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Resistance of *Pseudomonas aeruginosa* PAO to Nalidixic Acid and Low Levels of β-Lactam Antibiotics: Mapping of Chromosomal Genes

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Resistance to high concentrations of nalidixic acid in *Pseudomonas aeruginosa* PAO was due to mutations in one locus designated *nalA*, which was mapped by transduction between *hex-9001* and *leu-10*. The *nalA* mutants were cross-resistant to pipemidic acid, a nalidixic acid analog, at relatively low concentrations. Replicative DNA synthesis was resistant to both drugs in permeabilized cells of *nalA* mutants. A locus coding for low-level resistance to nalidixic acid, *nalB*, was cotransducible with *pyrB*, *proC*, and *met-28*. The *nalB* mutants were also resistant to low levels of pipemidic acid, novobiocin, and β-lactam antibiotics (e.g., carbenicillin, azlocillin, and cefsulodin), but not to other drugs, such as gentamicin, rifampin, kanamycin, or tetracycline. In *nalB* mutants, DNA replication showed wild-type sensitivity to nalidixic acid, whereas carbenicillin-induced filamentation required higher drug levels than in the wild-type strain. Thus, *nalB* mutations appear to decrease cell permeability to some antibiotics. The sensitivity of replicative DNA synthesis to nalidixic acid and novobiocin was very similar in *P. aeruginosa* and *Escherichia coli*; by contrast, the concentrations of these drugs needed to inhibit growth of *P. aeruginosa* were higher than those reported for *E. coli* by one or two orders of magnitude.

Pseudomonas aeruginosa is about 50 times more resistant to nalidixic acid (NAL) than are *Escherichia coli* and other enteric bacteria (12, 27). The minimal inhibitory concentration (MIC) of NAL usually ranges between 50 and 400 μg/ml in *P. aeruginosa* wild-type strains (27). This is an example of the intrinsic resistance of *P. aeruginosa* to many antibacterial agents (2).

In *E. coli*, NAL inhibits replicative DNA synthesis (8, 24); the target is the DNA gyrase subunit A (6, 7). Here we show that in permeabilized *P. aeruginosa* cells, DNA replication is sensitive to NAL; the NAL concentration needed to give 50% inhibition (ID_{50}) of DNA synthesis was very similar to that reported for permeabilized *E. coli* (24). Hence, it seems that the cell envelope critically influences the different susceptibilities of these bacteria to NAL. In mutants of *E. coli* that are resistant to high NAL levels, the DNA gyrase is not inhibited by the drug, in most instances because of a mutation in the *gyrA* (formerly *nalA*) gene (7, 12, 33). Mutations in the *nalB* locus cause low-level resistance to NAL by impeding the uptake of the drug (1, 12). *P. aeruginosa* mutants of the *nalA* type have been found in strains PAO (9) and PAT (31), and a *nalB* locus has been mapped in PAT (32). However, the physiological implications of these mutations have not been studied. In this paper, we characterize NAL-resistant mutants of PAO genetically and physiologically; in particular, we examine their susceptibilities to pipemidic acid (PIP; an NAL analog active against *P. aeruginosa* [15, 27]), novobiocin (an inhibitor of the DNA gyrase subunit B in *E. coli* [6, 7]), and β-lactam antibiotics.

Preliminary reports of this research have appeared (M. Rella and D. Haas, Experientia **36**:493, 1980; Soc. Gen. Microbiol. Quart. **8**:134, 1981).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. All strains are listed in Table 1. The phages F116L and G101 were used for transduction, phage G101c was used for strain construction, and phages E79, F116c, B3c, and B39 were used to test potential alterations of cell surface structure (13, 14). The plasmids FP2 and R68.45 were used for conjugation (9, 10, 14).

Media. Nutrient yeast broth, nutrient agar, minimal medium P, and minimal medium E were the same as previously described (9–11). The concentration of amino acids was 1 mM and that of pyrimidines was 0.1 mM when used as supplements. L-broth contained (per liter): 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 0.2% (wt/vol) glucose (21).

Isolation of mutants. Spontaneous or ethyl methane sulfonate-induced *nalA* mutants were obtained by plat-

TABLE 1. Strains of *P. aeruginosa*

Strain	Genotype[a]	Reference or derivation
PAO1	Wild type	14
PAO4	*pyrB52 arg-47*	Holloway collection
PAO25	*argF10 leu-10*	9
PAO177	*met-28 ilv-202 argB1 str-1*	9
PAO236	*met-28 trp-6 lys-12 his-4 pro-82 ilv-226 nalA2*	9
PAO303	*argB18*	11
PAO317	*argF10*	11
PAO372	*argH32 lys-58*	11
PAO477	*met-28 ilv-202 argB1 str-1 nalA12*	11
PAO505	*met-9011 amiE200*	29
PAO512	*argH32 lys-58 nalA7*	11
PAO513	*argB18 lys-60 nalA8*	11
PAO514	*argF10 nalA2*	Leu^+ NAL-resistant transductant of F116c. PAO236 × PAO25
PAO515	*met-9011 amiE200 nalA5*	NAL-resistant (≥2 mg/ml), PIP-resistant (50 μg/ml) derivative of PAO505
PAO643	*trpB4 car-9 proB64 nalA9*	10
PAO963	*met-9020 leu-9005 hex-9001 nalA16*	NAL-resistant (≥2 mg/ml), PIP-resistant (25 μg/ml) derivative of PAO1840
PAO969	*proC130*	Leisinger collection
PAO1819	*trpF9008 leu-9001*	20
PAO1840	*met-9020 leu-9005 hex-9001*	Matsumoto collection
PAO6001	*met-28 ilv-202 argB1 str-1 nalA12 pip-6001*	PIP-resistant (200 μg/ml) derivative of PAO477[b]
PAO6002	*met-9011 amiE200 nalB4*	PIP-resistant (25 μg/ml) derivative of PAO505[c]
PAO6003	*met-9011 amiE200 nalA5 pip-6003*	PIP-resistant (200 μg/ml) derivative of PAO515
PAO6004	*amiE200 pip-6003*	Met^+ $NalA^+$ derivative of PAO6003 × PAO303 (R68.45)
PAO6005	*proC130 nalB8*	PIP-resistant (25 μg/ml) derivative of PAO969[d]
PAO6006	*proC130 nalB9*	NAL-resistant (500 μg/ml) derivative of PAO969
PAO6007	*pip-6001*	Pro^+ PIP-resistant (25 μg/ml) transductant of G101c. PAO6001 × PAO969
PAO6008	*met-9020 leu-9005 hex-9001 nalB5*	PIP-resistant (50 μg/ml) derivative of PAO1840[d]
PAO6009	*met-9020 leu-9005 hex-9001 nalA16 nalB6*	PIP-resistant (200 μg/ml) derivative of PAO963
GMB123	*polA4* (=*mmsD4*)	19

[a] The genotype symbols are the same as those used for *E. coli*. *nalA* is used instead of *gyrA* because gyrase activity has not been determined in *P. aeruginosa*. *hex* designates hexose utilization, *ami* designates acetamide utilization, and *str* designates resistance to streptomycin.

[b] The parent strain PAO477 was resistant to 25 μg of PIP per ml.

[c] The parent strain PAO505 was resistant to 12.5 μg of PIP per ml.

[d] The parent strains PAO969 and PAO1840 were resistant to 6.25 μg of PIP per ml.

ing approximately 10^9 cells of a fresh overnight culture on nutrient agar plates containing 2,000 μg of NAL per ml (9). Spontaneous *nalB* and the *pip-6003* mutants were isolated similarly, except that the concentration of NAL was 500 μg/ml and that of PIP varied from 25 to 200 μg/ml.

Genetic crosses. Matings on the plate and transductions were performed as described previously (9–11). Scoring of *nalB* and *pip* loci in crosses was done by replica plating onto nutrient agar containing three PIP concentrations: 25, 50, and 100 μg/ml for $nalA^+$ strains and 50, 100, and 250 μg/ml for *nalA* strains.

Determination of MICs. Fresh overnight cultures were diluted in saline (0.9% [wt/vol] NaCl) to a concentration of 5×10^3 cells per ml, and 10-μl samples were spotted on solid media containing the drugs at twofold dilutions. All drugs were incorporated in nutrient agar except novobiocin, which gave sufficient growth inhibition only in supplemented minimal medium E. The MIC was read as the lowest concentration that caused complete growth inhibition after 24 h of incubation at 37°C.

Assay of DNA synthesis in vitro. Our method is based on that described by Vosberg and Hoffmann-Berling (30), who used ether to permeabilize *E. coli* cells suspended in basic medium (40 mM Tris-hydrochloride [pH 7.4], 80 mM KCl, 7 mM magnesium acetate, 2 mM EGTA, 0.4 mM spermidine·3HCl, and 0.5 M sucrose). The method used by Moses and Richardson (23), i.e., cell permeabilization with toluene and incubation in phosphate buffer, proved unsatisfactory for the assay of DNA synthesis in *P. aeruginosa*. The following procedure was adopted for strain PAO. An overnight culture was diluted 20-fold into 100 ml of L-broth and grown at 37°C with good aeration to approximately 2×10^8 cells per ml. A 50-ml portion of this culture was poured into 20 ml of cold basic medium (30), centrifuged, and washed with 7 ml of cold L-broth (five parts) plus basic medium (two parts). All further operations were carried out at 0 to 4°C. After

centrifugation, the cells were suspended in 1.5 ml of basic medium in a centrifuge tube containing a small magnetic bar. Cold ether (1.5 ml) was mixed with the cell suspension, using gentle magnetic stirring for 30 to 45 s (longer exposure resulted in loss of DNA synthesis activity). Then 2 ml of basic medium containing 0.8 M sucrose (instead of 0.5 M sucrose) was added to the bottom of the tube. Centrifugation at 1,100 × *g* for 10 min separated the cells from the upper ether-containing phases. The supernatant was sucked off completely, and the cell pellet was suspended gently in 0.45 ml of basic medium. The permeabilized cells (2×10^{10} cells per ml) were kept on ice and used the same day. They contained $\leq 10^6$ viable cells per ml as determined by plating on nutrient agar.

DNA synthesis was assayed in 1.0 ml of basic medium supplemented with dATP, dCTP, dGTP, [^{3}H]dTTP (at 40 μM each; specific activity of [^{3}H]dTTP was 1 μCi/40 nmol), 0.2 mM NAD, 2 mM ATP (omitted in controls measuring repair synthesis), and inhibitors as indicated. The reaction was started by the addition of 2×10^9 permeabilized cells. Incubation was at 37°C with gentle agitation. At 15-min intervals, 0.1-ml samples were removed and poured into 3 ml of ice-cold stop solution (10% [wt/vol] trichloroacetic acid, 0.1 M potassium pyrophosphate, and 0.05% [wt/vol] thymidine). After the samples were allowed to stand on ice for 30 min, the precipitate was collected on a Whatman GFC filter and washed three times with stop solution, once with 0.1 M HCl, and once with ethanol. The radioactivity on dried filters was determined in scintillation fluid (4 g of PPO [2,5-diphenyloxazole] plus 0.1 g of dimethyl-POPOP [1,4-bis-(5-phenyloxazolyl)benzene] in 1 liter of toluene), using a Beckman LS-250 scintillation counter; the counting efficiency was about 60%. Replicative DNA synthesis activity was calculated as total DNA synthesis (in the presence of 2 mM ATP) minus repair synthesis (without ATP).

Determination of ID_{50}. Replicative DNA synthesis activity was assayed after 30 min of incubation at four to six different inhibitor (NAL or PIP) concentrations. Double-logarithmic plots of the percentage of activity (relative to uninhibited controls) versus the inhibitor concentration were linear and served to determine the ID_{50} of DNA replication.

Chemicals. Carbenicillin was generously supplied by Beecham Pharmaceuticals, Worthing, England; PIP was a gift of Roger Bellon Laboratories, Neuilly-Paris, France; cefsulodin, ticarcillin, azlocillin, and piperacillin were kindly provided by W. Zimmermann, Basel, Switzerland. Novobiocin, nalidixic acid, streptomycin, tetracycline, gentamicin, and kanamycin were obtained from Sigma Chemical Co., St. Louis, Mo.; neomycin was obtained from Syntex Pharm AG, Allschwil, Switzerland; benzalkonium chloride was obtained from Fluka Laboratories, Buchs 5G, Switzerland; rifampin was obtained from Ciba-Geigy, Basel, Switzerland; and polymyxin B was obtained from Novo Industri A/S, Copenhagen, Denmark. Deoxyribonucleoside triphosphates and NAD were from Boehringer Mannheim Corp., Rotkreuz, Switzerland. [^{3}H]dTTP was supplied by New England Nuclear Corp., Boston, Mass. Habs O:5 antiserum (Institut Pasteur Production, Paris, France) was used according to the manufacturer's recommendations.

RESULTS

Mapping of mutations conferring NAL and PIP resistance. (i) Mutants highly resistant to NAL (≥2 mg/ml in nutrient agar or minimal medium) were obtained spontaneously at frequencies of 10^{-8} to 10^{-9}. The *nalA7* mutation (in strain PAO512) was mapped between *hex-9001* and *leu-9005* (*leu-10*) by FP2-mediated conjugation. The marker order was *amiE hex-9001 nalA leu-9005 met-9020*; the recipient strains were PAO505, PAO1819, and PAO1840 (data not shown). Three mutations, *nalA2* (in PAO514), *nalA5* (in PAO515), and *nalA7* (in PAO512), were mapped by transduction with phage F116L. They were all cotransducible with *leu-9005* or *leu-10* (0.8 to 1.4% linkage) and *hex-9001* (30 to 40% linkage), but not with *met-9020* or *amiE* (Fig. 1A). Experiments using phage G101 confirmed these data. However, no linkage between *nalA* and *leu-10* was detectable (Fig. 1A) in this case. All eight *nalA* loci examined (Table 1) gave similar cotransduction values (23 to 40%) with *hex-9001*. Previous failure to detect cotransduction between *nalA* and *hex* (14) appears to be due to anomalous expression of NAL resistance encoded by *nalA2; hex-9001*$^+$ *nalA2* transductants of PAO1840 grew only on nutrient agar containing NAL, but not on minimal media with NAL, which were normally used to score NAL resistance. Transductions with *nalA2* or *nalA7* in the donor and *nalA16* in the recipient gave no (<0.3%) NAL-susceptible segregants when selection was made for Hex$^+$. Thus, these *nal* mutations are very closely linked. Based on the biochemical evidence (shown below), all mutations described here that led to high-level NAL resistance were classified as *nalA*.

(ii) Since *nalA* mutations conferred cross-resistance to PIP (Table 2), they could also be obtained by selection for PIP resistance (on nutrient agar with 50 to 100 μg of PIP per ml) in *nalA*$^+$ strains (data not shown). A mutation (*pip-6003*) leading to increased PIP resistance (200 μg/ml) in the *nalA* mutant PAO515 (resistant to 100 μg of PIP per ml) was obtained and mapped near *nalA* (Fig. 1A). The linkage data do not allow us to decide whether *pip-6003* is allelic with, or just very close to, *nalA*.

(iii) Spontaneous mutants resistant to PIP (25 to 100 μg/ml) or low levels of NAL (500 μg/ml) were isolated at frequencies of 10^{-7} to 10^{-8}. Many of these mutants resemble genetically the *nalB* mutants of strain PAT described by Watson and Holloway (31). The marker order *nalB pyrB proC met-28* was the same in both strains (Fig. 1B; 31). In strain PAO, five mutations were tentatively grouped together in the *nalB* locus. They gave similar cotransduction values with

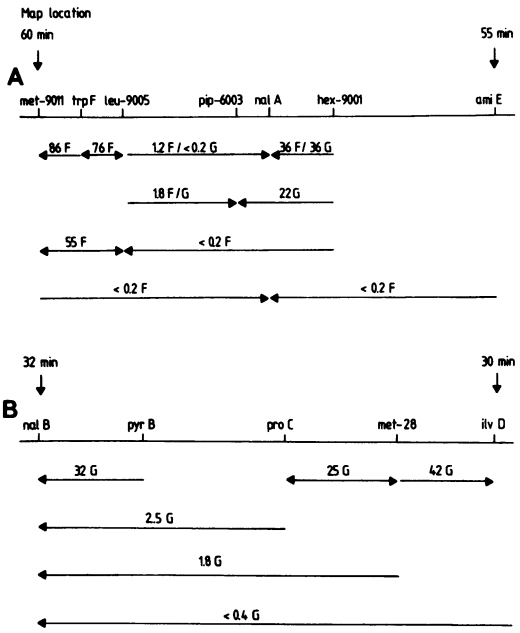

FIG. 1. Mapping of *nalA*, *pip-6003*, and *nalB* loci by transduction. Values indicate the percentage of cotransduction (mean of several experiments) with phage F116L (F) or G101 (G). From 400 to 600 recombinants were scored for coinheritance of unselected markers. Arrows point to the unselected marker. Map locations are taken from reference 14; closely linked markers are *leu-9005*, *leu-9001*, and *leu-10*, and *met-9011* and *met-9020* (Matsumoto, personal communication). *proC* and *pyrB* are 55% cotransducible (26). In strains carrying the *hex-9001* mutation, selection for auxotrophic markers was made on minimal medium P containing acetamide. (A) The following alleles were used to map *nalA*: *nalA2*, *nalA5*, and *nalA7*. A transductional cross, F116L. PAO6003 × PAO25, suggested the marker order shown, *leu-10 pip-6003 nalA5*. (B) The following alleles were used to map *nalB*: *nalB4*, *nalB5*, *nalB6*, *nalB8*, and *nalB9*.

pyrB (23 to 48%) and *proC* (2 to 4%), and conjugational crosses between them yielded no NAL-susceptible progeny. The *pip-6001* mutants PAO6001 and PAO6007 were phenotypically similar to the *nalB* mutants (Table 2), but *pip-6001* gave higher cotransduction with *proC* (13 to 18%), and cross PAO6005 ($FP2^+$) × PAO6001 produced 1.5% NAL-susceptible recombinants (selection for Met^+). Hence, *pip-6001* may lie between *nalB* and *pyrB*, but its precise location has not been determined.

Susceptibility of mutants to NAL, PIP, and other drugs. Six *nalA* mutants showed cross-resistance to PIP; however, some highly NAL-resistant strains remained susceptible to relatively low PIP concentrations (Table 2). This phenomenon of incomplete cross-resistance has already been observed in *E. coli* (15). The *nalA2* mutation gave a fourfold reduction in the MIC of

TABLE 2. MICs of NAL, PIP, novobiocin, and β-lactam antibiotics in *P. aeruginosa* mutants

Mutation	Strain(s) tested	NAL		PIP		Novobiocin		Carbenicillin[c]	
		MIC range[a]	Relative MIC[b]	MIC range	Relative MIC	MIC range	Relative MIC	MIC range	Relative MIC
Wild type	PAO1, PAO177, PAO317, PAO372, PAO505, PAO969, PAO1840	50	1	12.5–50	1	100–800[d]	1	12.5–50	1
nalA	PAO477, PAO512, PAO514, PAO515, PAO963	≥2,000	≥40	50–400	4–16	100–1,600	0.25–1[e]	12.5–50	1
pip-6003	PAO6004	400	8	100	4	50	0.25	25	1
nalB	PAO6002, PAO6005, PAO6006, PAO6008	800	16	50–100	4–8	800–1,600	4–8	200	4–8
pip-6001	PAO6007	800	16	50	4	1,600	4	200	8

[a] MIC range is expressed in micrograms per milliliter. Where single MIC values are given, the same MIC was found for all strains tested.
[b] Relative MICs are expressed as the ratio of the MIC for a mutant strain divided by the MIC for the parent strain.
[c] Other β-lactam antibiotics gave the same relative MICs. The parent strains had the following MICs: ticarcillin, 8 to 32 μg/ml; piperacillin, 2 to 8 μg/ml; azlocillin, 2 to 8 μg/ml; and cefsulodin, 2 to 4 μg/ml.
[d] MICs were determined in minimal medium; in nutrient agar, the MIC was >4,000 μg/ml.
[e] The 0.25 MIC was found for *nalA2* only.

novobiocin (Table 2). A range of other antimicrobial agents had essentially the same effect on *nalA* mutants and their parents.

The *pip-6003* mutation was unique in that it gave low-level resistance to NAL and PIP and increased susceptibility to novobiocin (Table 2). Kropinsky et al. (18) described rough, E79-resistant mutants of strain PAO defective in lipopolysaccharide that display low-level NAL resistance. Matsumoto (14, 20) has mapped the markers *eseA* (E79 resistance), *somA* and *somB* (somatic O antigen, a lipopolysaccharide) between *leu-9005* and *hex-9001*. Because the *pip-6003* mutation was mapped in the same region (Fig. 1A), we considered the possibility that this mutation might affect the lipopolysaccharide structure. However, strain PAO6004 (*pip-6003*) plated phage E79 normally and behaved similarly to *pip*$^+$ strains in an agglutination test with Habs O:5 antiserum. Hence, a gross lipopolysaccharide defect is unlikely in this mutant. Because the drug sensitivity of DNA replication was slightly altered in PAO6004 (Table 3), we assume that the *pip-6003* mutation affects a component of DNA synthesis.

Five *nalB* and the *pip-6001* mutants were four to eight times more resistant to NAL, PIP, novobiocin, and β-lactam antibiotics (carbenicillin, ticarcillin, piperacillin, azlocillin, and cefsulodin) than were the parent strains (Table 2). By contrast, these mutants showed no significant changes in their susceptibilities to other antimicrobial agents (streptomycin, neomycin, gentamicin, tetracycline, rifampin, polymyxin B, EDTA, benzalkonium chloride, and mercuric chloride) and phages E79, F116c, G101c, B3c, and B39. It appears that a single mutation in *nalB* was responsible for the pleiotropic resistance pattern observed. All PIP-susceptible (*nalB*$^+$) transductants of PAO6002, PAO6005, PAO6006, PAO6008, and PAO6009 obtained when selection was made for Pro$^+$ had acquired parent-type susceptibilities to carbenicillin and cefsulodin.

Inhibition of DNA synthesis by NAL and PIP. *P. aeruginosa* cells permeabilized with ether (30) were used to measure incorporation of [^{3}H]dTTP into DNA (cold trichloroacetic acid-precipitable material). Wild-type cells synthesized DNA in vitro for at least 1 h (Fig. 2A). About 70% of the activity was dependent on added ATP, was strongly inhibited by NAL (Fig. 2A), and presumably is due to replicative DNA synthesis as in *E. coli* (23, 28). About 30% of the activity was ATP independent, was noninhibitable by NAL (Fig. 2A), and reflects repair synthesis (23, 28). Strain GMB123, which is defective in DNA polymerase I (19), lacked ATP-independent DNA synthesis almost completely (Fig. 2B). Replicative DNA synthesis stopped after 15 to 30 min in this mutant, but inhibition by NAL occurred as in permeabilized wild-type cells (Fig. 2B and other data not shown). The proportion of ATP-independent DNA synthesis was greater in *P. aeruginosa* (Fig. 2A) than in *E. coli* (28). In the wild-type PAO strains, the NAL concentration required to bring about ID_{50} of replicative DNA synthesis was about 10 μg/ml (Table 3). The corresponding ID_{50}s reported for toluenized cells of *E. coli* and *Serratia marcescens* are 13.6 and 2.6 μg/ml, respectively (24). Thus, in vitro the replicative DNA syntheses of these three organisms show similar sensitivities to NAL. By contrast, for growth inhibition the MICs of NAL were 50 μg/ml in strain PAO (Table 2) and 1 to 3 μg/ml in *E. coli* (12, 15, 27). This implies that *P. aeruginosa* and *E. coli* differ in their permeability for NAL. PIP gave the same ID_{50}s as NAL for replicative DNA synthesis in *P. aeruginosa*, but was generally a more potent growth inhibitor than was NAL (Table 2), probably because PIP was taken up more readily. There was no obvious correlation between the genotypes of various PAO mutants and their susceptibilities to PIP, the MICs ranging from 12.5 to 50 μg/ml.

In vitro, replicative DNA synthesis in *nalA* mutants was resistant to both NAL and PIP; the

TABLE 3. Inhibition of replicative DNA synthesis in permeabilized *P. aeruginosa* cells

Mutation	NAL			PIP		
	ID_{50} range[a] (μg/ml)	No. of strains tested	Relative ID_{50}[b]	ID_{50} range (μg/ml)	No. of strains tested	Relative ID_{50}
Wild type	6–16	6	1	6–12	5	1
nalA	180–400	6	20–40	40–45	2	6
pip-6003	25	1	1.5	15	1	2
nalB	10	2	1	6–7	3	1
pip-6001	ND[c]	0	ND	6	1	1

[a] ID_{50}s were determined as described in Materials and Methods.
[b] Relative ID_{50}s are expressed as the ratio of the ID_{50} for a mutant strain divided by the ID_{50} for the parent strain.
[c] ND, Not determined.

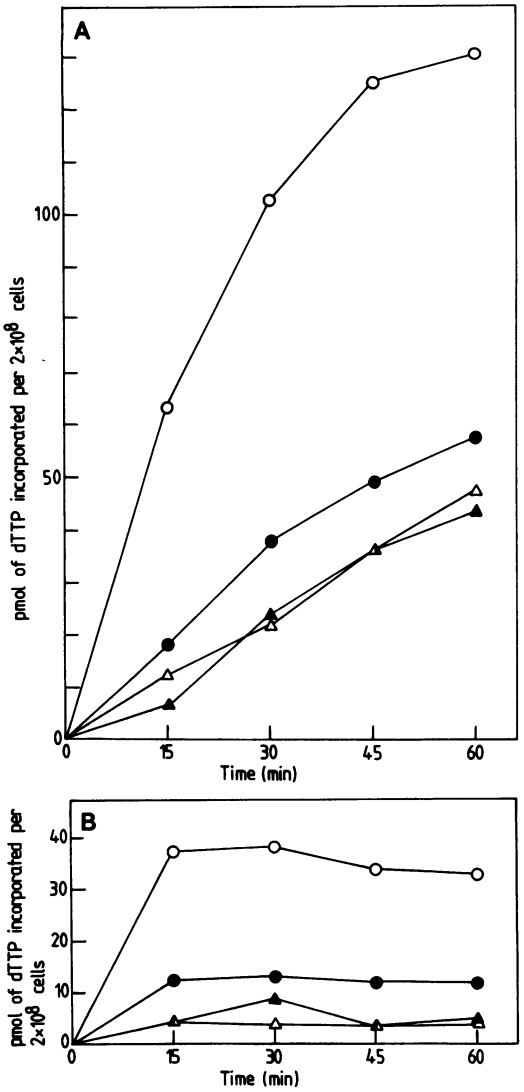

FIG. 2. DNA synthesis in permeabilized cells of *P. aeruginosa*. Standard assay conditions were used as described in Materials and Methods. (A) strain PAO1, (B) strain GMB123 (*polA4*). Symbols: ○, plus ATP; ●, plus ATP plus NAL (200 μg/ml); △, without ATP; and ▲, without ATP plus NAL (200 μg/ml).

pip-6003 locus conferred low-level resistance to both drugs (Table 3). By contrast, the *nalB* mutations had no influence on the inhibition of replication by both drugs, and the *pip-6001* locus in PAO6007 did not render replicative DNA synthesis resistant to PIP (Table 3). These results indicate that *nalA* and *pip-6003* affect DNA synthesis, whereas the findings for *nalB* and *pip-6001* are consistent with a reduced uptake of NAL and PIP.

Novobiocin strongly inhibited ATP-dependent synthesis in permeabilized wild-type cells (ID_{50} = 0.3 μg/ml); however, a biphasic inhibition curve indicated that there may exist two targets having different sensitivities to this drug (data not shown). Two *nalA* mutants (*nalA12* and *nalA5*) had wild-type sensitivities to novobiocin in permeabilized cells. Whole cells of *P. aeruginosa* PAO1 were intrinsically resistant to novobiocin; the MIC was approximately 400 μg/ml in minimal medium (Table 2) and >4 mg/ml in nutrient agar. These values are about 100 times higher than those reported for *E. coli* K-12 (4, 33). As in the case of NAL, the resistance of *P. aeruginosa* to novobiocin is probably due to a permeability barrier. However, an enzymatic inactivation of NAL and novobiocin by *P. aeruginosa* remains possible.

Inhibition of carbenicillin-induced filamentation in a *nalB* mutant. Sublethal concentrations of carbenicillin induce the formation of long filaments in *P. aeruginosa* (5). In liquid medium, strain PAO969 (*nalB*$^+$) began to form filaments (observed in a phase-contrast microscope) at 4 μg of carbenicillin per ml. Carbenicillin concentrations of ≥4 μg/ml resulted in a decrease of colony-forming units, whereas the cell mass (measured by the optical density) continued to increase exponentially (Fig. 3A). The *nalB* mutant PAO6005 behaved similarly, but filamentation and inhibition of colony-forming units started only at 16 μg/ml (Fig. 3B). Thus, it appears that a permeability barrier in the mutant prevented the antibiotic from reaching its target(s).

DISCUSSION

High-level NAL resistance mutations in strain PAO were characterized by several common features. (i) All mutations examined could be localized in the same chromosome region, and there was no evidence for more than one genetic locus. Thus, the previous classification into *nalA* and *nalC* loci (14), which was essentially based on the apparent lack of linkage of *nalA2* to *hex*, seems to be unnecessary, and we now use the symbol *nalA* for all high-level NAL resistance mutations mapped between *hex* and *leu-10*. (ii) The DNA replication was NAL resistant in permeabilized cells of *nalA* mutants. Because we have not measured gyrase activity, the designation *gyrA* (now used in *E. coli*) was not adopted. (iii) Our *nalA* mutants generally showed no pleiotropic effects on the susceptibility to other antibiotics. The *nalA2* mutation was exceptional; it conferred increased susceptibility to novobiocin and was reminiscent of the *nalA* mutation found in *E. coli* KL166 (4). (iv) The *nalA* mutants were cross-resistant to the NAL analog PIP, but as in *E. coli* (15), they were less resistant to PIP than to NAL. It would be interesting to test *nalA* mutants for their suscep-

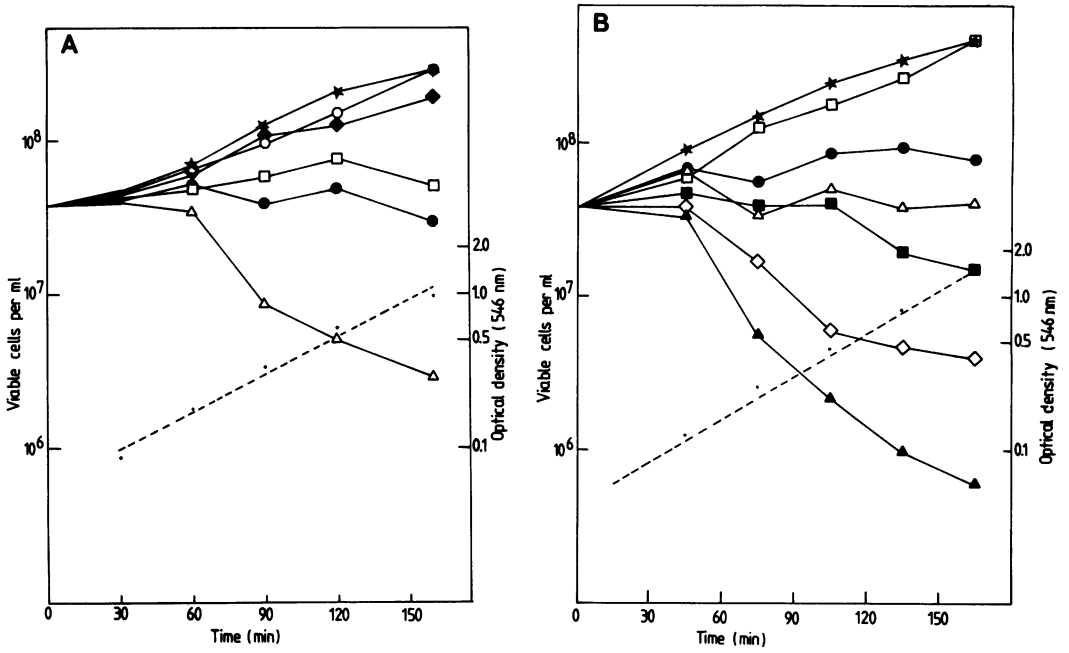

FIG. 3. Growth inhibition of strain PAO969 and its *nalB* derivative PAO6005 by carbenicillin. Growth of PAO969 (A) and PAO6005 (B) in nutrient yeast broth containing different carbenicillin concentrations at 37°C, with aeration, was assayed by measuring the viable counts (——) and the optical density (– – –). The increase in optical density was the same in all cultures regardless of carbenicillin concentration. Carbenicillin was added at time zero at the following concentrations: ★, 0 μg/ml; ○, 2 μg/ml; ◆, 4 μg/ml; □, 8 μg/ml; ●, 16 μg/ml; △, 32 μg/ml; ■, 64 μg/ml; ◇, 128 μg/ml; and ▲, 256 μg/ml.

tibility to AM-715, a NAL analog highly active against *P. aeruginosa* (17).

The *pip-6001* and *nalB* mutations were pleiotropic; they affected the susceptibility to NAL, PIP, novobiocin, carbenicillin, and other β-lactam antibiotics. The *nalB8* locus (in strain PAO6005), for example, caused resistance to PIP in whole cells but not in ether-permeabilized cells used for the assay of replication. Moreover, *nalB8* increased the concentration of carbenicillin needed to induce filamentation. The simplest explanation for these phenotypes is that *nalB8* modified cell permeability. There is evidence that outer membrane proteins can modulate the susceptibility of *P. aeruginosa* to β-lactam antibiotics (16, 22, 34). In *Salmonella* and *E. coli*, mutations in porin genes can change the permeation of these drugs (25). The *nalB* gene may therefore code for an outer membrane protein, but other explanations are also possible, e.g., alterations of penicillin-binding proteins in *nalB* and *pip-6001* mutants. Electron microscopic pictures of *nalB* mutants revealed no obvious defects of the outer membrane (H. Ebersold, personal communication).

All resistance mutations studied here were chromosomal. R plasmids specifying NAL resistance have not been described in the literature (3). Mutations of the *nalB* type were obtained spontaneously at a high frequency (10^{-7} to 10^{-8}) and may be of interest because they lead to resistance to clinically useful β-lactam antibiotics. Bryan (2) concluded that this type of low-level resistance may be of greater importance than high-level resistance encoded by R plasmids in the therapy of *P. aeruginosa* infections.

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