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Mutation of *Salmonella paratyphi* A Conferring Cross-Resistance to Several Groups of Antibiotics by Decreased Permeability and Loss of Invasiveness

L. GUTMANN,[1,2]* D. BILLOT-KLEIN,[1] R. WILLIAMSON,[1] F. W. GOLDSTEIN,[2] J. MOUNIER,[3] J. F. ACAR,[1,2] AND E. COLLATZ[1]

Laboratoire de Microbiologie Médicale, Université Pierre et Marie Curie, 15-21, rue de l'Ecole de Médecine, 75270 Paris, Cédex 06,[1] Laboratoire de Microbiologie Médicale, Hôpital Saint-Joseph, 75674 Paris, Cédex 14,[2] and Sérvice des Entérobactéries, U199 Institut National de la Santé et de la Recherche Médicale, Institut Pasteur, 75724 Paris, Cédex 15,[3] France

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A spontaneous one-step mutant of *Salmonella paratyphi* A selected on ampicillin showed cross-resistance to all β-lactam antibiotics except imipenem and to aminoglycosides, chloramphenicol, tetracycline, trimethoprim, and quinolones. It also grew as small colonies. Examination of the cell envelope of the mutant showed a quantitative decrease in three major outer membrane proteins of 40.6, 39.6 (presumably porins), and 24 kilodaltons and quantitative as well as qualitative modifications in the ladder pattern of lipopolysaccharide. Direct evidence for decreased permeability in the mutant included reduced uptake of [^{3}H]glucose and norfloxacin, reduced accessibility of aztreonam and benzylpenicillin to penicillin-binding proteins in whole cells, and decreased diffusion of lactose and cephaloridine into proteoliposomes that were reconstituted with outer membrane proteins from the mutant. There was also loss of invasiveness of the mutant into HeLa cells. We assume that a pleiotropic mutation was responsible for multiple alterations in the outer membrane components of the resistant mutant of *S. paratyphi* A.

Different types of cross-resistance to various antibiotics that are associated with decreased permeability and modification of outer membrane proteins have been reported in many gram-negative organisms (4) and in members of the family *Enterobacteriaceae* in particular (6, 11, 13, 33, 34). In those studies, resistance to various combinations of β-lactam antibiotics, quinolones, chloramphenicol, trimethoprim, tetracycline, and aminoglycosides was observed. Quantitative changes in major outer membrane proteins, which were either proven or supposed to be porins, were considered as the basic mechanism for resistance.

Salmonella paratyphi A, a highly pathogenic organism, is naturally susceptible to ampicillin, which is considered to be the treatment of choice for *S. paratyphi* infection. During routine susceptibility testing we found a mutant of *S. paratyphi* A which had become resistant in vitro to ampicillin and other antibiotics. To investigate this phenomenon further, under controlled conditions we selected a similar mutant which showed decreased susceptibility to all of the antibiotics mentioned above, in conjunction with the loss of invasiveness and changes in several components of the cell envelope.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A wild-type *S. paratyphi* A strain, 10(S), was isolated from a patient with endocarditis caused by this organism, (before the patient was treated with a β-lactam antibiotic) at the Hôpital Saint-Joseph in Paris. Spontaneous resistant mutants that were growing as small colonies were selected from this strain on agar plates that contained 8 μg of ampicillin per ml at a frequency of about 10^{-8}. Spontaneous antibiotic-susceptible revertants, which were easily distinguished as large colonies, were obtained from the resistant mutants at a similar frequency. One resistant mutant, 100(R), and its spontaneous revertant, 1000(Rev), were chosen for further study. All strains were grown with aeration at 37°C in Mueller-Hinton broth (Diagnostics Pasteur). The MICs of antibiotics were determined on Mueller-Hinton agar by using a multiple-inoculum replicator (5) and 10^4 CFU per spot. Growth of cultures was followed by measuring the turbidity at 650 nm with a Perkin-Elmer Junior III spectrophotometer (Coleman Instruments Division, Oak Brook, Ill.).

Antibiotics and reagents. The following antibiotics were kindly provided by the indicated companies: ampicillin, Beecham-Sevigné; chloramphenicol and cefotaxime, Roussel-Uclaf; aztreonam, E. R. Squibb & Sons (Princeton, N.J.); moxalactam, Eli Lilly & Co. (Indianapolis, Ind.); ceftazidime, Glaxo Pharmaceuticals, Ltd. (Greenford, United Kingdom); cefoxitin, imipenem, and norfloxacin, Merck Sharp & Dohme (West Point, Pa.); trimethoprim lactate, Wellcome Research Laboratories S. A. (Beckenham, England [Div. Burroughs Wellcome Co.]); flumequine and nalidixic acid, Winthrop Laboratories, Div. Sterling Drug Inc. (New York, N.Y.); colistin and pefloxacin, Roger Bellon; chloramphenicol and tetracycline, Diamant; streptomycin, Spécia; amikacin, Bristol Laboratories (Syracuse, N.Y.). *para*-[^{3}H]benzylpenicillin (0.66 TBq/mmol) was generously provided by Rhône-Poulenc Recherche (Vitry-sur-Seine, France) and was synthesized, as was [^{3}H]glucose (3.7 GBq/mmol), at the Service des Molécules Marquées, Commissariat à l'Energie Atomique (Gif-sur-Yvette, France).

Characterization of outer membrane proteins and lipopolysaccharide. Outer membranes were prepared by using 0.3% (wt/vol) *N*-laurylsarcosine (Sigma Chemical Co., St. Louis, Mo.), as described previously (13). Outer membrane proteins were separated by one- (21) or two-dimensional (29)

* Corresponding author.

polyacrylamide gel electrophoresis. Lipopolysaccharide (LPS) was extracted from cells and analyzed by polyacrylamide gel electrophoresis by the method described by Hitchcock and Brown (17).

Penicillin-binding protein labeling. Assays of penicillin-binding proteins (PBPs) with isolated membranes were performed as described previously (35), except that [^{3}H]benzylpenicillin was used. For comparison of in vitro and in vivo labeling, 20-ml samples of an exponential-phase cell culture were centrifuged at 25,000 × *g* for 5 min at 4°C. Cells were suspended in 80 μl of ice-cold 50 mM sodium phosphate buffer (pH 7). A first 40-μl sample that contained intact cells was incubated with [^{3}H]benzylpenicillin for 10 min at 37°C and washed twice with 3 ml of ice-cold buffer containing 1% (vol/vol) mercaptoethanol. The bacteria were suspended in 2 ml of buffer and broken by sonication. Cell envelopes were collected by centrifugation at 100,000 × *g* for 30 min at 2°C and suspended in 50 μl of ice-cold buffer containing 1% mercaptoethanol. The cells in a second 40-μl sample were sonicated immediately. The membranes, which were prepared as described above and suspended in 40 μl of buffer, were exposed to [^{3}H]benzylpenicillin concentrations that were similar to those used for whole cells. A similar procedure was used to assay the competitive binding of cefoxitin to PBPs, except that the samples were preincubated with different concentrations of cefoxitin. PBPs that were not acylated by this antibiotic were detected with 1 μg of [^{3}H]benzylpenicillin per 50 μl, which was sufficient to saturate all PBPs.

The relative intensities of the PBP bands on the fluorograms, as well as the amounts of the Coomassie blue-stained outer membrane proteins, were quantified with a Cliniscan (Helena Laboratories, Beaumont, Tex.) and were corrected for any variation in width.

Permeability assays. "Crypticity" factors were calculated by the determination of either the ratio of the amounts of labeled PBPs or the ratio of the β-lactamase activities in intact and sonicated cells. For the latter determination, a plasmid carrying the TEM-1 β-lactamase gene was introduced into the cells by conjugation.

The uptake of [^{3}H]glucose was measured essentially as described previously (13). Cells from 10 ml of minimal broth (Difco Laboratories, Detroit, Mich.) cultures were harvested at an optical density at 650 nm of ca. 0.350, and were washed three times with the same medium containing 50 μg of chloramphenicol per ml. Cells were suspended in 5 ml of minimal medium containing 1 mM glycerol but no glucose. A total of 1 ml of cell suspension was added to the labeled glucose and incubated at 25°C for 30 s, after which 125 μl of 40% (vol/vol) formaldehyde and 1 ml of 10% (wt/vol) ice-cold trichloroacetic acid were added. After 10 min in ice, the samples were filtered through GF/A filters (Whatman, Inc., Clifton, N.J.), rinsed with minimal medium containing 1 mM glycerol and then with ethanol, and subsequently dried before quantification of the radioactivity.

The uptake of norfloxacin was measured by a previously described method (16). Cells grown to the mid-log phase were centrifuged and suspended in the same medium at an optical density at 600 nm of ca. 0.7. Norfloxacin was added to a final concentration of 10 μg/ml, and incubation was continued with shaking at 37°C. At different time points, 10-ml samples were chilled, washed once, and then suspended in 2 ml of saline. The cell suspensions were incubated in boiling water for 7 min. Norfloxacin concentrations were measured in the supernatant by use of a bioassay with *Klebsiella pneumoniae* B480.

TABLE 1. MICs of different antibiotics for *S. paratyphi* A strains

Antibiotic	MIC (μg/ml) for strain:		
	10(S)	100(R)[a]	1000(Rev)
β-Lactams			
Imipenem	0.12	0.12 (1)	0.12
Cephaloridine	2	8 (4)	2
Ampicillin	2	16 (8)	1
Cefoxitin	4	64 (16)	2
Cefotaxime	0.12	2 (16)	0.06
Aztreonam	0.12	4 (32)	0.12
Moxalactam	0.06	4 (64)	0.06
Others			
Norfloxacin	0.25	1 (4)	0.25
Pefloxacin	0.25	1 (4)	0.25
Flumequine	0.5	4 (8)	0.5
Nalidixic acid	2	16 (8)	2
Colistin	1	1 (1)	1
Tetracycline	2	4 (2)	2
Chloramphenicol	4	16 (4)	4
Amikacin	0.5	4 (8)	0.12
Streptomycin	8	64 (8)	2
Fosfomycin	8	128 (16)	8
Timethoprim	0.5	16 (32)	0.5

[a] Values in parentheses are the fold increase.

Liposome swelling assay. Liposomes were made under previously described conditions (27), except that 2.4 μmol of acetone-extracted phosphatidylcholine and 0.1 μmol of dicetylphosphate were used. The amounts of total outer membrane protein used are indicated in Fig. 6. For the preparation of these proteins, outer membranes were washed three times with 5 mM Tris buffer (pH 7.4) and solubilized with 1% (wt/vol) sodium dodecyl sulfate (SDS) in the same buffer at room temperature. The SDS concentration in the protein solution, in which the phospholipid film was suspended, never exceeded 0.1%, a concentration which was also used during the preparation of the control liposomes. Proteoliposomes were formed in 0.6 ml of a 15% (wt/vol) solution of Dextran T40 (Pharmacia Fine Chemicals, Piscataway, N.J.) in 5 mM Tris (pH 7.4). Liposome swelling was monitored at 400 nm with a double-beam spectrophotometer (550S; Perkin-Elmer) coupled to a recorder (561; Perkin-Elmer). The full-scale deflection of the chart recorder was normally set to a range of 0.5 optical density units.

Virulence assay. Virulence was assayed by testing the bacterial invasion of HeLa cells (14).

RESULTS

Growth characteristics and antibiotic susceptibilities. The susceptible strains of *S. paratyphi* A 10(S) and 1000(Rev) had generation times in Mueller-Hinton broth of 34 and 33 min, respectively, in contrast to the resistant strain 100(R), which had a generation time of 44 min and formed smaller colonies. The size of the resistant cells was estimated to be 30 to 40% less than that of the susceptible strain 10(S), as determined by scanning electron microscopy (data not shown).

Strain 100(R) was resistant to several antibiotics and antibiotic groups, i.e., β-lactam antibiotics, aminoglycosides, tetracycline, chloramphenicol, trimethoprim, fos-

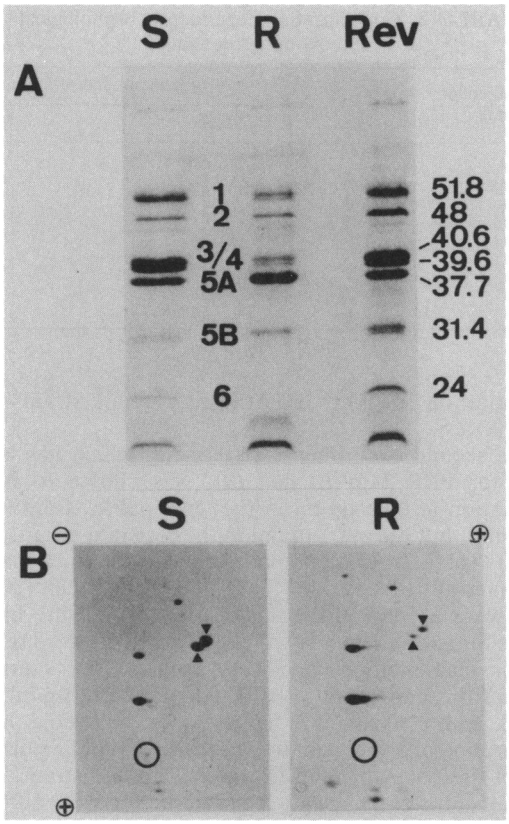

FIG. 1. (A) Outer membrane proteins from *S. paratyphi* A 10(S) (S), 100(R) (R), and 1000(Rev) (Rev). Proteins are numbered 1 through 6 (next to lane S; 5A and 5B are the two heat-modifiable forms of OmpA). Molecular masses (in kilodaltons) are indicated on the right. (B) Two-dimensional pattern of the outer membrane proteins from *S. paratyphi* A 10(S) (S) and *S. paratyphi* 100(R) (R). Symbols: +, Anode, −, cathode. The putative porins are indicated by arrowheads. The 24-kDa protein, which was present in *S. paratyphi* 10(S), is circled.

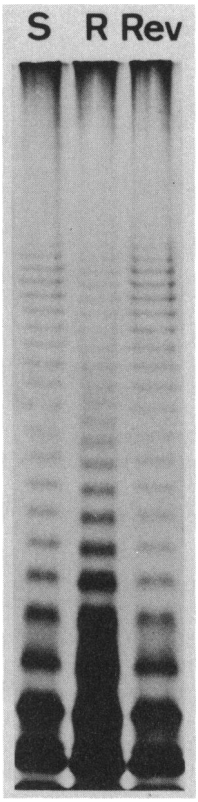

FIG. 2. LPS from whole-cell lysates of *S. paratyphi* A, silver-stained after SDS-polyacrylamide gel electrophoresis. Lanes: S, *S. paratyphi* A 10(S); R, *S. paratyphi* A 100(R); Rev, *S. paratyphi* A 1000(Rev).

fomycin, and quinolones, but not to colistin (Table 1). With respect to the β-lactam antibiotics, there was no change in the MIC of imipenem, whereas that of moxalactam was increased the greatest (64-fold). The MICs for the revertant strain 1000(Rev) were similar to those for the susceptible strain. In repeated experiments, however, strain 1000(Rev) appeared to be more susceptible to aminoglycosides and to ampicillin than did strain 10(S).

Characterization of outer membrane proteins and LPS. Quantification of the major outer membrane proteins (Fig. 1A) showed that only 10% of proteins 3 (40.6 kilodaltons [kDa]) and 4 (39.6 kDa) and less than 3% of protein 6 (24 kDa) were present in the resistant strain. Proteins 3 and 4 were probably porins, since they were solubilized at 100°C but not at 50°C in 1% SDS (23); and they migrated as the most acidic proteins in two-dimensional polyacrylamide gels (Fig. 1B). Proteins 5A and 5B (Fig. 1A) represented the two forms of the heat-modifiable protein OmpA (3), since an increase in the amount of the protein 5A band with a concomitant decrease of the protein 5B band was observed when samples were taken at various times during incubation of the outer membrane proteins in SDS at 100°C (data not shown).

The electrophoretic analysis of LPS (Fig. 2) extracted from whole cells showed a similar ladder pattern for strains 10(S) and 1000(Rev). In contrast, the LPS of the resistant strain showed a quantitative decrease of the high-molecular-mass polymers and an increase of those with a lower molecular mass. Also, the apparent size of the individual polymers was different in the resistant strain 100(R), which did not appear to be rough and continued to be agglutinable by the specific O1 and 2 antiserum.

PBP patterns. PBPs were examined after exposure of membranes to increasing concentrations of [^{3}H]benzylpenicillin (Fig. 3). Six PBPs were present that had similar

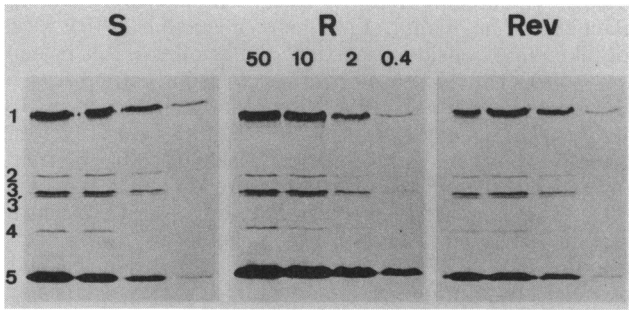

FIG. 3. PBP pattern of *S. paratyphi* A 10(S) (S), 100(R) (R), and 1000(Rev) (Rev). Membranes were exposed to the following increasing concentrations of [^{3}H]benzylpenicillin: 0.4, 2, 10, and 50 μg/ml. PBPs (indicated on the left) were numbered in order of decreasing size.

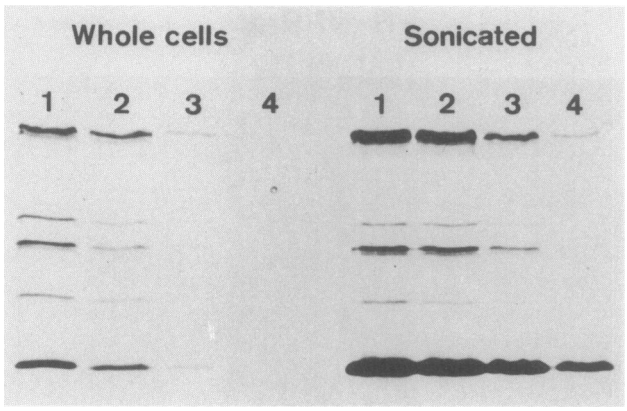

FIG. 4. Comparison of the binding of [^{3}H]benzylpenicillin to the different PBPs in whole and sonicated cells of *S. paratyphi* A 100(R). The concentrations used were as follows: 50 μg/ml (lanes 1); 10 μg/ml (lanes 2); 2 μg/ml (lanes 3); 0.4 μg/ml (lanes 4).

molecular masses to those of *Escherichia coli* (35), and no differences in affinities of the PBPs for benzylpenicillin were found for the various strains. The only noticeable difference was a ca. fourfold increase in the amount of PBP 5 in strain 100(R), and there was no change in affinity. Such an increase of a low-molecular-mass PBP has been reported previously (12) in a permeability mutant of *Serratia marcescens*. There is no evidence, however, that low-molecular-mass PBPs in members of the family *Enterobacteriaceae* participate in the modulation of the susceptibility to β-lactam antibiotics.

Evidence for decreased permeability of the outer membrane. The crypticity factor of the β-lactamase activity was compared for the different strains. Since the strains did not contain any detectable β-lactamase (probably due to the lack of the *ampC* gene [22]), a plasmid-borne TEM-1 enzyme was introduced into each strain. The crypticity factors for the susceptible [10(S)], the resistant [100(R)], and the revertant [1000(Rev)] strains were 9.1, 18.2, and 7.7, respectively. These numbers indicate that the resistant strain had a decreased outer membrane permeability. This is stated with caution, however, since there was some leakage of β-lactamasefrom intact cells, even in 100 mM phosphate buffer containing 5 to 50 mM magnesium. Therefore, two other experiments were designed to estimate crypticity.

In the first experiment, binding of [^{3}H]benzylpenicillin to PBPs was determined at different concentrations in paired samples of intact and sonicated cells (Fig. 4). The crypticity factor was defined as the ratio of the total amounts of [^{3}H]benzylpenicillin bound to PBPs 1 to 4 of sonicated and intact cells. The binding of [^{3}H]benzylpenicillin to PBP 5 was not taken into consideration since the amount of this protein was somewhat increased in the resistant strain. At a [^{3}H]benzylpenicillin concentration of 2 μg/ml, the crypticity factor was 4 for both the susceptible [10(S)] and revertant [1000(Rev)] strains and 8.5 for the resistant [100(R)] strain. At 10 μg/ml, the crypticity factors were 1.5 and 2.2, respectively. The decrease of the crypticity factor at a concentration of 10 μg/ml was not surprising since under these conditions the PBPs in the membranes of sonicated cells were already saturated, and more antibiotic diffused through the outer membrane of the intact cells. As was expected, at a concentration of 50 μg/ml, the crypticity factors were close to 1 for all three strains. Nevertheless, we consider it significant that at the lower [^{3}H]benzylpenicillin concentrations, the PBPs were more than twofold more cryptic in the resistant strain 100(R) than in the susceptible strains 10(S) and 1000(Rev).

TABLE 2. Concentration-dependent morphological effects of aztreonam

Aztreonam concn (μg/ml)	Filamentation of strain:	
	10(S)	100(R)
0.06	+	0
0.125	++	0
0.25	+++	0
0.5	+++	0
1	+++	+
2	+++	++
4	+++	+++

In the second experiment, aztreonam, which has a high affinity for PBP 3 in *E. coli* (8), was shown to induce filamentation at 0.06 μg/ml in the susceptible strain (MIC, 0.12 μg/ml), but only at 1 μg/ml in the resistant strain (MIC, 4 μg/ml) (Table 2). In a competitive assay in which isolated membranes of the sensitive [10(S)] and the resistant [100(R)] strains were used, PBPs 3 and 3′, which had the highest affinities for aztreonam and which we assumed were responsible for filamentation, had very similar 50% saturation values for this compound (PBP 3, 0.25 and 0.34 μg/ml; PBP 3′, 0.33 and 0.44 μg/ml). Thus, since a 16-fold higher concentration of aztreonam was necessary to obtain filamentation in the resistant than in the susceptible strain, while similar amounts of the drug were required for 50% saturation of PBP 3 or 3′ in both strains, we conclude that the access for β-lactam antibiotics to the PBPs was impeded in the resistant strain. This probably occurred because of a decreased permeability of the outer membrane. Additional supportive evidence for decreased permeability was the significantly reduced uptake of [^{3}H]glucose by the resistant strain (Fig. 5A) when the cells were exposed to increasing concentrations of this sugar. Similarly, reduced uptake of norfloxacin was observed in resistant strain 100(R) (Fig. 5B).

Role of the outer membrane proteins in the permeability defect. Because decreased permeability was the most likely cause of multiple resistance, and because of the demonstrated decrease of penetration of the β-lactam antibiotics, we evaluated the possible involvement of the outer membrane proteins in this particular resistance.

Proteoliposomes, into which equal quantities of total outer membrane proteins from the susceptible [10(S)] or the resistant [100(R)] strain were incorporated, were assayed for swelling in the presence of lactose or cephaloridine. A reduced swelling of the proteoliposomes that contained the

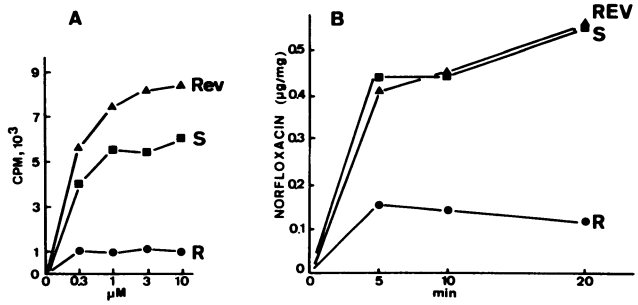

FIG. 5. Uptake of [^{3}H]glucose (A) and norfloxacin (B) in *S. paratyphi* A 10(S) (S), 100(R) (R), and 1000(Rev) (Rev).

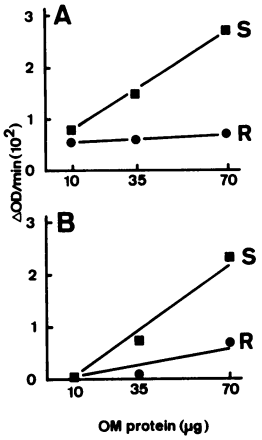

FIG. 6. Rates of proteoliposome swelling in the presence of cephaloridine (A) or lactose (B). Total outer membrane (OM) proteins were prepared from strains *S. paratyphi* A 10(S) (S) and 100(R) (R). ΔOD, Change in optical density.

total outer membrane proteins of the resistant strain 100(R) was observed (Fig. 6). Since identical quantities of proteins were used, smaller amounts of the 40.6-, 39.6-, and 24-kDa proteins would have been present in the proteoliposomes that were formed with the components of the resistant strain 100(R) (compare the electropherograms in lanes R and S in Fig. 1). It is probable that the decreased amount of presumed porins (40.6 and 39.6 kDa) was responsible for the lower rate of swelling. The possibility cannot be excluded, however, that modified LPS, which was not separated from the outer membrane proteins, may have contributed to the decreased swelling, even though porin function is thought to be unaffected by LPS (30). The 24-kDa protein did not have the characteristics of a porin, it was not acidic (Fig. 1B, lane S), and it was not peptidoglycan-associated (data not shown). Furthermore, it resembled a ca. 24-kDa protein of *Serratia marcescens* with similar characteristics which, when purified, does not support swelling of proteoliposomes (E. Collatz, unpublished data).

Bacterial invasion of HeLa cells. The susceptible [10(S)] and revertant [1000(Rev)] *S. paratyphi* A strains were shown to be very efficient at invading HeLa cells (about 10 to 30 bacteria per cell), and more than 95% of the HeLa cells were infected. In contrast, the resistant strain 100(R) was unable to invade HeLa cells; less than 1% of the HeLa cells were infected, and there were less than two bacteria per cell.

DISCUSSION

Cross-resistance toward different families of antibiotics in which alterations of outer membrane proteins are involved has been reported in several instances (4). In *E. coli*, resistance to β-lactam antibiotics with moderate cross-resistance to chloramphenicol, tetracycline, and quinolones has been associated with alterations of porin and, in some cases, of LPS as well (15, 16, 19). In *Klebsiella*, *Enterobacter*, and *Serratia* species, cross-resistance to chloramphenicol, trimethoprim, and different quinolones has been shown to be associated with low (13) or high (33) levels of resistance to β-lactam antibiotics and to be correlated with quantitative alterations of outer membrane proteins, presumably porins. In *Serratia marcescens*, quantitative alterations of outer membrane proteins, including porins, were associated with cross-resistance to β-lactam antibiotics and aminoglycosides (11, 12).

In the present study we have described the selection, at a frequency of 10^{-8}, of a mutant of *S. paratyphi* A that exhibited cross-resistance at different levels to multiple antibiotics. One peculiarity of this mutant is that it could be selected at a frequency that suggested a point mutation and that it was cross-resistant to a particularly large variety of antibiotics (Table 1). The simplest explanation for such an extensive cross-resistance would be a decrease in the membrane permeability. By measuring the uptake of [^{3}H]glucose and of norfloxacin, as well as the crypticity of β-lactamase and PBPs, we have demonstrated that the permeability of the resistant strain 100(R) is reduced when compared with that of the susceptible strains 10(S) and 1000(Rev). Examination of the outer membrane proteins in strain 100(R) showed a net quantitative decrease in at least three proteins, two of which (40.6 and 39.6 kDa) were probably porins. This observation, in conjunction with the reduced swelling of proteoliposomes when prepared with strain 100(R) proteins, strongly suggests that the alteration of the outer membrane proteins, and of the porins in particular, is the basis for the resistance, at least to β-lactam antibiotics. Similar observations have been made for other members of the family *Enterobacteriaceae* (4, 20, 25, 28).

There appeared to be a correlation between the extent of increase of the MIC and the charges of the β-lactam antibiotics. Thus, in the resistant strain, the MICs of the dianionic compounds aztreonam and moxalactam were more noticeably higher than were the MICs of the monoanionic compounds cefotaxime and cefoxitin, which, in turn, were more noticeably higher than the MICs of cephaloridine, ampicillin, and imipenem, which are zwitterions. This observation is in agreement with that of an inverse relationship between negative charge and *trans*-porin diffusion rates of the β-lactam antibiotics (39), and further implicates porin alterations in the resistance of strain 100(R) to these compounds. The only moderate increase of the MIC of ampicillin and the unchanged MIC of imipenem might also be explained by the existence of diffusion pathways for penicillins other than porins, which has been suggested previously (38).

It is likely that the decreased susceptibility to tetracycline, chloramphenicol, and trimethoprim was also linked to the decrease in the amounts of porins in the resistant strain. A similar observation has been made for several other species (13, 16, 33, 34). The same may also apply to the quinolones (13, 16, 19, 33), although there is some controversy about this, since different porin-deficient mutants that originated from *E. coli* KL16 were reported to have very different susceptibilities to quinolones (16, 19, 31). It does not seem likely that the modification of LPS would be responsible for the decreased permeation of β-lactam antibiotics, since modifications of LPS in members of the family *Enterobacteriaceae* are not thought to increase the MICs of β-lactam antibiotics, except in very rough mutants of *Salmonella*, which have a concomitant loss of porins (28). As far as quinolones are concerned, LPS-deficient mutants of *Salmonella typhimurium* have been shown to become susceptible to hydrophobic quinolones (15). If such LPS alterations were to interfere with the quinolone susceptibility of the resistant strain 100(R), one would expect an increase rather than a decrease of susceptibility to the more hydrophobic quinolones nalidixic acid and flumequine.

Non-enzyme-mediated cross-resistance to β-lactam antibiotics and aminoglycosides has been described only in *Pseudomonas aeruginosa* (32) and *Serratia marcescens* (11). In *Serratia marcescens*, quantitative porin alterations were associated with reduced amounts of a 24-kDa outer mem-

brane protein (E. Collatz, unpublished data). There are two possibilities to account for the low-level resistance to aminoglycosides. The first could be a decreased diffusion through the outer membrane if fewer porins were present or if the LPS was modified, which has been suggested to occur in other organisms (6, 10). However, we selected mutants of *S. paratyphi* A that were resistant exclusively to low levels of aminoglycosides and that lacked only the 24-kDa outer membrane protein (data not shown). These results resemble those obtained with similar mutants of *Serratia marcescens* that lacked a 24-kDa protein which did not appear to function as a porin (see above). An alternative possibility would be an impairment of the energy-dependent accumulation of aminoglycosides across the cytoplasmic membrane, as described previously (1, 2, 26) for small colony variants of gram-negative and gram-positive bacteria that are resistant to low levels of aminoglycosides.

The acquisition of multiple resistance in strain 100(R) was accompanied by the loss of its capability to invade HeLa cells. A similar loss of pathogenicity, which was associated with the acquisition of multiple drug resistance and modification of outer membrane proteins, has been reported in *Aeromonas salmonicida* (37) and *Enterobacter cloacae* (36). It is unlikely that the LPS modifications in strain 100(R) affect its capability to invade HeLa cells since rough mutants of *Salmonella typhimurium* maintain this characteristic (9). However, since outer membrane proteins in *Shigella* species have been considered to be involved in the invasion of HeLa cells (24), it is conceivable that the membrane proteins, which are altered in strain 100(R), normally participate in the process of invasion.

What appears to be most remarkable about the apparent one-step mutation that is responsible for the phenotype of strain 100(R) is the extent of pleiotropy. It encompasses, as demonstrated above, alterations of the LPS and of several outer and inner membrane proteins. It results in slow growth; multiple drug resistance as a consequence of reduced drug diffusion across the outer membrane; probably, reduced drug accumulation across the inner membrane; and finally, loss of invasiveness into HeLa cells. A pleiotropic mutation in *E. coli* that entails cross-resistance to multiple antibiotics, including quinolones, and that is associated with a decreased amount of OmpF has been observed in a regulatory gene, *marA* (7, 18).

The selection of the mutant 100(R) was not particular to one susceptible strain of *S. paratyphi* A, since similar mutants were selected in vitro from three other clinical isolates of the same species (data not shown). It is possible that the clinical relevance of such mutants resides in the loss of pathogenicity. Although a mutation of the type described here is likely to affect bacterial penetration into the cells of the digestive tract, this is difficult to assess formally without an animal model. Also, we know nothing about the pathophysiological consequences of an altered bacterial surface if the mutational alteration would happen to occur during therapeutic treatment after completion of the invasive step.

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