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Salicylate-Inducible Antibiotic Resistance in *Pseudomonas cepacia* Associated with Absence of a Pore-Forming Outer Membrane Protein

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The most common mechanism of antibiotic resistance in multiply resistant *Pseudomonas cepacia* is decreased porin-mediated outer membrane permeability. In some gram-negative organisms this form of antibiotic resistance can be induced by growth in the presence of weak acids, such as salicylates, which suppress porin synthesis. To determine the effects of salicylates on outer membrane permeability of *P. cepacia*, a susceptible laboratory strain, 249-2, was grown in 10 mM sodium salicylate. Antibiotic susceptibility and uptake, as well as outer membrane protein patterns, were compared between strain 249-2 grown with and without salicylates. The MICs of chloramphenicol, trimethoprim, ciprofloxacin, and ceftazidime were compared between organisms grown in standard and salicylate-containing medium and are as follows: chloramphenicol, 12.5 versus 100 μg/ml; trimethoprim, 0.78 versus 3.125 μg/ml; ciprofloxacin, 0.4 versus 1.56 μg/ml; ceftazidime, 3.125 versus 3.125 μg/ml. The permeability of β-lactam antibiotics was calculated from the rate of hydrolysis of the chromogenic cephalosporin, PADAC. There was no significant difference between strains grown in the presence and absence of salicylate. By using high-pressure liquid chromatography quantitation of loss from culture medium, the effect of 10 mM salicylate on the cellular permeability of chloramphenicol was measured in strain 249-2 by introduction of a plasmid which encodes production of chloramphenicol acetyltransferase. After 1 h of incubation, 18.5% ± 1.54% versus 70.1% ± 3.52%, and after 2 h, 4.20% ± 1.65% versus 41.90% ± 2.16% remained in supernatants from organisms grown in the absence and presence of 10 mM salicylate, respectively. Outer membrane protein pattern analysis demonstrated the absence of a protein of apparent molecular weight of 40,000 when strain 249-2 was grown in the presence of 10 mM salicylate. To determine whether this protein functioned as a porin, reconstituted membrane vesicles were constructed to assess antibiotic permeability. Vesicles constructed with this salicylate-suppressible outer membrane protein (OpcS) were permeable to chloramphenicol but not to penicillin G. These findings suggest that OpcS is a selective, antibiotic-permeable porin which can be suppressed by growth in the presence of salicylate. Further investigation will be required to determine the biochemical effects of salicylate on porin synthesis.

Pseudomonas cepacia is a ubiquitous, highly antibiotic-resistant gram-negative bacterium which has resulted in outbreaks of nosocomial infections (12, 20, 26) and may cause severe pulmonary infections in children and young adults with cystic fibrosis (22, 37). The organism is often quite difficult to eradicate, even in the face of appropriate antimicrobial therapy (14, 15), and susceptible strains readily develop resistant phenotypes. Studies of specific antibiotic resistance mechanisms in *P. cepacia* have demonstrated antibiotic-inactivating enzymes such as β-lactamase (3, 9, 21, 36) and resistant antibiotic targets such as dihydrofolate reductase (7); however, in the majority of multiply resistant strains, decreased porin-mediated outer membrane permeability is the mechanism of resistance (2, 6, 35). In addition, intrinsic resistance to polycationic antibiotics such as polymyxin and the aminoglycosides appears to be related to the outer membrane organization of *P. cepacia* (29).

Porins are major outer membrane proteins which form transmembrane pores that permit the diffusion into bacterial cells of hydrophilic compounds including sugars, amino acids, and antibiotics (see reference 17 for a review). In some gram-negative organisms, antibiotic resistance can be induced by growth in weak acids, such as salicylates, which suppress porin synthesis (13, 38, 40). Because many *P. cepacia* strains demonstrate intrinsic decreased porin-mediated permeability, we investigated the effects of growth in salicylates on antibiotic susceptibility and uptake and on outer membrane proteins and their function.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. cepacia* 249-2, a Lys^- Pen^s laboratory strain (5) was used in all experiments. Plasmid pJLB2, a 10.7-kb, broad-host-range, mobilizable plasmid encoding resistance to tetracycline, ampicillin (production of TEM-type β-lactamase), and chloramphenicol (production of chloramphenicol acetyltransferase [CAT]) (6), was introduced into 249-2 for use in β-lactam and chloramphenicol penetration studies. The plasmid was maintained in culture by growth in tetracycline to eliminate the possibility that ampicillin or chloramphenicol in the culture medium would interfere with uptake assays.

Media. The liquid media used in this study were Mueller-Hinton broth for MIC determinations and outer membrane protein preparation and M9 Casamino Acids medium (25) for chloramphenicol uptake experiments. Broth cultures were incubated at 37°C and shaken at 150 cycles per min. Plate cultures were incubated at 37°C on OFPBL medium (45).

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Strains were stored at −80°C in skim milk and inoculated onto fresh agar medium 16 to 40 h prior to use.

MICs. Determination of the MICs of chloramphenicol, trimethoprim, ciprofloxacin, and ceftazidime were performed by standard broth dilution technique with an inoculum of 10^4 CFU (43). For each antibiotic, MICs were determined under standard conditions and in the presence of 10 mM sodium salicylate.

β-Lactam penetration. The permeability of β-lactam antibiotics was calculated from the rate of hydrolysis of the chromogenic cephalosporin, PADAC (Calbiochem, La Jolla, Calif.) (31) in intact bacterial cells grown under standard conditions or in the presence of 10 mM sodium salicylate (2). Cell-free lysates of strain 249-2(pJLB2) were assayed to determine the K_m and maximum rate of metabolism (V_{max}) for the reaction in the presence and absence of salicylate. PADAC concentrations used for cell lysates were 0.005, 0.01, 0.02, and 0.05 mM. The extracellular concentration (S_o) of PADAC in intact cells was 0.01 mM; the Zimmermann and Rosselet equation (46), $S_e = (V_{intact} \times K_m)/(V_{max} - V_{intact})$, was used to calculate the intracellular concentration (S_e).

Chloramphenicol uptake. The cellular permeability of chloramphenicol into organisms grown under standard conditions or in the presence of 10 mM sodium salicylate was measured in strain 249-2(pJLB2) by using high-pressure liquid chromatography (HPLC) quantitation of the decrease of chloramphenicol concentration in culture medium (6, 8). The plasmid pJLB2, which encodes production of CAT, was introduced into 249-2 for these experiments because CAT catalyzes the acetylation of the chloramphenicol within the periplasmic space to maintain the concentration gradient across the outer membrane. Briefly, organisms were grown to mid-logarithmic phase ($3.05 \times 10^8 \pm 2.2 \times 10^8$ CFU/ml) in M9 Casamino Acids broth with or without 10 mM salicylate, chloramphenicol was added (initial concentration in the culture medium was 100 μg/ml), the culture was incubated at 37°C, and the medium was sampled immediately and 1 and 2 h following addition. The effect of salicylate on CAT activity was assayed by using the spectrophotometric assay of Shaw and Brodsky (41).

Outer membrane proteins. Outer membrane proteins were isolated as previously reported (6) from strain 249-2 grown in standard Mueller-Hinton broth and broth containing 10 mM sodium salicylate. The proteins were analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (24) and visualized by staining in 0.1% Coomassie brilliant blue (16). This preparation was used for gel purification of specific outer membrane proteins.

The porin fraction used in functional assays was prepared by using a modification of the technique of Parr et al. (35). The procedure was identical with the exception that the sample was not subjected to gel filtration after acetone precipitation. The resultant porin fraction contained the 81-kDa oligomer described by Parr et al. (35) which dissociated to subunits of approximate molecular weights of 36,000 and 27,000.

Gel purification of outer membrane proteins. Outer membrane proteins of interest were isolated by preparative SDS–10% polyacrylamide gel electrophoresis and electroeluted from gel slices into 0.1% SDS–0.025 M Tris (pH 8.3)–0.192 M glycine by using a BRL model 422 Electro-Eluter according to the manufacturer's instructions.

Reconstituted membrane vesicles. Reconstituted membrane vesicles to test the pore-forming ability of outer membrane proteins were prepared by the method of Hancock and Nikaido (18). Vesicles were constructed as described by using 0.5 μmol of commercially available dioleylphosphatidyl choline (Sigma, St. Louis, Mo.), 0.04 μmol of purified *P. cepacia* lipopolysaccharide (11), and 30 μg of the outer membrane protein of interest. Control vesicles were reconstituted from dioleylphosphatidyl choline and lipopolysaccharide without added outer membrane proteins to ensure that apparent antibiotic permeability did not result from diffusion through lipid membranes. The vesicles were formed in the presence of an antibiotic and either sucrose (a porin-permeable sugar) or inulin (a porin-impermeable sugar), each radiolabelled with a different isotope (either ^3H or ^{14}C; 2.5×10^5 cpm of each was added). Radioisotopes used included [^{14}C]chloramphenicol (specific activity, 60 mCi/mmol; New England Nuclear, Wilmington, Del.), [^{14}C]sucrose (specific activity, 3.6 mCi/mol; New England Nuclear), [^3H]inulin (specific activity, 288 mCi/g; New England Nuclear), and [^3H]penicillin (specific activity, 21 Ci/mmol; Amersham). After separation from unenveloped counts by gel filtration, the radiolabel remaining within the vesicles was quantitated by scintillation spectroscopy, the ratio of retained ^{14}C and tritium was normalized by comparison with the ratio in the initial buffer, and the results were expressed as the ratio between the ^{14}C-labelled and tritiated compounds.

TABLE 1. Antibiotic susceptibility in the presence and absence of 10 mM sodium salicylate

Antibiotic	MIC (μg/ml)[a]	
	−Salicylate[b]	+Salicylate[c]
Chloramphenicol	12.5	100
Trimethoprim	0.78	3.125
Ciprofloxacin	0.4	1.56
Ceftazidime	3.125	3.125

[a] The MICs were determined by broth dilution technique with a 10^4 CFU inoculum.
[b] MICs performed in MH broth.
[c] MICs performed in MH broth with the addition of 10 mM sodium salicylate.

RESULTS

Antibiotic susceptibility. The MICs of chloramphenicol, trimethoprim, ciprofloxacin, and ceftazidime in the absence and presence of 10 mM sodium salicylate are listed in Table 1. The MIC of the first three antibiotics increased in the presence of salicylate; however, the MIC of ceftazidime remained unchanged.

Antibiotic uptake. β-Lactam penetration into intact cells was compared in 249-2(pJLB2) grown with and without 10 mM sodium salicylate. The β-lactamase encoded by pJLB2 hydrolyzes the β-lactam ring of the chromogenic cephalosporin and, when assayed in intact and disrupted cells, permits the quantitation of outer membrane penetration of β-lactams. The Zimmermann-Rosselet equation (46) was used to calculate the intracellular concentration of PADAC (S_e); the extracellular concentration (S_o) was 0.01 mM. For 249-2(pJLB2) grown without salicylates, $S_e/S_o = 0.66$; with salicylate, $S_e/S_o = 0.65$.

Chloramphenicol penetration into intact cells was compared in 249-2(pJLB2) grown in the presence and absence of 10 mM sodium salicylate. HPLC was used to quantitate chloramphenicol entry into the periplasmic space in strain 249-2 expressing CAT which is encoded by pJLB2. Sampling

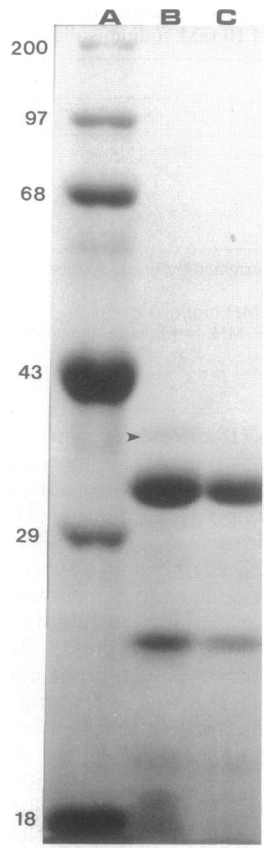

FIG. 1. Coomassie blue-stained polyacrylamide gel of outer membrane proteins of strain 249-2 grown under standard conditions and in the presence of 10 mM sodium salicylate. Lane A, molecular weight markers; lane B, standard conditions; lane C, plus 10 mM salicylate. Arrowhead denotes the protein not visualized when the organism was grown in the presence of salicylate.

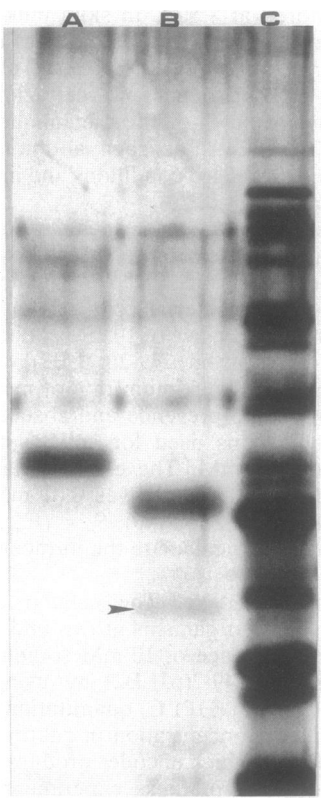

FIG. 2. Silver-stained SDS-polyacrylamide gel of proteins electroeluted from a preparative gel. Lane A, electroeluted OpcS (it is the only band present in this lane); lane B, electroeluted OpcP (the 36-kDa porin subunit appears to be contaminated with a small amount of the 27-kDa subunit [arrowhead]); lane C, the same outer membrane preparation of strain 249-2 as seen in Fig. 1, lane B; more bands are visible because the silver stain is more sensitive than the Coomassie blue stain.

at 1 and 2 h after addition of 100 μg of chloramphenicol per ml was compared between strains grown with and without 10 mM sodium salicylate. After 1 h of incubation, 18.50% ± 1.54% and 70.13% ± 3.52% of the original chloramphenicol was remaining in culture supernatants of strains grown in the absence and presence of salicylate, respectively. At 2 h, 4.20% ± 1.65% and 41.90% ± 2.16% was remaining, respectively. Thus, at 2 h, 10-fold-less uptake was demonstrated when the organism was grown in the presence of salicylate. There was no significant effect of 10 mM salicylate on either the production or the enzymatic activity of CAT determined by using the spectrophotometric assay.

Outer membrane proteins. A Coomassie blue-stained SDS-polyacrylamide gel of the outer membrane proteins of strain 249-2 grown under standard conditions and in the presence of 10 mM sodium salicylate is shown in Fig. 1. A minor outer membrane protein of apparent molecular weight of 40,000 is not demonstrated when the organism is grown in the presence of salicylate. We have termed this protein OpcS (*P. cepacia* salicylate-suppressible outer membrane protein). Gel-purified OpcS and the 36-kDa porin (which we termed, OpcP) which were used in functional studies are shown in Fig. 2.

Function of OpcS in vesicles. The electroeluted OpcS was tested in reconstituted membrane vesicles to determine whether it had pore-forming function comparable to that of the electroeluted porin, OpcP. The transmembrane penetration of chloramphenicol and penicillin G was tested in comparison with the penetration of inulin (porin impermeable) and of sucrose (porin permeable). Control experiments used vesicles constructed with lipopolysaccharide and phospholipid without the addition of outer membrane proteins to ensure that diffusion through phospholipid bilayers could be distinguished from diffusion through porin channels. Control vesicles containing no outer membrane proteins demonstrated a ratio between sucrose and inulin of 0.92 ± 0.03 and for chloramphenicol and inulin of 1.18 ± 0.16. Vesicles containing OpcS demonstrated ratios of 0.64 ± 0.07 and 0.32 ± 0.09 and vesicles containing OpcP demonstrated ratios of 0.82 ± 0.04 and 0.35 ± 0.15 for sucrose and inulin and chloramphenicol and inulin, respectively. Thus, OpcS-containing vesicles demonstrated chloramphenicol permeability equivalent to that of vesicles containing the previously characterized porin, OpcP, and markedly different from that of vesicles constructed without outer membrane proteins. No differences in the permeability to penicillin G were demonstrated between OpcS and OpcP-containing vesicles and those constructed without outer membrane proteins (data not shown). These results suggest that OpcS functions in this system as a transmembrane pore and the selectivity for chloramphenicol correlates with findings with intact cells.

DISCUSSION

Porin-mediated decreased outer membrane penetration of hydrophilic antibiotics is a common mechanism of antimicrobial agent resistance in gram-negative organisms (see references 17 and 32 for a review). In clinical isolates this may be the result of stepwise mutations and may be reversible when the selective pressure of growth in antibiotic-containing medium is removed. This may be caused by regulation of porin synthesis or function.

Several laboratories investigating *P. cepacia* porins have identified a pore-forming outer membrane fraction which contains an oligomer of apparent molecular weight of 81,000 which dissociates into a major and a minor polypeptide species of apparent molecular weights of 36,000 and 27,000, respectively (2, 6, 35). Both proteins when isolated from preparative SDS-polyacrylamide gels have pore-forming properties in model membranes (5b); however, whether one or both of these proteins function as the transmembrane pore in intact bacterial cells is not fully resolved.

Osmotic regulation of the expression of *Escherichia coli* porin proteins, OmpF and OmpC, has been the subject of extensive investigation. Synthesis of OmpF is favored in media of low osmolarity, OmpC is favored in media of high osmolarity, and the relative production of each is modulated so that the total amount of both porins in the outer membrane remains constant (27, 30, 34). Regulation of the *ompF* and *ompC* genes occurs at both the transcriptional level and the translational level. EnvZ and OmpR, gene products of the *ompB* operon, are positive regulatory factors for the transcriptional osmotic regulation of *ompF* and *ompC* (23, 28, 42, 44). Translation of *ompF* mRNA is regulated by the product of the *micF* gene, an antisense RNA which is complementary to the 3′ end of *ompF* mRNA, binding to it in vivo and inhibiting its translation (1).

The original reports of salicylate-inducible antibiotic resistance were from experiments with *E. coli* (38). Reversible resistance to chloramphenicol, ampicillin, tetracycline, and nalidixic acid was described in organisms grown in 5 mM salicylate as well as other weak acids such as acetylsalicylate, acetate, and benzoate. Investigation of the mechanism of this resistance demonstrated decreased permeation of cephalosporins into the periplasmic space (13). Further characterization of the molecular mechanism identified repression of porin synthesis in *E. coli* K-12 strains by growth in the presence of salicylate (39). Synthesis of the OmpF porin of *E. coli* is highly and reversibly inhibited by 5 mM salicylate; synthesis of the OmpC porin is also weakly inhibited. These findings are consistent with reports that OmpF pores function more efficiently in the transport of β-lactam antibiotics, chloramphenicol, and tetracycline than do OmpC pores (10, 33).

Recent studies by Rosner et al. (39) have identified the site of regulation of porin synthesis by salicylates. Examination of the transcription and translation of the *ompF* gene by using fusion products revealed that growth in sodium salicylate had little effect on transcription of the gene while translation of *ompF* mRNA was markedly inhibited. Growth in sodium salicylate was found to stimulate expression of *micF* RNA, resulting in decreased translation of *ompF* mRNA and decreased synthesis of the OmpF porin.

In addition, Aumercier et al. (4) have identified potentiation of the susceptibility of *E. coli* to aminoglycoside antibiotics by growth in the presence of salicylate. To determine whether the action of salicylate was the result of its function as a weak acid or its salicyl structure, experiments using either acetate (another weak acid) or salicyl alcohol were carried out. The results of those experiments suggested that both effects contribute to the synergy between aminoglycosides and salicylates. The authors hypothesize that the weak acid effect results in an increase in the membrane potential.

Few studies studying the effects of salicylates on other gram-negative organisms have been performed. In both *Klebsiella pneumoniae* and *Serratia marcescens*, suppression of the synthesis of one or both of the porin proteins has been detected when the organisms were grown in media containing 5 mM salicylate (40). Further characterization of the physiological effects or genetic regulation has not been reported. Hancock and Wong (19) demonstrated that 1.2 mM acetylsalicylate increased the outer membrane permeability of *Pseudomonas aeruginosa* to the β-lactam nitrocefin but not to lysozyme or the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine. This enhancement of permeability was antagonized by the addition of 1 mM Mg^{2+}, suggesting that the action of salicylate in this organism might be as a weak chelator. This is a very different action than that proposed for salicylate in *P. cepacia*.

We have demonstrated effects of sodium salicylate on the outer membrane permeability of *P. cepacia* which are similar to those reported for *E. coli* and the other members of the family *Enterobacteriaceae*. However, in *P. cepacia* some unique differences have been identified: (i) the susceptibility of the organism to chloramphenicol, trimethoprim, and ciprofloxacin is decreased, while the susceptibility to a β-lactam agent, ceftazidime, is unchanged; (ii) the outer membrane penetration of chloramphenicol is decreased, while that of a β-lactam indicator compound, PADAC, is not; and (iii) there is no apparent decrease in the amount of the 36-kDa porin, OpcP, in the outer membrane of bacteria grown in the presence of salicylate. Significantly, a 40-kDa minor outer membrane protein, OpcS, is no longer detectable in the outer membrane of bacteria grown in medium containing salicylate and is also not present in the outer membranes of some chloramphenicol-resistant clinical isolates (5a). These findings suggest that OpcS may function as a selective, antibiotic-permeable porin which can be suppressed by growth in the presence of salicylate. Further investigation will require further isolation and characterization of OpcS and demonstration of its role in outer membrane permeability of *P. cepacia*.

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