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Clinical Isolate of a Porinless *Salmonella typhi* Resistant to High Levels of Chloramphenicol

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We studied a clinical isolate of *Salmonella typhi* (strain 1895) characterized by resistance to 200 μg of chloramphenicol per ml despite the absence of chloramphenicol-inactivating activity. The outer membrane protein profile analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a deficiency of one of the major protein species which may serve as a porin for entry of chloramphenicol. When the strain was transformed with a plasmid encoding chloramphenicol acetyltransferase, chloramphenicol added to the culture was not inactivated, suggesting a drastic reduction of permeability towards the drug. Moreover, transformants bearing a plasmid coding for the *Escherichia coli* OmpF porin became considerably more susceptible to chloramphenicol (40 μg/ml). On the other hand, transformants carrying a plasmid encoding the *Salmonella typhi ompC* gene remained as resistant to the drug as the parental strain, even though they overexpressed OmpC. These findings indicate that the lack of OmpF plays a major role in the resistance to chloramphenicol in strain 1895.

Gram-negative bacteria allow the influx of low-molecular-weight hydrophilic solutes by water-filled channels or pores interspersed in the outer membrane (OM) (19). Such pores are formed by trimers of proteins termed porins (18).

Two functionally homologous porins, OmpF and OmpC, found in *Escherichia coli* and *Salmonella typhimurium* are directly involved in cell permeability, mediating a passive and nonspecific diffusion of small nutrients and drugs (23, 31).

Several reports have demonstrated that porin-deficient mutants are more resistant to antibiotics than are isogenic strains with the full complement of major outer membrane proteins (OMP). In fact, a decreased susceptibility to certain β-lactams, chloramphenicol, and tetracycline was observed in *E. coli* mutants deficient in OmpF (5, 11, 27). Moreover, mutant strains of *S. typhimurium* deficient in either porin OmpC or OmpD presented a reduced permeability to cephaloridine (22). Less-characterized porin-deficient mutants of *Proteus mirabilis* and *Enterobacter cloacae* exhibited resistance to tetracycline and to tetracyline and chloramphenicol, respectively (27). More recently, cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol was associated with a decreased amount of at least one major OMP of *Klebsiella*, *Enterobacter*, and *Serratia* species (8).

Although permeability mutants spontaneously arise in vitro at rather high frequencies (9), this has not been the case for the emergence of resistant enteric bacteria of nosocomial origin (26). Apparently these mutants either go undetected or are overcome by the antibiotic treatment. Nevertheless, clinical isolates of *Serratia marcescens* (7), *Haemophilus influenzae* (3), *S. typhimurium* (16), and *Pseudomonas aeruginosa* (25) were found to present a reduced amount of a major OMP associated with a decreased susceptibility to antibiotics.

Recently, OMP from *S. typhi* Ty2 were characterized as porins by planar lipid bilayer studies (13) and they were recognized as the most abundant proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4). Furthermore, the cloning and sequencing of the *S. typhi* Ty2 *ompC* gene confirmed the close similarity among OmpC porins from members of the family *Enterobacteriaceae* (1, 33, 34).

In this work, we describe a clinical isolate of a *S. typhi* strain resistant to high levels of chloramphenicol which was shown to be deficient in one major OMP, OmpF.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *E. coli* 711 is a multiresistant clinical isolate producing plasmid-mediated chloramphenicol acetyltransferase (CAT). *S. typhi* Ty2 is a standard strain from the World Health Organization, supplied by the Instituto de Salud Pública de Chile. *S. typhi* 1895 and *S. typhi* R2 are described in this work. Plasmids used were pBR325, a pBR322 derivative encoding CAT and TEM-1 β-lactamase (Bethesda Research Laboratories, Inc.); pSTP1-3 harboring a *S. typhi ompC* gene and Amp^r, obtained from Alejandro Venegas (34); and pTUN8-F, a low-copy-number plasmid containing *E. coli ompF* and Amp^r, provided by Takeshi Mizuno (17). Bacterial cultures were grown overnight in nutrient broth purchased from Difco Laboratories or in Luria broth (LB) (15). Chloramphenicol, carbenicillin, or ampicillin (Sigma Chemical Co.) was added when required.

MIC. Antibiotic susceptibility was determined by twofold serial dilution of the drug in nutrient broth. A 50-μl portion of an overnight bacterial culture was added to the test tubes, and they were incubated at 37°C for 14 to 18 h. MIC was defined as the lowest concentration of drug preventing bacterial growth.

Plasmid extraction. Alkali denaturing methods described by Kado and Liu (12) and Birnboim and Doly (2) were used.

Determination of CAT activity. CAT was assayed by monitoring A_{232}, which reflected the acetyl coenzyme A consumption due to chloramphenicol acetylation (29), and by detection of the HS-coenzyme A released from acetyl coenzyme A with 5,5′-dithiobis-2-nitrobenzoic acid (20).

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These assays were performed in cell extracts prepared by the method of Okamoto and Suzuki (24).

Bioassays to estimate chloramphenicol concentrations. Chloramphenicol concentration remaining in LB supernatants, prepared by the method of Gaffney et al. (6), was measured by two bioassays. In one of them, the inhibition halos produced by paper disks soaked with supernatant were determined by using *E. coli* K-12, a chloramphenicol-susceptible strain. In the second bioassay, increasing amounts of the supernatants were added to 1 ml portions of nutrient broth inoculated with *E. coli* K-12. Chloramphenicol concentration was deduced from a parallel assay by using LB supplied with a known amount of chloramphenicol.

Preparation of spheroplasts. Spheroplasts were prepared by the procedure of Irvin et al. (10) and then pelleted by low-speed centrifugation (5,000 × *g* for 20 min at 4°C) and resuspended in LB.

OMP preparation. OMP were obtained as described by Schnaitman (28). Bacteria were sonicated and centrifuged at 5,000 × *g* for 5 min. The supernatant obtained (crude extract) was centrifuged at 100,000 × *g* for 45 min, and the pellet was suspended in 10 mM Tris hydrochloride, pH 7.8–10 mM $MgCl_2$–2% Triton X-100. The suspension was centrifuged, and the insoluble pellet contained the OMPs. A suspension of intact bacteria was designated as whole cells.

SDS-PAGE and immunoblot. SDS-PAGE was performed in 12% polyacrylamide slabs as described by Laemmli (14). All samples were heated at 100°C in the sample buffer before electrophoresis (4). Transfer of proteins from polyacrylamide gel to nitrocellulose was accomplished as described by Towbin et al. (32). Anti-*S. typhi* porin rabbit serum prepared by using copurified OmpC and OmpF (4) was diluted 1:500 for immunodetection. Thereafter, the nitrocellulose sheet was incubated with protein A-alkaline phosphatase (Sigma Chemical Co.) diluted 1:1,000. Finally the immunoblot was developed with 0.16 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml and 0.33 mg of Nitro Blue Tetrazolium per ml.

Transformations. Cells were made competent by the procedure of Maniatis et al. (15). Selective antibiotic concentrations were as follows: chloramphenicol, 40 μg/ml; and ampicillin, 100 μg/ml.

Poly(U)-directed poly(Phe) synthesis. This assay was accomplished as described by Nierhaus and Dohme (21) by using 150 μg of S_{150} fraction from *E. coli* D10 ($RNase^-$ Met^-) to test 140 μg of *S. typhi* ribosomes. Chloramphenicol was added (100 to 1,000 μg/ml) when required. [^{14}C]Phe was purchased from Amersham Corp. (specific activity, 513 mCi/mmol). Bradford reagent from Bio-Rad Laboratories was used for protein measurements.

RESULTS

During the study of *S. typhi* clinical isolates displaying multiple antibiotic resistance, we detected one strain, designated 1895, which thrived in up to 200 μg of chloramphenicol per ml in nutrient broth. It was also resistant to carbenicillin and ampicillin but susceptible to most other antibiotics regularly tested in plasmid-bearing *S. typhi* (Table 1).

A plasmid-coded enzyme is often found responsible for the bacterial resistance to chloramphenicol. This is CAT activity, which inactivates the drug by 3-O-acetylation with acetyl coenzyme A (30). However, neither plasmid nor CAT activity that could be involved in chloramphenicol resistance was detected in strain 1895. *E. coli* 711 (CAT^+) was used as a positive control. In addition, we did not detect any other hypothetical chloramphenicol-inactivating activity in strain 1895, as revealed by two bioassays.

TABLE 1. MICs for *S. typhi* strains[a]

Antibiotic	MIC (μg/ml) for strain:					
	Ty2	1895	1895 (pTUN8-F)	1895 (pSTP1-3)	R2	R2 (pTUN8-F)
Chloramphenicol	2	220	40	220	4	2
Tetracycline	2	8	8	8	2	2
Streptomycin	2	2	2	2	2	2
Kanamycin	2	2	2	2	2	2
Nalidixic acid	2	2	2	2	2	2
Ampicillin	2	16	ND	ND	4	ND
Carbenicillin	8	64	ND	ND	64	ND
Cephaloridine	2	8	ND	ND	ND	ND
Cefazolin	2	8	ND	ND	ND	ND
Cephalosporin C	4	4	ND	ND	ND	ND

[a] Determined in supplemented nutrient broth after 14 to 18 h of incubation at 37°C. ND, Not determined.

The possibility that resistance to chloramphenicol resulted from a ribosomal mutation was also considered. In vitro studies of ribosome-mediated polyuridylic acid-directed polyphenylalanine synthesis revealed that *S. typhi* Ty2 as well as strain 1895-derived ribosomes were inhibited at the same extent by chloramphenicol.

To demonstrate that strain 1895 could be a permeability mutant, we studied the chloramphenicol-inactivating capacity of strain 1895(pBR325) (CAT^+) by the bioassays described in this work. These assays indicated that active antibiotic concentrations in LB supernatants remained unchanged after growth of the transformant, suggesting that chloramphenicol was precluded from reaching the cytoplasm, where CAT activity was located. In contrast, CAT activity was detected in cell extracts prepared from strain 1895(pBR325). As a control, pBR325 was used to transform *S. typhi* Ty2, susceptible to 2 μg of chloramphenicol per ml. The transformant obtained was in fact resistant to 80 μg of chloramphenicol per ml, and the presence of CAT activity as well as the inactivation of chloramphenicol was demonstrated in this strain.

It has been described that a conjugative R plasmid confers chloramphenicol resistance in *E. coli* by creating a permeability barrier to the drug, due to a putative change in the cytoplasmic membrane (6). However, this is not the case for strain 1895, since in our studies no plasmid was ever detected and partial removal of the OM changed 1895 cells into chloramphenicol-susceptible spheroplasts unable to regenerate proliferating cells in a growth medium supplemented with chloramphenicol (25 μg/ml). In contrast, spheroplasts incubated without chloramphenicol recovered after 4 h into actively proliferating bacteria.

Since in gram-negative organisms an increased resistance to some drugs may be exhibited by mutants deficient in one or more of the major OMP, the OMP profile of strain 1895 was analyzed by SDS-PAGE. We observed that this strain is deficient in OmpF (Fig. 1, lane b), one of the otherwise most abundant proteins in *S. typhi* Ty2 (Fig. 1, lane a). The porin left in the OM of strain 1895 is shown comigrating with OmpC (Fig. 1, lane c). OmpC porin was obtained from *E. coli* UH 302 containing the cloned *S. typhi ompC* gene (34). The absence of OmpF from strain 1895 was confirmed by immunoblot developed with rabbit anti-*S. typhi* Ty2 porins serum against whole cell preparation (Fig. 2, lane d), crude extract (Fig. 2, lane e), and OMP preparation (Fig. 2, lane f).

Thus far, our results suggest that in strain 1895, one of the

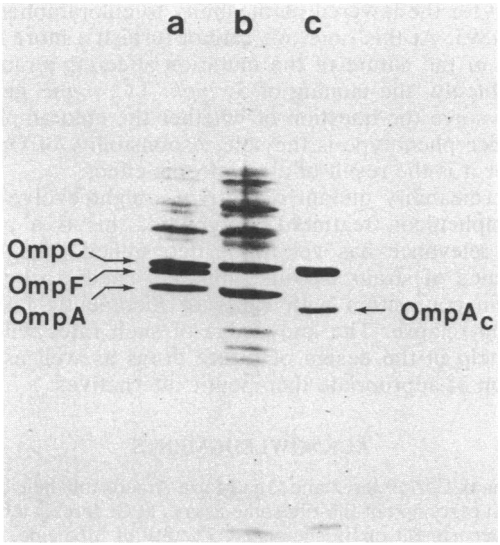

FIG. 1. SDS-PAGE of OMP. Lane a, *S. typhi* Ty2; lane b, *S. typhi* 1895; and lane c, *E. coli* UH302(pSTP1-3) expressing *S. typhi* OmpC. *E. coli* OmpA is indicated as $OmpA_c$.

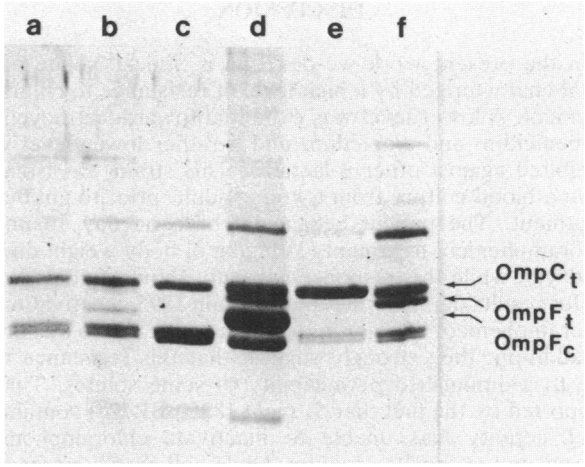

FIG. 3. SDS-PAGE of OMP to detect expression of *E. coli* OmpF in *S. typhi* strains. OMP preparations were obtained as follows: lane a, strain 1895; lane b, 1895(pTUN8-F); lane c, *S. typhi* R2; lane d, *S. typhi* R2(pTUN8-F); lane e, 1895(pSTP1-3); and lane f, *S. typhi* Ty2. OmpC and OmpF porins from *S. typhi* are indicated as $OmpC_t$ and $OmpF_t$, and OmpF from *E. coli* is indicated as $OmpF_c$.

porins is playing a critical role in the resistance to chloramphenicol. Therefore, we decided to obtain transformants that express either OmpC or OmpF at the OM. These transformations were accomplished by using plasmids pSTP1-3 carrying the *S. typhi* Ty2 *ompC* gene (34) and pTUN8-F, a plasmid containing the *E. coli ompF* gene (17). So far, *S. typhi ompF* has not been cloned.

When strain 1895(pSTP1-3) was tested against chloramphenicol, it remained resistant and it was shown by SDS-PAGE to express an increased amount of OmpC (Fig. 3, lane e). In contrast, strain 1895(pTUN8-F) expressing *E. coli* OmpF (Fig. 3, lane b) was completely restricted from growth at 40 μg of chloramphenicol per ml (Fig. 4), remaining resistant to lower concentrations of the drug.

We also studied *S. typhi* R2 (Fig. 3, lane c), an $OmpF^-$ *S. typhi* Ty2 spontaneous mutant selected by its resistance to carbenicillin (9). In addition, the mutant strain was resistant to chloramphenicol (4 μg/ml) and, after transformation with pTUN8-F, it became susceptible to 2 μg of the drug per ml. The expression of *E. coli* OmpF was confirmed by SDS-PAGE (Fig. 3, lane d). Determination of carbenicillin susceptibility was prevented in 1895(pTUN8-F) and R2(pTUN8-F) by the β-lactamase encoded by the plasmid.

Growth curves obtained for the transformants expressing *E. coli* OmpF revealed that they presented a higher growth rate than the corresponding isogenic strains. Furthermore, growth characteristics exhibited by R2(pTUN8-F) very closely resembled those of the wild-type strain, whereas this was not the case for strain 1895(pTUN8-F).

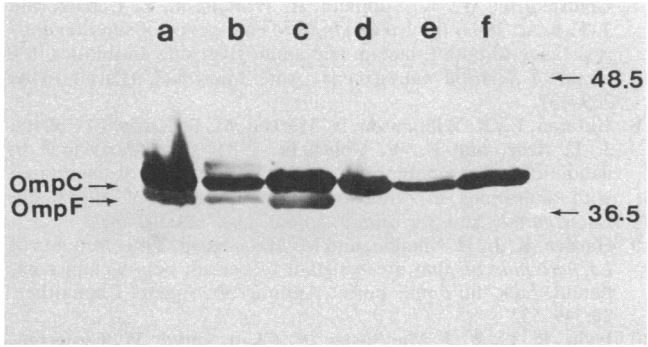

FIG. 2. Immunoblot of OmpF porin developed with an anti-*S. typhi* Ty2 porin rabbit serum. From *S. typhi* Ty2: lane a, whole cell preparation; lane b, crude extract; lane c, OMP preparation. From strain 1895: lane d, whole cell preparation; lane e, crude extract; lane f, OMP preparation. Molecular size standards (kilodaltons) are indicated by arrows.

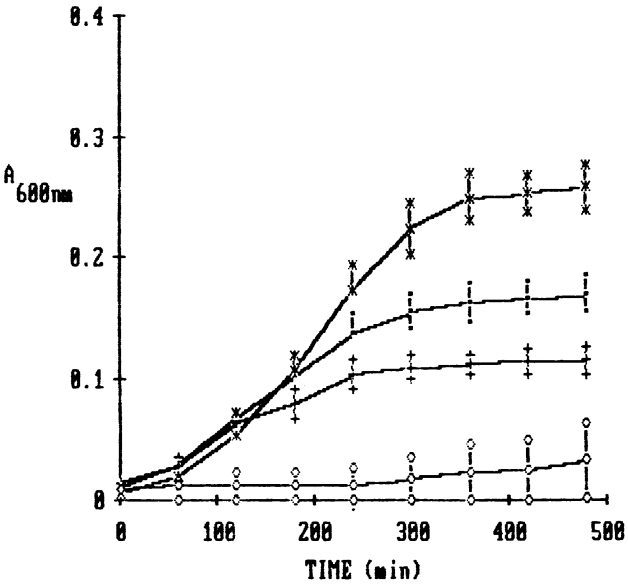

FIG. 4. Effect of chloramphenicol on strain 1895 and 1895 (pTUN8-F) growth. The bacterial cultures were incubated at 37°C, and the A_{600} was monitored. The plots represent the average of at least five growth curves. Bacterial growth in LB without chloramphenicol, strain 1895 (■) and strain 1895(pTUN8-F) (*); bacterial growth in LB supplemented with 40 μg of chloramphenicol per ml, strain 1895 (+) and strain 1895(pTUN8-F) (○).

DISCUSSION

In the present work we describe a clinical isolate of *S. typhi* characterized by a high level of resistance to chloramphenicol. A lower level was exhibited towards tetracycline, carbenicillin, and ampicillin, and a rather lower level was exhibited against other β-lactams. This strain was isolated from a blood culture from a young adult, prior to antibiotic treatment. The patient became afebrile on day 10 under chloramphenicol treatment (25 mg/kg of body weight during 15 days), while this response normally shows on day 4.

Our results demonstrate that in strain 1895, inactivation of chloramphenicol was not involved in resistance to this drug. In addition, they strongly suggest that this resistance was due to a diminished permeability to some solutes. This is supported by the fact that *S. typhi* 1895(pBR325) containing CAT activity was unable to inactivate chloramphenicol present in the growth medium, while cell extracts prepared from the same strain were able to do so.

We believe that strain 1895 displays a permeability barrier to chloramphenicol located in the OM, and according to SDS-PAGE, it is associated to the lack of one porin species. We propose that the missing protein should correspond to OmpF, since our results demonstrated that *S. typhi* R2, a carbenicillin-resistant spontaneous mutant, and strain 1895, which is also resistant to carbenicillin, are deficient in the same protein species. This conclusion is supported by the finding reported in *E. coli* K-12, in which the carbenicillin resistance phenotype is associated with the lack of OmpF (9).

Furthermore, when strain 1895 was transformed with a plasmid encoding *E. coli ompF*, we observed that such a gene product was found in the OM, that strain 1895 (pTUN8-F) was considerably more susceptible to chloramphenicol, and that it grew faster than the isogenic strain. All together, these findings support the role of OmpF in the permeability to chloramphenicol and nutrients. Nevertheless, 1895(pTUN8-F) was resistant to chloramphenicol at concentrations below 40 μg/ml. Perhaps *E. coli* OmpF does not fulfill all the functions provided by the *Salmonella* porin. Susceptibility of transformants towards carbenicillin and other β-lactams was not determined due to the β-lactamase encoded by plasmid pTUN8-F.

Although several attempts were made to demonstrate that strain 1895 displayed a reduced uptake of [^{14}C]chloramphenicol compared with that of *S. typhi* Ty2, we detected no difference between them. It is known that determinations such as these are seriously interfered with by a nonspecific binding of chloramphenicol to cells and filters used in the assays (30).

Even though strain 1895 and *S. typhi* R2 are OmpF$^-$ mutants equally resistant to carbenicillin, there exist sharp differences between them. After transformation with plasmid pTUN8-F, both expressed *E. coli* OmpF, although to clearly distinct extents. Moreover, in its behavior against chloramphenicol, *S. typhi* R2 doubles the resistance shown by *S. typhi* Ty2, while our clinical isolate is 100 times more resistant than the wild-type strain. A likely explanation might be that in addition to the lack of OmpF, strain 1895 displayed an anomalous OmpC. However, we have ruled out this possibility because strain 1895(pSTP1-3), in spite of overexpressing OmpC, was not susceptible to chloramphenicol. Alternatively, one might consider the clinical isolate as a legitimate OmpF-less strain, while the in vitro mutant might be some sort of leaky one. Finally, it is probable that the absence of OmpF is necessary but not sufficient to account for the lowered permeability to chloramphenicol in strain 1895. At this time, we cannot furnish a more precise picture of the nature of the mutation affecting strain 1895. Undoubtedly, the cloning of *S. typhi* Ty2 *ompF* gene will help to solve the question of whether the chloramphenicol resistance phenotype is the sole responsibility of OmpF or whether it is the result of a pleiotropic effect.

A permeability mutant of *S. typhi* might evolve during chloramphenicol treatment of typhoid; this is a problem whose relevance has yet to be determined. The rate of emergence of strain 1895-like mutants needs to be established in connection with cases of therapeutic failure or infection relapse. The knowledge of such rates will be of much help in the design of future drugs as well as in the selection of appropriate therapeutic alternatives.

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