 5×10^{-4} per viable cell plated after ethyl methane sulfonate mutagenesis of strain PAO 503. One of the resulting phenotypes failed to grow anaerobically in TSA with 0.4% KNO_3 and has previously been described (4). Two other phenotypes (PAO 2402, PAO 2403) could be differentiated by their level of gentamicin resistance (Table 2) and their growth anaerobically with nitrate and nitrite. Strain PAO 2403, the more resistant strain, grew poorly or not at all and accumulated large amounts of nitrite when grown with nitrate (Table 3). Strain PAO 2402 grew almost as well as PAO 503 and

TABLE 2. *MICs of gentamicin, tobramycin, and carbenicillin for R^+ and R^- P. aeruginosa PAO 503, PAO 2403, and PAO 2402*

Strain	MIC[a] (μg/ml)		
	Gentamicin	Tobramycin	Carbenicillin
PAO 503	6	1.5	50
PAO 2403	100	20	50
PAO 2402	25	6	50
PAO 503(pLB130)[b]	150	1.5	50
PAO 2403(pLB130)	750	20	50
PAO 2402(pLB130)	200	6	50
PAO 503(pLB151)[b]	150	12.5	400
PAO 2403(pLB151)	>750	150	400
PAO 2402(pLB151)	250	25	400

[a] MICs performed on TSA by multiple inoculator method with 10^5 cells per inoculated spot.

[b] pLB130 specifies an aminoglycoside acetyltransferase (3) [AAC(3)] which does not significantly modify tobramycin; pLB151 specifies an aminoglycoside nucleotidyltransferase (2″) [ANT(2″)], which modifies gentamicin and tobramycin, and a β-lactamase.

accumulated less nitrite (Table 3) than PAO 2403. Doubling times (minutes) in TSB and CMM, respectively, at 35°C aerobically were: PAO 503, 42 and 51; PAO 2403, 82 and 90; and PAO 2402, 67 and 120.

No change in resistance was detected by disk diffusion and agar dilution minimal inhibitory concentrations (MICs) to a number of non-aminoglycoside antibiotics. These included tetracycline, chloramphenicol, nalidixic acid, rifampin, sulfonamides, colistin, carbenicillin, and ticarcillin. However, PAO 2403 and PAO 2402 had increased resistance to all aminoglycosides tested including amikacin, netilmicin, kanamycin, and neomycin.

Reversion of PAO 2403 and PAO 2402 to a fully wild-type phenotype occurred at a frequency of 10^{-5} per viable cell. Due to the high rate of reversion, large initial inocula were used for all experiments, and at the end of each experiment the extent of reversion was determined. Experiments were accepted if revertants were less than 1 per 10^5 cells. The high reversion frequency to wild type indicates that these are single-point mutations.

Genetic mapping by linkage analysis and time by entry studies did not allow accurate mapping of the PAO 2403 and PAO 2402 loci except to demonstrate that both loci are in the late, poorly defined regions of the PAO chromosome. Ribosomes of PAO 2403 and PAO 2402 showed no difference from PAO 503 in ribosomal binding of [^{3}H]dihydrostreptomycin. No enzymic nucleotidylation, phosphorylation, or acetylation was detected in any of the three strains.

Nitrate and nitrite reductase activity. Nitrite reductase activity is markedly reduced in PAO 2403 and moderately decreased in PAO 2402 (Table 4). This is consistent with the nitrite accumulation shown by these strains (Table 3). Cytochrome absorption spectra demonstrated no detectable change in any cytochromes except in cytochrome *d* (Fig. 1). The absorption maximum at 634 nm seen in PAO 503 was absent in both PAO 2402 and PAO 2403 grown anaerobically with nitrate. Aerobically grown PAO 503 had a much reduced content of cytochrome *d*.

Components of electron transport and energy coupling. In addition to cytochrome absorption spectra, the ubiquinone content, Mg^{2+}-Ca^{2+}-ATPase activity, succinate and NADH dehydrogenases, ATP synthesis of starved whole cells, and P/O ratios produced by membrane fractions were determined for PAO 503, PAO 2403, and PAO 2402. No significant difference from our previously published values for PAO 503 (4) was detected for either PAO 2403 or PAO 2402. PAO 2403 membranes consumed 20% and 35% as much oxygen with suc-

TABLE 3. *Anaerobic growth of P. aeruginosa strains PAO 503, PAO 2402, and PAO 2403*

Strain	Growth conditions			
	CMM agar		TSA	TSB
	0.4% KNO_3	0.1% $NaNO_2$	0.1% $NaNO_2$	0.4% KNO_3 (μmol of nitrite formed in 8 h)
PAO 503	4+[a]	2+[a]	3+[a]	0.04
PAO 2403	1-2+	0	0	7.85
PAO 2402	4+	2+	2+	1.03

[a] Cultures were grown in CMM or TSB with 0.05% $NaNO_2$ overnight. An inoculum sufficient just to produce confluent growth was spread on agar medium and incubated in an anaerobic jar with a gas pack (Oxoid Ltd.). Growth was scored as follows: 4+, confluent; 3+, heavy but not confluent; 2+, light; 1+, very faint; 0, no growth.

TABLE 4. *Nitrate and nitrite reductase activities in P. aeruginosa PAO 503, PAO 2403, and PAO 2402*

Strain	Nitrate reductase (μmol of NO_2 produced per min per mg of protein)[a]	Nitrite reductase (nmol of NO_2 consumed per min per mg of protein)[a]
PAO 503	0.115	1.19
PAO 2403	0.105	0.10
PAO 2402	0.11	0.60

[a] Cells were grown anaerobically in TSB with 0.4% KNO_3.

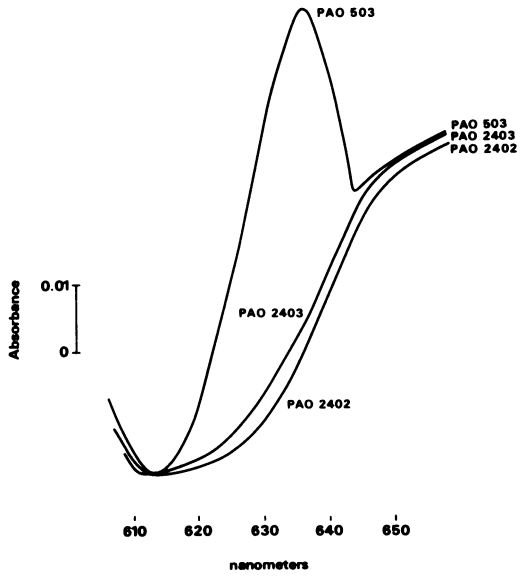

FIG. 1. *Difference spectra of reduced versus oxidized membranes extracted from P. aeruginosa PAO 503, PAO 2403, and PAO 2402 cells grown anaerobically on TSB with 0.4% KNO_3. The spectra were recorded after the addition of 1 mM sodium dithionite to the sample cuvette and no addition to the reference cuvette. Membranes were present at a concentration of approximately 5 mg of protein per ml. Aerobically grown PAO 503 had a spectrum identical to PAO 2403 and PAO 2402.*

cinate and NADH, respectively, as did strain PAO 503. Oxygen consumption with NADH is resistant to inhibition by 2 mM KCN, whereas that with succinate is as sensitive as PAO 503. Oxygen consumption by PAO 2402 is very similar to that of PAO 503.

Transport of antibiotics and other compounds. Figure 2 demonstrates that PAO 2403 accumulated less gentamicin than PAO 503 at both antibiotic concentrations shown. PAO 2402 showed reduced gentamicin uptake only at the lower gentamicin concentration tested. This is consistent with the higher aminoglycoside resistance of PAO 2403. Similar differences for dihydrostreptomycin uptake were also detected (data not shown). PAO 2403 and PAO 2402 also showed reduced gentamicin uptake in TSB and aminoglycoside uptake medium (7).

Transport of a series of other compounds was also measured. These included proline (proton motive-dependent transport in *E. coli*), glutamine (ATP-dependent transport in *E. coli*), arginine and lysine (positively charged amino acids), glutamic acid (negatively charged amino acid), spermidine (a polyamine), and glucose.

PAO 2403 exhibited reduced transport of spermidine, proline, lysine, and arginine (Fig. 3) and slightly reduced transport of glucose (Fig. 4) compared with PAO 503. This mutant consistently accumulated more glutamine (Fig. 4) and showed no difference in glutamic acid transport (not shown). Mutant PAO 2402 showed no difference in proline, lysine, arginine, and spermidine transport (Fig. 3) but had a markedly reduced rate of glucose transport (Fig. 4). Like PAO 2403, PAO 2402 showed no change in glutamic acid transport (not shown) and accumulated more glutamine than PAO 503 (Fig. 4).

MICs of aminoglycosides in R^+ and R^- strains. The MIC of a strain possessing an R plasmid-specified aminoglycoside-modifying enzyme has been proposed to be the result of competition between rates of aminoglycoside transport and enzymic modification (8). This prediction was further supported in the case of PAO 2403 and PAO 2402 (Table 2). PAO 2403 had the lowest aminoglycoside transport rate and the highest MIC without R plasmids or with pLB130 [AAC(3)] or pLB151 [ANT(2″)] enzymes. The parameters altering rates of enzymic modification (K_m, V_{max}, total enzyme) did not

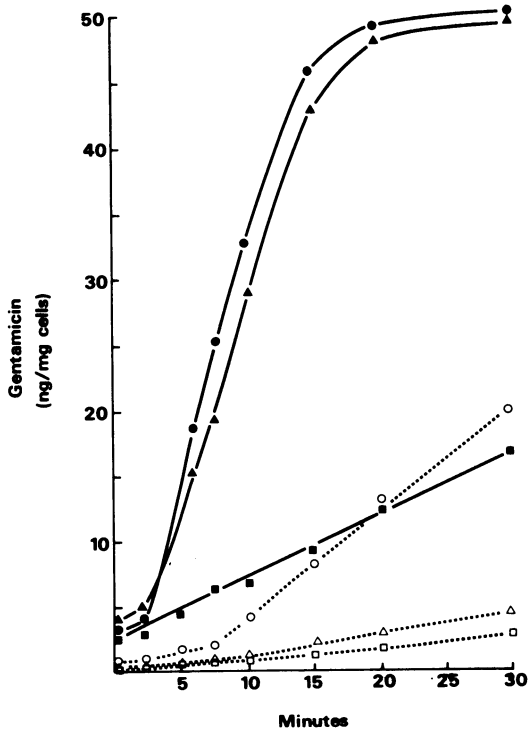

FIG. 2. *Accumulation of gentamicin at 37°C over time at 1 and 0.3 μg of gentamicin per ml in NB. At 1 μg of gentamicin per ml: PAO 503 (●); PAO 2403 (■); PAO 2402 (▲). At 0.3 μg of gentamicin per ml: PAO 503 (○); PAO 2403 (□); PAO 2402 (△).*

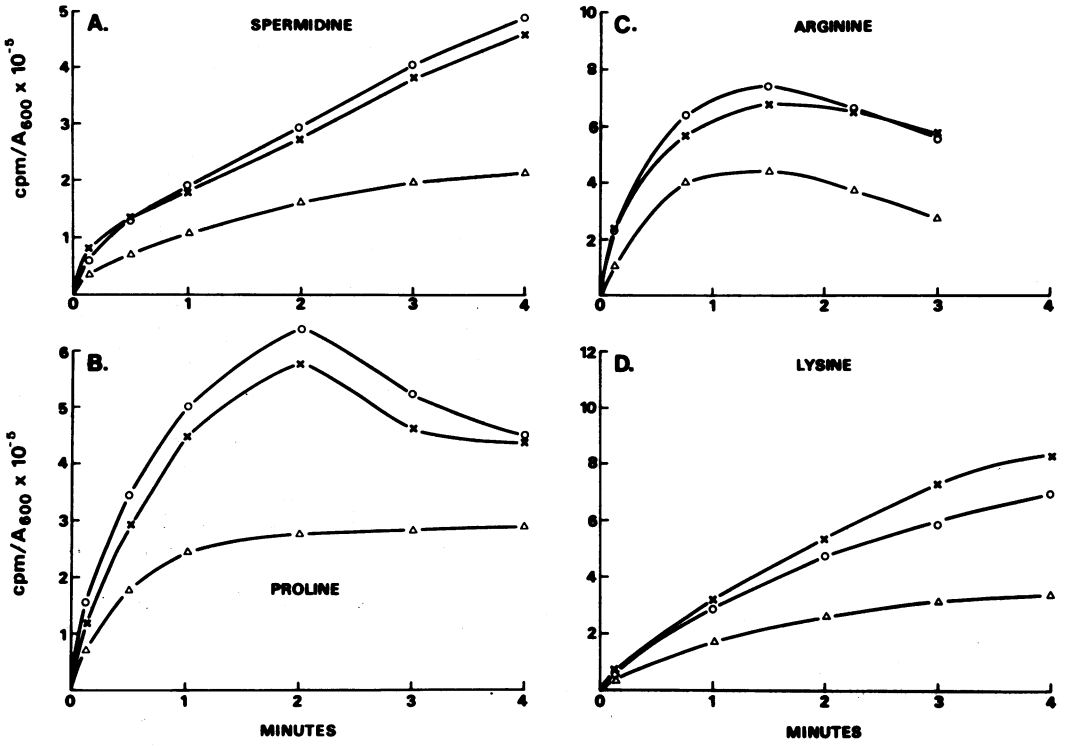

FIG. 3. *Uptake of (A) spermidine, (B) proline, (C) arginine, and (D) lysine by whole cells of PAO 503* (×), *PAO 2403* (△), *and PAO 2402* (○) *in CMM with methionine.*

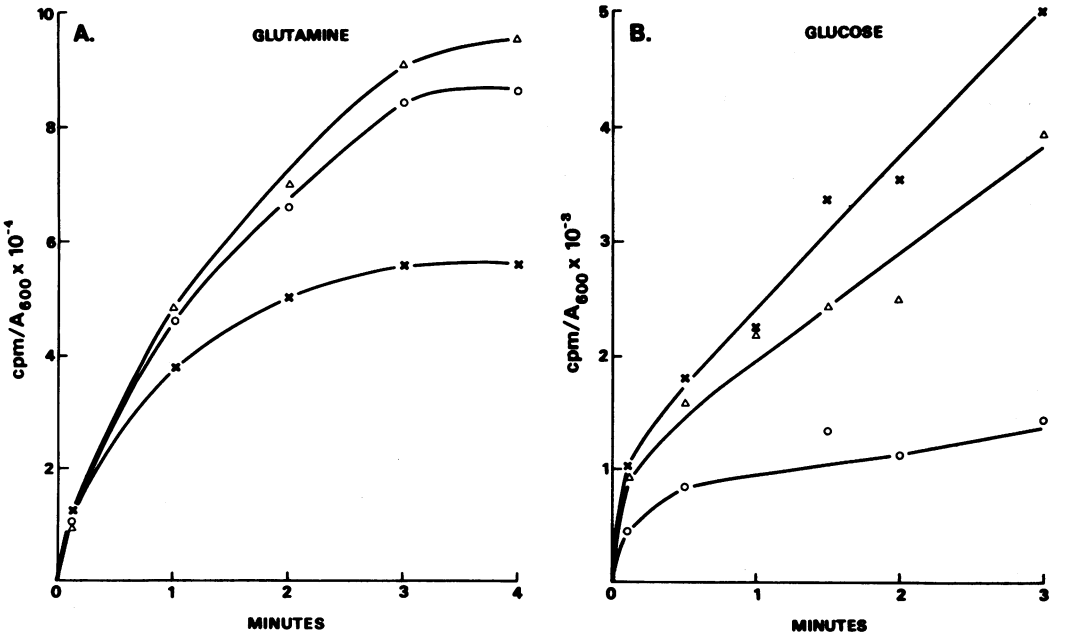

FIG. 4. *Uptake of (A) glutamine and (B) glucose by whole cells of PAO 503* (×), *PAO 2403* (△), *and PAO 2402* (○) *in CMM with methionine for glutamine and NB for glucose.*

change for either enzyme in any of the three strains. Thus, the increased MICs of PAO 2403 and PAO 2402 are due to lowered aminoglycoside transport. β-Lactam resistance was not altered by these mutations (Table 2).

DISCUSSION

The characterization of mutants PAO 2403 and PAO 2402 completes the description of the three gentamicin-resistant phenotypes obtained after ethyl methane sulfonate mutagenesis of PAO 503. These two mutants, like previously characterized mutant PAO 2401 (4), did not affect oxidative phosphorylation. No proton leaky, uncoupled mutants like those obtained with *E. coli* have been detected. It seems probable that uncoupled mutants are lethal in *P. aeruginosa*, which uses respiratory energy both aerobically and anaerobically.

The more gentamicin-resistant strain, PAO 2403, like the previously described mutant, PAO 2401, did reduce the rate of oxygen consumption and thus electron transport. However, PAO 2402, which is less resistant, did not significantly decrease oxygen consumption. The effects on transport were different among the three mutants. PAO 2401 resulted mainly in a specific decrease in aminoglycoside transport (4). PAO 2403 showed mainly a general decrease in transport rate for positively charged amino acids and the polyamine spermidine. This is consistent with PAO 2403's having a reduced electrical potential ($\Delta\psi$) component of the proton motive force (12). Transport of glutamic acid has been reported in some bacteria (11) to be driven mainly by the ΔpH component of the proton motive force, and its transport rate is normal. This is fully consistent with previous data supporting the proposal that aminoglycosides act as polycations and their transport is driven by $\Delta\psi$ (4).

PAO 2402 showed a marked decrease in glucose transport but no difference in transport of cationic materials other than aminoglycosides and no change in glutamic acid. Thus, it is likely that both $\Delta\psi$ and ΔpH components of the cellular proton motive force are normal in this strain. Aminoglycosides are sugars as well as polycations, but they show much structural diversity in the group and from glucose. Aminoglycoside and glucose transports are not competitive (7). Glucose is transported by an active transport system in *P. aeruginosa* rather than by group translocation (9). This is the first mutant described showing resistance to aminoglycosides that has an abnormality of sugar transport (3).

Glutamine transport was enhanced in PAO 2403 and PAO 2402. It was also increased in PAO 2401. It is not known whether glutamine transport is ATP dependent in *P. aeruginosa* as it is in *E. coli*. The results reported here would be consistent with ATP dependence in view of the difference in glutamine transport relative to transport of the other compounds studied, which are most likely dependent on cellular proton motive force. If glutamine transport is ATP dependent, then all three mutants have enhanced ATP-dependent transport.

Mutants PAO 2403 and PAO 2402 most likely involve a single mutation in view of their reversion rates to a fully wild-type phenotype. Thus, the decrease in cytochrome *d* and nitrite reductase is the result of a mutation affecting a common component of these two properties. *P. aeruginosa* has a nitrite reductase (ferrocytochrome c_{551} oxidoreductase, EC 1.9.3.2) with a molecular weight of 120,000 composed of two identical subunits, each possessing a heme c and heme d1. The enzyme functions primarily as a nitrite reductase but can also act as a cytochrome oxidase. It accepts electrons from reduced cytochrome c_{551} or azurin and is only formed in cells grown anaerobically with nitrate or nitrite (1, 14, 15). Nitrite reductase mutants of *P. aeruginosa* deficient in cytochrome *d* have been previously reported (13). However, the effect on transport of aminoglycosides, other compounds, or other components of electron transport and oxidative phosphorylation was not reported for these mutants.

PAO 2403 and PAO 2402 have similar functional abnormalities in nitrite reductase and cytochrome *d* deficiency but show markedly different transport abnormalities. It is not clear whether this is due to a different protein being altered or to the different degrees of the functional defects in the two mutants. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membranes of anaerobically nitrate-grown cells of PAO 2403, PAO 2402, and PAO 503 did not provide an answer to this problem.

The deficiency of nitrite reductase and cytochrome *d* in strain PAO 2403 caused a change in transport by aerobically grown cells. The reason for this is not clear, but oxygen consumption was reduced in aerobically grown PAO 2403, showing that the rate of terminal cytochrome oxidase activity was reduced. This suggests that some portion of the anaerobic cytochrome oxidase-nitrite reductase does affect aerobic electron transport and the development or maintenance of the driving force for, particularly, cationic solute transport. The PAO 2403 mutant is of particular interest because it shows a specific transport defect for cationic amino acids, polyamines, and aminoglycosides aerobically as a

result of a deficiency in a component of anaerobic electron transport.

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