A Major Outer-membrane Protein Functions as a Porin in *Haemophilus influenzae*

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Porins are pore-forming outer-membrane proteins which serve as a non-specific pathway for the entry of hydrophilic molecules into Gram-negative bacteria. We studied four strains of *Haemophilus influenzae* that had decreased permeability to chloramphenicol associated with diminished quantities of a 40 kDa major outer-membrane protein. Isogenic pairs of organisms containing and lacking this protein were compared. The latter strains grew more slowly and were less permeable to sucrose and raffinose. They were also more resistant to multiple hydrophilic antibiotics than an isogenic strain containing the 40 kDa protein and were less permeable to penicillin G and chloramphenicol. We conclude that the 40 kDa outer-membrane protein functions as a porin in *H. influenzae*.

INTRODUCTION

Haemophilus influenzae is an important Gram-negative pathogen. *H. influenzae* type b is the leading cause of bacterial meningitis in the United States, resulting in nearly 10000 cases per year; other systemic infections including epiglottitis, bacteraemia, pneumonia, septic arthritis and cellulitis afflict an additional 8000 children annually (Band *et al.*, 1984; Granoff & Basden, 1980). In addition, numerous serious infections in healthy and immunocompromised children and adults are caused by unencapsulated and non-type b capsulated *H. influenzae* (Bartlett *et al.*, 1983; Liston & Foshee, 1982; Shann *et al.*, 1984).

H. influenzae has special nutritional requirements for growth including the addition of haem and β -NAD to culture media (Kilian, 1985). However, despite its fastidious growth requirements, *Haemophilus* shares many features with other Gram-negative organisms, including an outer membrane which regulates cell permeability. Pore-forming transmembrane proteins (porins) serve as a non-specific pathway for the entry of hydrophilic compounds (including nutrients and antibiotics) into Gram-negative bacterial cells. Porins from the *Enterobacteriaceae* are organized as trimers in the outer membrane with subunit molecular masses ranging from 34 to 42 kDa (Nikaido & Vaara, 1985).

We recently reported four clinical isolates of *H. influenzae* which demonstrate decreased accumulation of chloramphenicol in association with markedly diminished quantities of a 40 kDa outer-membrane protein (Burns *et al.*, 1985). To test the hypothesis that this protein functions as a porin which regulates the influx of chloramphenicol and other hydrophilic compounds into the periplasmic space, we examined the growth rate, oligosaccharide uptake, antibiotic susceptibility and penicillin permeability of strains containing and lacking the protein.

METHODS

Bacterial strains and plasmids. The strains utilized have been previously characterized (Burns *et al.*, 1985). Strain MAP is a multiple antibiotic-resistant laboratory strain (Catlin *et al.*, 1972) which is chloramphenicol susceptible. Strains 77-1040, 76-81739, 76-79268 and C435 are chloramphenicol-resistant clinical isolates (Burns *et al.*, 1985). Transformant strains TF 77-1040-8, TF 76-81739-7, TF 76-79268-1 and TF C435-2 were constructed by

transformation of strain MAP with whole-cell DNA from the chloramphenicol-resistant clinical isolates; these strains are deficient in the 40 kDa outer-membrane protein (Burns *et al.*, 1985). All are strains of *H. influenzae*, as determined by an absolute requirement for haem and β -NAD (Kilian, 1985), and all are unencapsulated as determined by agglutination with Difco typing sera. Bacterial strains were stored at -80°C in vials of skim milk and freshly subcultured on solid media prior to each experiment.

Plasmid RSF007 (de Graaff *et al.*, 1976), a 30 MDa conjugative plasmid encoding β -lactamase production, was used to introduce β -lactamase activity into strain MAP and TF 76-81739-7. Strain RSF007 was the source of this plasmid and was kindly provided by Dr Stanley Falkow, Department of Microbiology, Stanford University, Calif., USA.

Media. Liquid medium was brain heart infusion broth (Difco) supplemented with $10\text{ }\mu\text{g}$ haemin, $10\text{ }\mu\text{g}$ L-histidine and $10\text{ }\mu\text{g}$ β -NAD ml^{-1} (sBHI). Solid medium was sBHI agar. Broth cultures were incubated at 37°C with shaking at $200\text{ cycles min}^{-1}$. Plate cultures were incubated at 37°C in room air supplemented with 5% (v/v) CO_2 .

Antibiotics and chemicals. Chloramphenicol, tetracycline.HCl and ampicillin sodium were obtained in crystalline form from Sigma. Cephalothin, cefaclor, cefamandole and moxalactam were supplied by Eli Lilly & Co., cefuroxime by Glaxo, and cefotaxime by Hoechst-Roussel. [^3H]Raffinose (specific activity $288.6\text{ GBq mmol}^{-1}$) and [^{14}C]sucrose (specific activity $133.2\text{ GBq mmol}^{-1}$) were purchased from New England Nuclear. Other chemicals, salts and buffers were of the purest form available from J. T. Baker Chemical Co. and Mallinckrodt Inc.

Growth rate determination. Flasks of sBHI broth (100 ml) were inoculated with 1 ml overnight broth culture and incubated at 37°C . Using a Spectronic 20 spectrophotometer (Bausch & Lomb), OD_{600} was recorded initially and at 30 min intervals once an increase in optical density was detected.

Determination of minimal inhibitory concentrations (MICs). MICs of nine hydrophilic antibiotics were determined by agar dilutions using a Steers replicator (Steers *et al.*, 1959). The inoculum used was 10^5 c.f.u. (Syriopoulou *et al.*, 1979). Plates were incubated for 18–24 h and results were then recorded.

Conjugation. β -Lactamase activity was introduced into strains MAP and TF 76-81739-7 by conjugation. The technique used was a filter paper mating (Burns *et al.*, 1985) with selection of transconjugant colonies by plating dilutions on sBHI agar containing ampicillin ($5\text{ }\mu\text{g ml}^{-1}$) and chloramphenicol ($2\text{ }\mu\text{g ml}^{-1}$). Transconjugants were examined for β -lactamase activity by the chromogenic cephalosporin assay (O'Callaghan *et al.*, 1972).

β -Lactam permeability assay. The β -lactam permeability assay (Mendelman *et al.*, 1984) compared spectrophotometrically the hydrolysis of penicillin G by periplasmic β -lactamase in whole cells to that in sonicated cell extracts. K_m and $V_{\max}$ were calculated for the hydrolysis of penicillin G in cell sonicates. The rate of hydrolysis in intact cells (V_{intact}) at a substrate concentration of 1 mM (S_0) was recorded and the penicillin concentration inside the cells (S_e) was calculated from the equation of Zimmermann & Rosselet (1977):

$$S_e = [V_{\text{intact}}/(V_{\max} - V_{\text{intact}})] \times K_m$$

Each assay was done in duplicate and repeated three times.

Uptake of oligosaccharides. A modification of the technique of Decad & Nikaido (1976) was used to examine the uptake of [^{14}C]sucrose (M_r 342.3) and [^3H]raffinose (M_r 504.5). A 500 ml broth culture of each organism was grown to mid-exponential phase (about 5×10^8 c.f.u. ml^{-1}) and harvested by centrifugation. The organisms were washed and resuspended in 2 ml 0.025 M-sodium phosphate buffer (pH 7.0). To 400 μl cell suspension was added 1.5 μCi (55.5 kBq) [^{14}C]sucrose and 0.5 μCi (18.5 kBq) [^3H]raffinose; unlabelled sucrose and raffinose were added at a final concentration of 0.01 M and 0.02 M, respectively. Cells were plasmolysed by the addition of 0.3 M-NaCl. Samples were vortexed briefly, incubated for 5 min at room temperature and centrifuged at 12000 g for 3 min. The supernatant (S_1) was retained, the tube was carefully dried, and the pellet was resuspended in 1 ml phosphate buffer. After 10 min at room temperature, the cells were again pelleted and a sample of supernatant was retained (S_2). The radioactivity in S_1 and S_2 for each strain was separately determined by double-label counting in a Packard Tricarb scintillation spectrometer and the permeable space for sucrose and raffinose was calculated for each strain:

$$\text{Permeable space} = \frac{\text{Total radioactivity in } S_2}{\text{Radioactivity } \mu\text{l}^{-1} \text{ in } S_1}$$

Colony counts were done following washing and resuspension of the original culture to enable interstrain comparison.

RESULTS

Growth rate. The growth rates of strain MAP and three transformant strains deficient in the 40 kDa outer-membrane protein (40K OMP $^-$) were compared (Fig. 1). In five separate experiments, the transformants consistently grew more slowly and achieved a lower OD_{600} than strain MAP.

Fig. 1. Comparison of growth rate of strain MAP (●) with three 40K OMP-deficient strains: TF C435-2 (□), TF 76-81739-7 (△) and TF 77-1040-8 (○). Culture flasks containing 100 ml sBHI were inoculated with 1 ml from an overnight culture of each organism. OD₆₀₀ was monitored and recorded over time.

Table 1. MIC values

Measurement was by the agar dilution method. A 10⁵ c.f.u. inoculum was applied by using a Steers replicator. Cm, chloramphenicol; Tet, tetracycline; Amp, ampicillin; Cef, cephalothin; Clr, cefaclor; Man, cefamandole; Crx, cefuroxime; Ctx, cefotaxime; Mox, moxalactam.

Strain	MIC (μg ml ⁻¹)								
	Cm	Tet	Amp	Cef	Clr	Man	Crx	Ctx	Mox
MAP	1	2	1	5	20	1	1	0.1	1
77-1040*	20	5	50	10	100	10	5	1	0.1
76-81739	20	5	2	10	50	5	5	0.1	0.1
TF 77-1040-8	5	20	5	20	20	5	2	0.1	0.1
TF 76-81739-7	5	20	5	20	50	5	5	0.1	0.1
TF 76-79268-1	5	20	5	20	50	5	2	1	1
TF C435-2	5	20	5	20	20	5	2	0.1	0.1

* β-Lactamase-producing strain.

MICs. MICs of nine hydrophilic antibiotics for strain MAP, the chloramphenicol-resistant clinical isolates and the 40K OMP⁻ transformants are listed in Table 1. The strains deficient in the 40 kDa protein were less susceptible to chloramphenicol, tetracycline, ampicillin and first- and second-generation cephalosporins than strain MAP. Susceptibility to cefotaxime and moxalactam was equivalent. These results suggest a non-specific permeability barrier to many hydrophilic antibiotics in addition to chloramphenicol.

Permeability to penicillin G. The permeability of the outer membrane to penicillin G was determined for strains MAP(RSF007) and TF 76-81739-7(RSF007). Each assay was done in duplicate and repeated three times. The calculated ratio of internal to external concentration of penicillin G (S_e/S_o , mean ± SD) was 0.76 ± 0.27 for MAP(RSF007) and 0.05 ± 0.03 for TF 76-81739-7(RSF007). The calculated minimum internal (periplasmic) concentration of penicillin G for strain TF 76-81739-7 compared with that for strain MAP suggests an outer-membrane impermeability to β-lactam antibiotics as well as to chloramphenicol.

Permeability to oligosaccharides. The relative permeability to [¹⁴C]sucrose and [³H]raffinose of *H. influenzae* strains MAP and TF 76-81739-7 was compared; *Escherichia coli* strains C600 (Bachmann, 1972) and CmlB, a porin-deficient, chloramphenicol-resistant mutant (Reeve, 1968), served as controls (Table 2). Experiments were done in duplicate. Strains TF 76-81739-7 appeared to have decreased penetration of both sucrose and raffinose compared with strain MAP, supporting the hypothesis of a generalized permeability barrier to hydrophilic

Table 2. *Permeability to radiolabelled oligosaccharides*

Permeability was determined by the technique of Decad & Nikaido (1976). Each value was calculated from two determinations of the permeable space for radiolabelled oligosaccharides in a cell pellet containing 4×10^{10} c.f.u.

Strain	[¹⁴ C]Sucrose-permeable space (μ l per 4×10^{10} c.f.u.)	[³ H]Raffinose-permeable space (μ l per 4×10^{10} c.f.u.)
<i>H. influenzae</i>		
MAP	64.0	53.4
TF 76-81739-7	46.7	37.5
<i>E. coli</i>		
C600	71.2	51.6
CmlB*	49.7	34.3

* A porin-deficient *E. coli* mutant (Reeve, 1968).

compounds. In addition, for all four strains examined there was relatively lower permeability to raffinose than to the smaller saccharide, sucrose. However, in the *H. influenzae* strains, the difference was not as striking as in the *E. coli* strains. This is consistent with the existence of a higher exclusion limit in *H. influenzae*.

DISCUSSION

Porins were first identified in the *Enterobacteriaceae*, and porin physiology has been defined in *E. coli* and *Salmonella typhimurium*. There are two major porin proteins in *E. coli* K12, OmpF and OmpC (Lutkenhaus, 1977; Lugtenberg *et al.*, 1975); in *S. typhimurium* there are three, 36K, 35K and 34K (Nakae & Ishii, 1978). The proteins function as non-specific pores facilitating the diffusion of small hydrophilic molecules including saccharides, amino acids, nucleotides and antibiotics (Heuzenroeder & Reeves, 1981; Nakae & Nikaido, 1975; Nikaido *et al.*, 1977; van Alphen *et al.*, 1978). Nikaido and co-workers (Decad & Nikaido, 1976; Nakae, 1976) determined the exclusion limit for enterobacterial porins to be 600–800 Da.

The role of porins in antibiotic susceptibility has been well characterized in the *Enterobacteriaceae*: porin-deficient mutants with altered outer-membrane permeability have been selected using antibiotic resistance as a marker. *E. coli* mutants deficient in OmpF, OmpC, or both, exhibit decreased penetration of hydrophilic antibiotics including β -lactams, tetracycline and chloramphenicol, with resultant phenotypic resistance (Chopra & Eccles, 1978; Nikaido *et al.*, 1983; Reeve, 1968). *S. typhimurium* mutants deficient in the 36K and 34K proteins, together or singly, have decreased permeability to β -lactams (Nikaido *et al.*, 1977).

Vachon *et al.* (1985) reported a 40 kDa protein present in outer-membrane fractions that formed transmembrane permeability channels in *H. influenzae* type b. When this protein was introduced into reconstituted vesicles, they became permeable to sucrose, raffinose and stachyose (M_r 666) but fully retained dextrans larger than 1500 Da.

We previously reported four unencapsulated clinical isolates of *H. influenzae*, having decreased permeability to chloramphenicol associated with the apparent absence of a 40 kDa major outer-membrane protein (Burns *et al.*, 1985). The data we present here confirm the hypothesis that this protein functions as a porin *in vivo*. Strains lacking the protein exhibit a slower growth rate and a relative decrease in accumulation of saccharides in addition to a generalized decrease in antibiotic susceptibility and a specific decrease in penicillin G permeability. Thus, the 40K OMP⁻ strains are deficient in two porin-mediated functions: nutrient uptake and antibiotic accumulation.

We conclude that the 40 kDa major outer-membrane protein functions as a porin in *H. influenzae*. The growth of 40K OMP-deficient strains in the laboratory and their ability to infect patients suggest that alternative porins (either constitutive or inducible) function in these strains.

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