Variation in the Composition and Pore Function of Major Outer Membrane Pore Protein P2 of *Haemophilus influenzae* from Cystic Fibrosis Patients

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**We investigated the relationship between susceptibility to β-lactam antibiotics and variation in the major
outer membrane protein P2 (OmpP2; also called porin) of persistent nonencapsulated *Haemophilus influenzae*
isolated from cystic fibrosis patients. Nine OmpP2 variants were selected from two distinct *H. influenzae* strains
from two patients extensively treated with β-lactam antibiotics. The variants differed in their susceptibilities
to at least two β-lactam antibiotics. By detergent extraction and column chromatography, OmpP2 was purified
from two variants that were derived from strain 70 and that differed notably in their susceptibilities to β-
lactam antibiotics. The proteins were reconstituted into black lipid membranes for measurement of porin
function. OmpP2 from the more resistant isolate (isolate 70b) had a smaller channel conductance than OmpP2
of the more susceptible isolate (isolate 70f). DNA sequencing of *ompP2* of these isolates revealed single non-
synonymous base differences; there were changes in the amino acid sequence corresponding to surface-exposed
loops 4, 5, 6, and 8. Changes in loops 4, 5, and 6 were previously shown to result in antigenic differences. Beside
these mutations, variants of strain 70 showed additional mutations in loop 1 and nonexposed loop 3. Taken
together, our results suggest that in variants of strain 70, nonsynonymous point mutations accumulated both
in the sequences of *ompP2* coding for antigen-variable loops and in other loops, notably, loops 1 and 3. The
latter changes are suggested to affect the permeability of the porin channel.**

Haemophilus influenzae colonizes the human nasopharynx
and is an important pathogenic bacterium in humans. Encap-
sulated strains (type b) cause systemic disease, including men-
ingitis, epiglottitis, and cellulitis, in nonvaccinated individuals.
Nonencapsulated *H. influenzae* is a frequent cause of respira-
tory tract infections including otitis media, sinusitis, pneumo-
nia, and persistent infections in chronic bronchitis and cystic
fibrosis (CF) patients (24).

Compared to *H. influenzae* type b, nonencapsulated *H. in-
fluenzae* shows much greater strain-to-strain diversity in the
amino acid sequence of its major outer membrane proteins
(Omps) including OmpP2 (48). OmpP2 has a molecular weight
ranging from 35,000 to 42,000 (15, 48). The diversity of the
amino acid sequence is found predominantly in surface-ex-
posed loops 2, 4, 5, 6, and 8 (12).

During persistent infections in chronic bronchitis patients,
the amino acid composition of OmpP2 of nonencapsulated
H. influenzae was shown to vary due to the accumulation of
point mutations (13). These changes were mapped in *ompP2* to
regions coding for loops 5 and 6 of the protein (2, 13, 19, 44).
These changes in amino acid composition resulted in antigenic
drift (13, 14, 18). More extensive amino acid variations have
been observed when nonencapsulated *H. influenzae* strains
persisted in the lower respiratory tracts of CF patients (29).

OmpP2 of *H. influenzae* type b is a member of the porin
superfamily (22). Porins form nonspecific outer membrane dif-

fusion channels for small water-soluble solutes including anti-
biotics (35). Several porin structures have been determined at
the atomic level and have served to establish a consensus
folding pattern of porins (7, 36, 40). This folding pattern con-
sists of a β barrel made up of 16 or 18 membrane-spanning
antiparallel β strands that are joined by short connecting turns
on the periplasmic side of the membrane and loops of various
lengths on the extracellular surface. Also, part of the consensus
folding pattern is the folding of loop 3 to form a constriction in
the barrel (45, 54). Topological models of OmpP2 of *H. influ-
enzae* place loop 3 at the channel constriction (2, 44). Recently,
Srikumar et al. (43) demonstrated that deletions in loop 4
resulted in an increased channel conductance and have pro-
posed that loop 4 may serve to narrow the channel entrance.
Mutations in the gene encoding the OmpF porin of *Escherichia
coli* were detected as small changes in amino acid composition
and in susceptibility to various antibiotics, particularly to β-lac-
tams (3).

Because CF patients are extensively treated with antibiotics
and especially β-lactam antibiotics (31), we wished to deter-
mine whether (i) differences in susceptibility to β-lactam anti-
biotics were associated with variations in OmpP2 of *H. influ-
enzae* isolates from CF patients and (ii) variations in OmpP2
correlated with changes in pore properties. The *H. influenzae*
OmpP2 variants differed in their susceptibilities to various
β-lactam antibiotics and to chloramphenicol. OmpP2 proteins
were purified from two variants and were incorporated into
black lipid membranes to determine their channel properties
(9). DNA sequencing of the genes encoding OmpP2 identified
the differences between variant OmpP2 proteins.

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MATERIALS AND METHODS

***H. influenzae* strains from CF patients.** A total of 247 nonencapsulated *H. influenzae* isolates were obtained from sputum samples from 39 CF patients (age range, 0.2 to 36 years; median age, 13 years) participating in a 2-year follow-up study (29). These isolates were characterized phenotypically by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of their cell envelope proteins (Omp analysis) (29) and genotypically by randomly amplified polymorphic DNA (RAPD) analysis (51). Strains were found to differ in both their Omp and their RAPD patterns (29). Variants differed only in their Omp patterns but not in their RAPD patterns. These Omp variants were isolated from several patients over time (29). The antibiotics administered and the duration of antibiotic treatment of the patients were recorded. *H. influenzae* ATCC 49766 was used as a standard for β-lactam antibiotic sensitivity tests (1).

Strains were routinely plated on chocolate agar plates or were grown in brain heart infusion broth supplemented with 10 μg of hemin (X factor) per ml and 10 μg of NAD (V factor) per ml at 37°C in 5% CO_2 for 18 h.

Antibiotic susceptibility testing. All 247 *H. influenzae* isolates were tested for β-lactamase activity by using nitrocefin as the chromogenic cephalosporin (37). For the β-lactamase-negative isolates, susceptibility to ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, cefaclor, and imipenem was determined by disk diffusion on standard Haemophilus test medium (HTM) plates (diameter, 9 cm; Becton Dickinson, Erembodegem, Belgium) as described by Doern et al. (10). Sensidisks (also from Becton Dickinson) were used according to the method described by the National Committee for Clinical Laboratory Standards (approved standard M2-A4) (34). Selected isolates were further tested for susceptibility to penicillin, cephalothin, and chloramphenicol.

The broth microdilution test was used to assess the MICs of the three β-lactam antibiotics ampicillin, penicillin, and cephalothin and the MIC of chloramphenicol. Bacteria were grown overnight for 18 h (normally growing bacteria) or 36 h (slowly growing bacteria). A bacterial suspension of 2×10^8 to 8×10^8 CFU/ml was prepared in phosphate-buffered saline. According to the standard method of the National Committee for Clinical Laboratory Standards (33), 10 μl of the suspension was transferred to 10 ml of HTM broth to prepare the inoculum for the test. To each well of sterile microtiter plates (Hospidex, Nieuwkoop, The Netherlands) 50 μl of serial twofold dilutions of ampicillin, penicillin, cephalothin, or chloramphenicol in HTM was added. The concentrations of ampicillin ranged from 0.03 to 16 μg/ml, those of penicillin ranged from 0.006 to 3 μg/ml, those of cephalothin ranged from 0.125 to 64 μg/ml, and those of chloramphenicol ranged from 0.06 to 32 μg/ml. Control wells of HTM without antibiotics were included in each experiment. To each well 1×10^8 to 4×10^8 CFU/ml was added. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 18 h. When the medium in the control wells was not turbid, the plates were incubated for another 18 h. All MIC determinations were done in triplicate. The MIC was defined as the lowest antibiotic concentration that inhibited the growth of *H. influenzae* in the microtiter plates. *H. influenzae* ATCC 49247 (1) and QC2174 (10) were included in each test series for reference.

Sequence analysis of *ompP2*. Fragments of DNA containing approximately 300 bp of *ompP2* were obtained by PCR with chromosomal DNA and three *ompP2*-specific primer sets as described by Duim et al. (12). The PCR products were sequenced by direct sequencing of double-stranded DNA (Applied Biosystems) with dyed primers. All fragments were sequenced in both directions and in duplicate from at least two independently obtained PCR products. The analysis was performed in an automated fluorescent DNA sequencer (model 370A; Applied Biosystems). The sequences were analyzed with the computer programs included in the program package PC/GENE (IntelliGenetics, Inc., 1991). The DNA sequences were aligned with the program CLUSTAL by the method developed by Higgins and Sharp (20).

OmpP2 purification. OmpP2 of *H. influenzae* was isolated by the method described by Munson et al. (32). Bacterial strains were cultured in brain heart infusion broth supplemented with 10 μg of both X and V factors per ml. The bacteria were harvested by centrifugation and were suspended in 10 mM HEPES (pH 7.4). The bacterial cell envelope fraction was isolated from the bacterial cells by ultrasonic treatment, followed by centrifugation as described by van Alphen et al. (49). The cytoplasmic membrane proteins were solubilized by treating the cell envelopes with 2% (wt/vol) sodium lauryl sarcosinate in 10 mM HEPES (pH 7.4). The insoluble fraction was collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 8) containing 2% sodium deoxycholate, 200 mM sodium chloride, and 5 mM EDTA, centrifuged again, and washed once in the same buffer. OmpP2 was then solubilized from the pellet in 0.4% (wt/vol) Zwittergent Z-3,14 buffered with 25 mM imidazole at pH 6.5. The insoluble fraction was removed by centrifugation. The crude OmpP2 was collected from the supernatant by precipitation with ethanol at −20°C, followed by centrifugation at 1,700 × g for 30 min. The proteins were solubilized in 50 mM Tris-HCl (pH 8) containing 0.05% Zwittergent Z-3,14. OmpP2 was further purified by high-pressure liquid chromatography with a MonoQ HR 5/5 column (42). The salt concentration of the elution buffer (0.05% Zwittergent Z-3,14 in 50 mM Tris-HCl [pH 8]) was raised to 0.35 M with a 2-ml gradient. When no more protein was eluted with 0.35 M NaCl as detected by UV absorption, the salt concentration was raised in a gradient fashion to 1.0 M. Highly purified OmpP2 eluted with between 0.4 and 0.7 M NaCl. It was collected by precipitation with ethanol at −20°C, followed by centrifugation at 1,700 × g for 30 min. Purified protein was

solubilized in 50 mM Tris-HCl (pH 8)–0.1% Zwittergent Z-3,14. The amount of protein was estimated with the dye-binding reagent of Bio-Rad; the purity of OmpP2 was assessed by SDS–11% PAGE (26), followed by Coomassie blue staining for proteins or by SDS–16% PAGE (41) followed by silver staining (46) for lipopolysaccharide. Purified *H. influenzae* lipopolysaccharide was prepared as described previously (50) and was used as a standard.

Pore function measurement. Channel conductance was measured by the method described by Dahan et al. (9). Two Teflon chambers were separated by a thin Teflon foil with a small circular aperture. Lipid bilayers were formed across this aperture with a solution of 2.5% glyceryl monooleate (Sigma) dissolved in *n*-decane. The porin sample was added to the aqueous phase either before or after membrane formation. The conductance across the membrane was measured by determination of a fixed transmembrane potential.

Penicillin-binding assay. The penicillin-binding assay method described by Mendelman et al. (27, 28) was used to test the binding of [³H]penicillin to exponentially growing or stationary-phase cells. Stationary-phase bacteria were obtained by cultivating bacteria on chocolate agar plates overnight, followed by resuspension in 5 ml of 50 mM Tris-HCl–1 mM $MgCl_2$ (pH 7.8) to an optical density at 600 nm of 0.6. The bacteria were centrifuged and concentrated 10-fold in the same buffer. Logarithmic-phase bacteria were obtained by growing the cells in brain heart infusion broth supplemented with 10 μg of both X and V factors per ml until an optical density at 600 nm of 0.7 was achieved. The bacteria were spun down and resuspended in 0.5 ml of the Tris-HCl–$MgCl_2$ buffer. For each bacterial suspension, 95 μl was incubated with 5 μl of [³H]penicillin (22 ng/ml, 44.5 μCi/μg; Amersham) for 30 min at 37°C. Penicillin-binding proteins were analyzed by SDS-PAGE, followed by autoradiography.

Nucleotide sequence accession numbers. The nucleotide sequence data can be retrieved from the EMBL/Genbank/DDBJ Nucleotide Sequence Databases under the accession nos. AF052541 to AF052553.

RESULTS

Antibiotic susceptibilities of *H. influenzae* isolates. Isolates (total = 247) were obtained from sputum samples from 39 CF patients, and 16 isolates produced β-lactamase. By the disk diffusion method for susceptibility testing, 47 (20.3%) of the 231 non-β-lactamase producing isolates showed intermediate or high levels of resistance to one or more of the following clinically useful β-lactam antibiotics: ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, cefaclor, and imipenem. On the basis of the results of Omp and RAPD analyses, the 47 isolates included 4 strains and 13 OmpP2 variants. Thirty additional isolates of these four strains were obtained at intervals of at least 1 month following initial isolation. OmpP2 variants that differed in their susceptibilities to more than one of the β-lactam antibiotics were isolated from only 3 of the 39 patients (patients 27, 28, and 30). Patient 27 was treated with β-lactam antibiotics for 12 weeks and with non-β-lactam antibiotics for 54 weeks. Patient 28 was treated with β-lactam antibiotics, mainly amoxicillin, for 2 years, and patient 30 was treated with β-lactam antibiotics for 3 months. The *H. influenzae* isolates from these patients included OmpP2 variants of strains 67, 69, 70, and 77. Isolates from each of these series had different OmpP2 proteins.

To determine whether differences in antibiotic susceptibility coincided with OmpP2 variation, *H. influenzae* isolates from patients 27, 28, and 30 were selected for further susceptibility testing and OmpP2 analysis. In addition, two related OmpP2 variants (82e and 82g) from patient 32, who was not treated with antibiotics during the study period, were included as controls. *H. influenzae* 70a, 70b, 70c, 70e, and 70f isolated from sputum specimens from patient 28 and *H. influenzae* isolates 77a, 77b, 77c, and 77f isolated from samples from patient 30 differed in their susceptibilities to at least two of the tested β-lactam antibiotics and to chloramphenicol. Two genotypically as well as phenotypically different isolates from sputum samples from patient 27 (isolates 67d and 69a) differed in their susceptibilities to ampicillin, penicillin, and cephalothin. Control OmpP2 variants 82e and 82g from CF patient 32 were similar with respect to their antibiotic susceptibilities.

The antibiotic susceptibilities of all 13 isolates were further analyzed by the more precise MIC microdilution method and

TABLE 1. MICs of ampicillin, penicillin, cephalothin, and chloramphenicol for 13 *H. influenzae* isolates from four CF patients and antibiotic therapy of the patients

Patient no.	Isolated *H. influenzae* strain[a]	MIC (μg/ml)				Antibiotic treatment of patient[b]
		Ampicillin	Penicillin	Cephalothin	Chloramphenicol	
27	67d	8	3	4	0.5	β-Lactams, 12 wk; non-β-lactams, 54 wk
	69a	0.5	0.2	0.5	0.5	
28	70a	8	3	2	0.06	β-Lactams, 104 wk; non-β-lactams, 11 wk
	70b	8	3	2	0.5	
	70c	4	3	ND[c]	ND	
	70e	8	3	2	0.25	
	70f	1	0.75	1	0.125	
30	77b	2	1.5	10	1	β-Lactams, 13 wk; non-β-lactams, 5 wk
	77a	4	1.5	2	2	
	77c	4	1.5	4	1	
	77f	1	0.75	1	0.5	
32	82e	0.125	0.1	0.25	0.125	None
	82g	0.25	0.2	0.25	0.25	

[a] The numbers indicate distinct strains that vary in their Omp and RAPD patterns; letters indicate the variants (identical RAPD patterns and different Omp compositions).
[b] Number of weeks that the patients were treated with β-lactam antibiotics or with non-β-lactam antibiotics during the period that the strains were isolated.
[c] ND, not done.

are summarized in Table 1. Differences in the MICs of at least two of the three β-lactam antibiotics (ampicillin, penicillin, and cephalothin) were observed between OmpP2 variants isolated from patients 28 and 30 and between the distinct strains from patient 27. Also, differences in susceptibility to chloramphenicol were found for the *H. influenzae* isolates obtained from patients 28 and 30. The MICs of all tested antibiotics were similar for the OmpP2 variants of the strain from patient 32, who was not treated with antibiotics. These results show that for isolates from patients extensively treated with antibiotics, OmpP2 variation was associated with fourfold differences in the MICs of a variety of antibiotics including β-lactam and non-β-lactam antibiotics.

Pore properties of purified OmpP2. Since the greatest intrastrain differences in antibiotic susceptibility occurred between isolates 70b and 70f, we purified the OmpP2 proteins from these two isolates (Fig. 1) and subjected them to pore function analysis. The OmpP2 purification procedure described by Munson et al. (32) was followed by chromatography on a MonoQ column. A protein of 40 kDa was obtained from both strain 70b and strain 70f (Fig. 1). Densitometric scanning of silver-stained gels revealed that lipopolysaccharide contamination was 5 and 7% for the two protein samples, respectively.

The channel properties of the OmpP2 preparations from variants 70b and 70f were characterized by determining single-channel conductances. By using approximately 15 ng of the porin and a transmembrane potential of 10 mV, stepwise increases in transmembrane conductance were recorded; this is characteristic for porins spontaneously inserting into the bilayer. The amplitudes of the conductance steps for the porins of variants 70b and 70f were measured, and the data are displayed as histograms (Fig. 2). Analysis of the conductance of purified OmpP2 of variant 70b revealed two channel populations (Fig. 2A): one with a conductance range of 0.65 to 0.75 nS and an average conductance of 0.71 nS and the other with a conductance range of 0.95 to 1.05 nS and an average conductance of 1.01 nS. Analysis of the purified OmpP2 of strain variant 70f showed two channel populations as well (Fig. 2B). However, these two populations had conductances significantly higher than those of their 70b counterparts. One population of

channels showed a conductance range of 0.75 to 0.90 nS and an average conductance of 0.83 nS, and the other showed a conductance range of 1.05 to 1.35 nS and an average conductance of 1.20 nS.

***ompP2* DNA and protein sequences.** By DNA sequence analysis of *ompP2*, differences in the sizes of the OmpP2 proteins
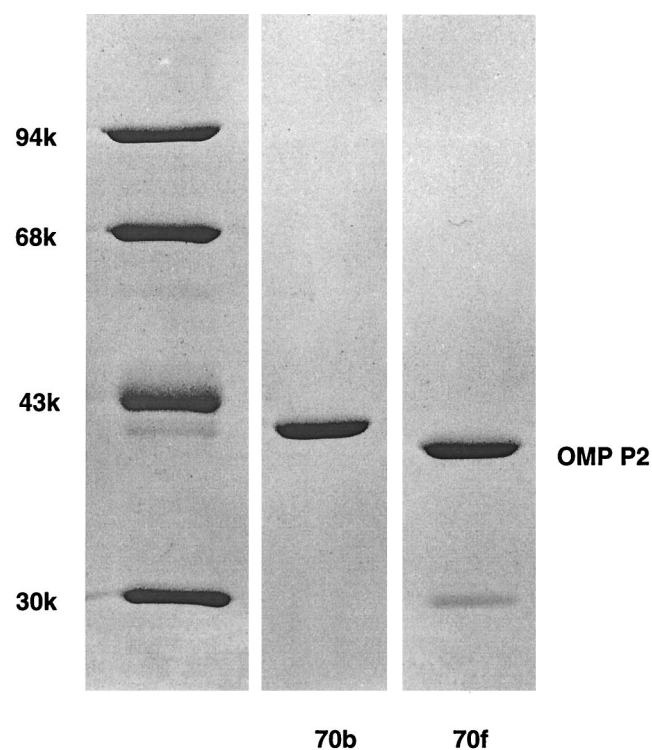

FIG. 1. Analysis of porins from variants strains *H. influenzae* 70b and 70f. A Coomassie brilliant blue-stained SDS-polyacrylamide gel is shown. The left contains a low-molecular-weight marker (in thousands, as indicated); lane 70b, 2 μg of protein of strain 70b; lane 70f, 1.5 μg of protein of strain 70f.
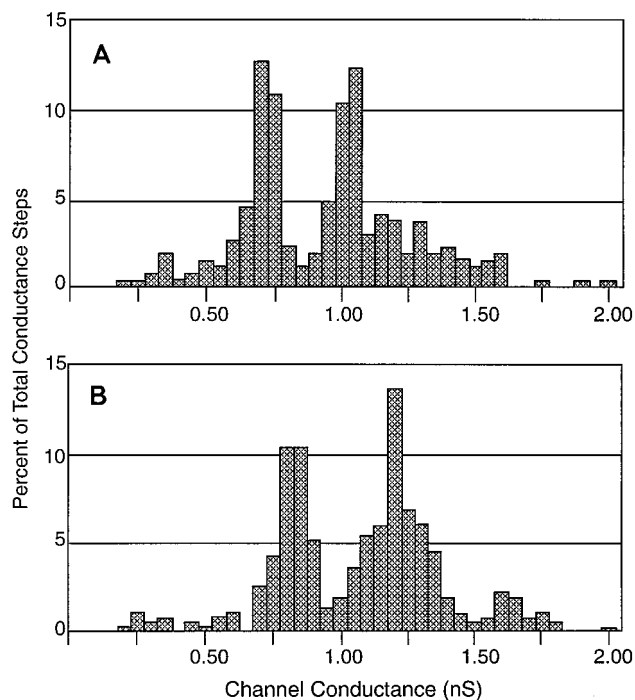
FIG. 2. Comparison of channel conductance as measured in planar bilayers for porins from *H. influenzae* OmpP2 strain variants 70b (A) and 70f (B). Conductance steps were recorded at a transmembrane potential of 10 mV and with 1 M KCl as the electrolyte. The bilayer was formed with monoolein dissolved in *n*-decane. The two porins were diluted with 50 mM Tris-HCl (pH 8.0) to 15 ng/µl. Approximately 1 µl of the diluted sample was added to the Teflon chamber. The total number of conductance steps analyzed was 257 for strain variant 70b and 349 for strain variant 70f.

of the five *H. influenzae* strains (different RAPD patterns and different Omp patterns) and their OmpP2 variants (identical RAPD patterns and different Omp patterns) were identified. Isolates showed interstrain diversity in regions of the gene coding for loops 2, 4, 5, 6, 7, and 8 (Fig. 3). The sequence changes that we identified resulted in substitutions, insertions, or deletions of amino acids. Sequence changes in loops 5 and 6 were apparently not related to antibiotic treatment, since the loops of the two variants of strain 82, isolated from a patient who was not treated with antibiotics, showed similar changes. Variations in loops 5 and 6 are known (6, 9) to be characteristic of antigenic variants of OmpP2. The membrane-spanning regions showed only a few conservative changes (data not shown). The *ompP2* DNA sequences of the *H. influenzae* strains isolated from patient 27 (strains 67d and 69a) showed as much diversity as those of the *H. influenzae* strains isolated from different CF patients.

The five OmpP2 variants of strain 70 also showed amino acid substitutions in the regions corresponding to surface-exposed loops 4, 5, 6, 7, and 8. This variability was apparently unrelated to the antibiotic susceptibility pattern. In addition to these amino acid changes, the susceptible OmpP2 variant 70f showed the substitution Val30→Ile within loop 1 and the substitution Arg46→His at the border between loop 1 and β strand 2; resistant variants 70a, 70b, 70c, and 70e had an arginine in the same position. Variant 70c, which showed only a slight increase in susceptibility, also showed variation in loop 1: Val32→Asp. Among the OmpP2 variants of strain 70, changes in loop 3 were only partly associated with differences in the susceptibilities of these variants to the antibiotics tested. Multiple changes in loop 3 were detected only in variants 70f and 70c. None of the

variants of strains 77 or 82 showed variation in this part of the sequence, suggesting that these alterations were not involved in antigenic drift but may influence antibiotic susceptibility.

Composition of penicillin-binding proteins. Despite numerous attempts, we did not succeed in transforming variant 70f with *ompP2* of variant 70b. This may have been due to the poor growth of the strains and the fact that the difference in MIC was only 6 µg/ml. To exclude the possibility that other factors influenced antibiotic susceptibility, we tested the binding of radiolabeled penicillin to penicillin-binding proteins. *H. influenzae* 70, including variants of strains 70, 77, and 82, were incubated with ^{3}H-labeled penicillin and analyzed by SDS-PAGE, followed by autoradiography. The results are shown in Fig. 4. *H. influenzae* Rd was included as control since it was previously shown to express all penicillin-binding proteins, three of which bind ampicillin with high affinities (27, 28).

Although the penicillin-binding protein binding patterns of OmpP2 variants 70a, 70b, 70c, 70e, and 70f were not identical, differences between the patterns were not correlated with differences in penicillin or ampicillin susceptibility. Although strain 70f was more susceptible to ampicillin than strain 70b, several penicillin-binding proteins of strain 70f showed reduced penicillin binding activity. A similar variation in the penicillin-binding pattern was observed for OmpP2 variants of strain 77. Similar penicillin-binding protein profiles were observed for exponentially growing and stationary-phase bacterial cultures, indicating that changes in the growth phase of the bacteria did not affect penicillin binding.

DISCUSSION

Of all β-lactamase-negative *H. influenzae* isolates examined in this study, one-fifth had reduced susceptibility to one or more β-lactam antibiotics. This frequency is within the range of resistance previously reported by others (11, 20, 22, 32, 38, 39). While OmpP2 variants were isolated from 23 of 40 patients (58%), only a minority of the variants showed reduced susceptibility to various broad-spectrum antibiotics (29). Furthermore, these resistant variants were isolated from only a small percentage (8%) of the CF patients. We previously reported (30) that among *H. influenzae* isolates from CF patients, there was no strong correlation between OmpP2 variation and antibiotic susceptibility. The isolated OmpP2 variants that differed in broad-spectrum antibiotic susceptibility were infrequently observed. As documented in this report, they were isolated only from patients extensively treated with antibiotics (Table 1). The disk diffusion test and the microdilution antibiotic susceptibility tests may not be sufficiently sensitive to detect differences in susceptibility that are attributable to differences in the diffusion rate of antibiotics through porins. However, *E. coli* mutants with increased permeability through the OmpF porin were identified by a similar agar diffusion method (3). Furthermore, Burns et al. (5) used the agar dilution test for MIC determinations and demonstrated reduced amounts of OmpP2 in *H. influenzae* isolates and a corresponding reduced susceptibility to chloramphenicol and β-lactam antibiotics. They also showed that these mutants had a lower outer membrane permeability for β-lactam antibiotics. In the microdilution test, differences in growth rate between resistant and susceptible strains could not have affected the susceptibility test readings since the test results were read after 24 h and again after another 18 h.

Our results suggest that the differences in the broad-spectrum antibiotic susceptibilities of *H. influenzae* strains from patients extensively treated with antibiotics can be correlated with changes in OmpP2 sequence and in the channel proper-

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          LOOP 1                            LOOP 2                    LOOP 3                          LOOP 4
 P.  S.   23                 46   69                 86   105                   133   158                   177
 27  67d-R AEQSSNTLDDQEQQHGALRN QDSR FVTD---ITKDAADHFGDITT RAKTIADGITSAEDKEYGVLNNSKYVPTN GRNPTNLTDPAGEVSPQSIS
     69a-S ...........K........ .G.. L..NPNGF.HEDR.G...... ............................. KYDN.GGN.--...QK....

 28  70a-R AEQSSNTVDVQKQQHGALRN QESR LVTEGTQDNADHFGDITT   RAETIADGITSAEDKEYGVLNNSKYVHTN QRFHNGQGVTLNKGGEVYPQAIS
     70b-R .................... .... .................     ............................. .........A.............
     70c-R .........D.......... .G.. .................     ..K.......................R.. ..L.SN...II............
     70e-R .................... .... .................     ..K.......................... .........A.............
     70f-S .......I............ .G.H .................     ..K.......................R.. .....N...A.............

 30  77a-R AEQSSNTLDDQKQQHGALRN QGSR FVTDITKHYSDHFGDITT   RAKTIADGITSAEDKEYGVLNNSEYIHTN ERHPTTHTDPVGELVSQTIS
     77b-R ..................·. .... .................     ............................. .....K....A.........
     77c-R .................... .... .................     ............................. .....K.............
     77f-S .................... .... .................     ............................. .....K....A.........

 32  82e-S AEQSSNTLDDQKQQHGALRNQGS R FVTDITKNHSDHFGDITT   RAKTIADDITSAEDKEYGVLNNSKYIRTN ERHPTNHTDPVGELVPQTIS
     82g-S ......................... . .................   ............................. ......Y.............

          LOOP 5          LOOP 6                      LOOP 7           LOOP 8
          202       216   245               267       291  299        326           342
 27  67d  YKERLRQNAVDPRRT KTKNHKEPAGNNRNPAYDEKRYF     TSVNQGQNT       RTKTNDKGKT---EKTEKGK
     69a  ...--DNSPSASK.K ......QYKAAAA-.........     N.SD..KK.       .P...G..Q.GKP.....E.

 28  70a  YRENTTGNLHMISDRK KTKNHTIT-TT-GQQKTKPACDEKRYF TSSDEGEKT       RTKTNDKGKPEKTEKEK
     70b  ............... ........T..A.P......Y......   .........       .................
     70c  .........R..PG.. ............----.KE....Y...... ......K..     ......D.A.......
     70e  ............... ............A..P......Y......   .........       .........A.......
     70f  ......DV.RI..G.. ......T.I..TNLKQ....Y......   ......K..       ......D.A.....G.

 30  77a  YKANMRISFGAPGRK KTKNHKQVKDYNTAYRDNKPAYDEKRYF TSSDEGKKT       RTKTNNTGKPEKTEKGK
     77b  ............... .........G...TH.............   .........       ...............E.
     77c  ............... ................H...........   .........       ...............E.
     77f  ............... ..............HH............   .........       ...............E.

 32  82e  YKENMRASVLQPDRK KTKNHKQVKSTATAA-TNKPACDEKRYF TSSDEGKKT       RTKTNNTGNPEKTEKGK
     82g  .....KI..VE.G.. .........V.TA.TT.....Y......   ...G.....       .................
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FIG. 3. Alignment of the deduced amino acid composition of the variable regions of OmpP2 corresponding to surface-exposed loops according to the topology model of OmpP2 of nonencapsulated *H. influenzae* (3). The OmpP2 sequences of five strains and eight variants of these strains isolated from four CF patients are shown. The amino acid sequences of strain variants 67d, 70a, 77b, and 82e are used as the consensus sequences. The amino acid positions of the loops of strain 67d are indicated above the sequence. Dots indicate identical amino acids, and dashes indicate deletions. Amino acids are given as one-letter codes. Abbreviations: P, patient; S, strain numbers for OmpP2 variants; R, antibiotic-resistant variant; S, antibiotic-susceptible variant.

ties of these porins (Fig. 2 and 3). We cannot exclude the possibility that other determinants of antibiotic susceptibility may be changed in these variants as well. Changes in specific targets for the antibiotics are very unlikely, since the variants showed altered susceptibilities to a variety of unrelated antibiotics. The penicillin-binding proteins of the resistant and susceptible isolates obtained from patient 30 indeed varied in their reactivities with ampicillin, the drug administered to the patient (Fig. 4). However, since the susceptible strain showed less binding than the more resistant one, the resistance is not caused by reduced antibiotic binding to the penicillin-binding proteins. In addition, similar variations in binding to penicillin-binding proteins was observed among strains with identical susceptibilities.

The surface-exposed loops 2, 4, 5, 6, 7, and 8 of the *ompP2* sequences of unrelated *H. influenzae* strains isolated from persistently infected CF patients differed predominantly (Fig. 3). Loops 2, 4, 5, and 6 of OmpP2 also showed strong diversity among *H. influenzae* isolates from sputum samples from patients with chronic bronchitis, and loop 8 showed diversity among isolates from patients with otitis media (12). The most extensive diversity, including amino acid insertions, was found in loop 6. Since the strong diversity in loop 6 has now been demonstrated for *H. influenzae* isolates that persist in both CF and chronic bronchitis patients, the variation in loop 6 may therefore be associated with the persistence of *H. influenzae* in the inflamed lower respiratory tract. Such variation may be a

result of strong selective pressure, which is important for escape from antibody-mediated defense mechanisms of the host (13, 14, 17, 53). The sequences of the *ompP2* genes of *H. influenzae* variants isolated from CF patients differed (Fig. 3) by single point mutations and often by one or more codon insertions, mainly in loops 4, 5, and 6. In isolates from CF patients, loop 6 was the most variable part of OmpP2 and was the longest loop, a result that matched the information for *H. influenzae* variants from chronic bronchitis patients. According to the secondary-structure models of Srikumar et al. (44) and Bell et al. (2), the amino acid substitutions may be positioned at the tip of the loop. Variation in loops 5 and 6 resembled strongly the antigenic variations observed among OmpP2 variants of *H. influenzae* isolates from chronic bronchitis patients (13, 14). In comparison with isolates from chronic bronchitis patients, the OmpP2 variants from CF patients had many more amino acid changes in loops 5 and 6 and also in several other loops (loops 1, 3, 4, 7, and 8) of OmpP2. Amino acid changes in OmpP2 loops 1, 3, 7, and 8 observed among variants isolated from sputum samples from CF patients were rarely found among *H. influenzae* variants from chronic bronchitis patients (13, 14).

Various considerations support the idea that some of these changes may influence the porin channel and consequently the susceptibility to β-lactam antibiotics (4, 6, 35). *H. influenzae* variants of strain 70 isolated from CF patient 28, who had been extensively treated with β-lactam antibiotics, showed substitu-
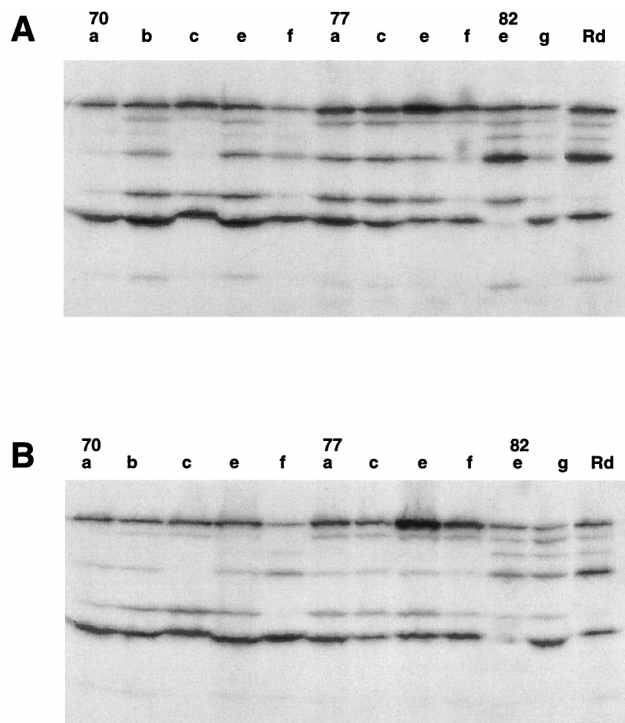
FIG. 4. Autoradiogram of the binding of [³H]penicillin to the penicillin-binding proteins of strains Rd, 70, 77, and 82, including OmpP2 variants (indicated by the lowercase letters), after the bacteria in the exponential (A) and stationary (B) phases of growth were harvested. The proteins were analyzed by SDS–11% PAGE before autoradiography.

tions in loop 3. According to the topological models of OmpP2 in the outer membrane (2, 44), the third surface-exposed loop folds into the channel and plays an essential role in determining cross-sectional diameter (43, 45, 52). Changes in loop 1 occurred only among the OmpP2 variants that differed in their antibiotic susceptibilities to the three tested β-lactam antibiotics as well as to chloramphenicol. Because Benson et al. (3) showed that changes in the amino acid sequences of porins were correlated with differences in uptake through the outer membrane, the variations in loop 1 may have affected pore function. In support of this hypothesis, channel conductance measurements showed significantly lower conductance values for the OmpP2 porin from the more resistant variant (variant 70b) than for the porin from the more susceptible variant (variant 70f) (Fig. 3). We propose that the smaller channel conductance of *H. influenzae* variant 70b OmpP2 could explain its lower susceptibility to β-lactam antibiotics and chloramphenicol compared to that of variant 70f.

Channel conductance measurements of OmpP2 purified from variants 70b and 70f revealed two channel populations (Fig. 3). An effect of lipopolysaccharide can be excluded since the amounts of lipopolysaccharide were low and lipopolysaccharide was not required for the pores to be functional (8). Contamination of the OmpP2 porin preparations (Fig. 1) with another pore-forming protein is an unlikely explanation for this phenomenon, because both channel populations of the porin from variant 70b showed a 14 to 16% smaller conductance than those of the corresponding populations of porin from variant 70f. It would be a very unlikely coincidence for two different proteins to change so that their conductances are altered to the same degree. In addition, the amount of protein used for the conductance measurements is low, and therefore,

the concentration of the contaminants is almost negligible. Other explanations for the bimodal distribution merit consideration: the two populations of channel conductances may represent different porin conformational states, or the two channel populations may have been arisen from different subunit associations.

We conclude that the majority of variations observed in loops 4, 5, and 6 from OmpP2 proteins from strains from of CF patients likely cause antigenic differences and are not related to differences in pore function (of OmpP2). The results of channel conductance measurements, the amino acid analysis of OmpP2 variants, and the observed differences in antibiotic susceptibilities of the variants (Table 1) suggest that in some isolates OmpP2 variation might be responsible for the observed alteration in broad-spectrum antibiotic susceptibility.

Sequence analysis of the *ompP2* (OmpP2) genes of *H. influenzae* isolates from CF patients showed that nearly all amino acid changes in the protein were, as in isolates from chronic bronchitis patients (13), the result of nonsynonymous point mutations. This observation indicates that variants are appearing under strong selective pressure. Changes in loops 5 and 6 have been associated with antigenic drift as a consequence of immune selection (13, 14). The changes in loops 1 and 3 may have been selected by the antibiotic therapy of the CF patient. The sequence variation in *H. influenzae* during persistent infections in CF patients was more extensive than that in *H. influenzae* during persistent infections in chronic bronchitis patients. This increased variability is most likely the consequence of the observed extensive persistence of the infection in CF patients (29), since persistence is sufficient for the appearance of variants (53). The very viscous mucus produced as a consequence of the chloride channel defect in CF patients (16) may provide an environment where *H. influenzae* is sheltered from both immune defense mechanisms and the bactericidal effect of antibiotics.

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