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Chromosomal DNA, Iron-Transport Systems, Outer Membrane Proteins, and Enterotoxin (Heat Labile) Production in *Salmonella typhi* Strains

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We examined a representative collection of *Salmonella typhi* strains from Chile, Peru, Mexico, India, and England for the presence of several properties. All strains had a conserved pattern of outer membrane proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrophoresis profiles of chromosomal DNA digested with *Eco*RI and *Pst*I restriction enzymes were similar for all the strains. A conserved pattern of hybridization was observed when digested chromosomal DNA was hybridized with DNA probes for the 36-kilodalton porin, enterobactin synthesis, and enterobactin receptor genes. All the strains produced enterobactin but not aerobactin in bioassays. None of the strains produced heat-labile toxin, as measured by an enzyme-linked immunosorbent assay. Colony and Southern hybridizations with DNA probes for aerobactin synthesis and its receptor and heat-labile toxin genes were negative. These results indicate that *S. typhi* strains from different origins have similar phenotypic and genetic properties and, as has been suggested, constitute a clone.

Typhoid fever has almost totally disappeared as a cause of morbidity in industrialized countries because of improved sanitary conditions in the preparation of food and water, sewage disposal, and treatment of carriers (8). Nonetheless, typhoid fever continues to be an important public health problem in some developing countries, where the transmission of the disease is characterized by epidemics superimposed on an endemic background of *Salmonella typhi* infections (8). Massive outbreaks of typhoid fever during the past 17 years, with hundreds of thousands of cases, in Mexico City, Mexico, Santiago, Chile, and Lima, Peru (5), have usually been due to breakdowns in the treatment of water, contaminated food, and improper disposal of sewage (5).

The conserved antigenic and metabolic properties among different isolates of *S. typhi*, as well as the recent demonstration of a conserved isoenzyme pattern (17, 18), have suggested that all *S. typhi* strains worldwide are clonally related. Nonetheless, rRNA gene restriction pattern analysis has recently provided a tool to differentiate among *S. typhi* strains (3). To understand *S. typhi* virulence, to elucidate the host immune response to its antigens, and to design new vaccines and diagnostic assays, it is important to determine if other *S. typhi* properties are constant among different *S. typhi* isolates (19). Included in these properties are the presence of genes coding for putative virulence factors and surface antigens. Two such genes are those that code for the outer membrane proteins of the enterochelin-mediated iron uptake system and the 36-kilodalton (kDa) porin (6, 11). Both the porin and the iron uptake proteins are important human immunogens and evoke high titers of antibody in typhoid fever patients (6, 11). We have used suitable DNA probes and assays to determine whether these genes and their products are conserved among *S. typhi* strains of different geographical origin.

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MATERIALS AND METHODS

The *S. typhi* strains used in this study were blood isolates from patients with typhoid fever; the strains were usually grown in L broth or agar at 37°C and kept at −20°C in 70% glycerol. The isolates include strains isolated 15 years ago (Mexican and Indian strains) and strains isolated in the last 3 years (Peruvian and Chilean isolates) and were as follows: Ty2, a laboratory strain; TyIN1, 803, 2047, 2531, 4224, and 4693 from Chile; D1776 from England; D1067, D1176, and D1163 from India; H34, H39, and H47 from Mexico; and 26, 132, 255, 295, 309, 349, 351, 610, and 1334 from Peru. Susceptibility to colicin B and cloacin DF13 were assayed as described previously (9, 11). The production of enterochelin and aerobactin was detected in bioassays with *Escherichia coli* BN3040 and LG1522, respectively (9, 11). The production of heat-labile toxin (LT) was detected with a GM1-based enzyme-linked immunosorbent assay and CHO, Y1, and Vero cell monolayers (13, 20). Plasmid DNA was extracted by the Birnboim-Doly or Kado-Liu methods, and clear lysates were run in cesium chloride-ethidium bromide gradients and analysed by agarose gel electrophoresis (1, 11). Chromosomal DNA was extracted from whole-cell lysates as described previously (15). Outer membrane proteins were extracted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1, 11). Colony DNA hybridization and Southern hybridization of restriction enzyme-digested chromosomal DNA were performed as previously described (2, 11, 12). The DNA probe for the 36-kDa porin gene was an intragenic *Pvu*II-*Pst*I DNA fragment of 800 base pairs from plasmid pST13 (1). The aerobactin synthesis and receptor DNA probes were a 2-kilobase *Ava*I DNA fragment of plasmid pABN-5 and a 2.3-kilobase *Pvu*II DNA fragment of plasmid pABN-1, respectively (12). The probe for the enterochelin synthesis genes was a 1,200-base-pair *Hpa*I-*Pvu*II DNA fragment containing regions of the *entA* and *entB* genes from pITS12 (9, 16). The probe for the enterochelin receptor gene *fepA* was a 400-base-pair *Kpn*I-*Pvu*II fragment from pITS1 (11). The LT probe was a *Hin*dIII DNA fragment from plasmid pEWD299 encoding primarily the B subunit of

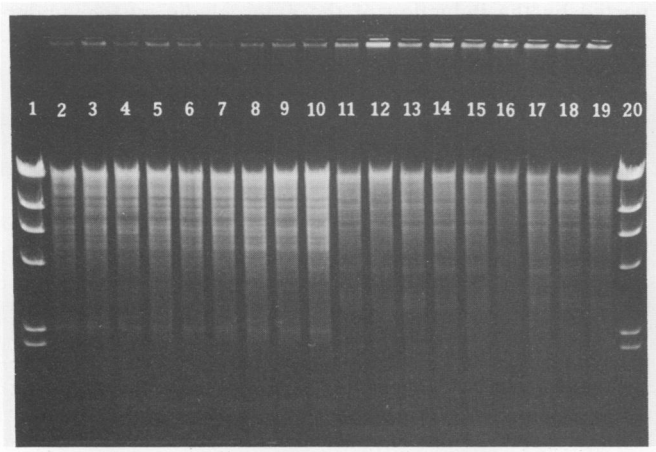

FIG. 1. Agarose gel electrophoresis of chromosomal DNA from different *S. typhi* strains digested with *Eco*RI and *Pst*I restriction enzymes. Lanes: 2 and 11, Ty2; 3 and 12, TyIN1; 4 and 13, 803; 5 and 14, 351; 6 and 15, 26; 7 and 16, 349; 8 and 17, D1776; 9 and 18, D1067; 10 and 19, H39; 2 to 10, *Eco*RI digests; 11 to 19, *Pst*I digests; 1 and 20, lambda DNA digested with *Hin*dIII.

the LT molecule (2, 7). The restriction enzymes were from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as suggested by the manufacturer. The DNA probes were prepared by electroelution of the restricted DNA fragments and labeled with [α-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.) by nick translation (9, 11, 12).

RESULTS

Restriction fragment length polymorphism of chromosomal DNA of *S. typhi*. Agarose gel electrophoresis of the chromosomal DNA of different *S. typhi* strains digested with *Eco*RI and *Pst*I showed conserved banding patterns in all the strains (Fig. 1). Southern hybridization of the *S. typhi* chromosomal DNA digested with *Pst*I with an intragenic probe for the 36-kDa porin gene revealed a single hybridization band among different *S. typhi* strains, with the exception of strain 1163 (Fig. 2). A similarly conserved pattern of hybridization was seen when *Eco*RI- and *Pst*I-digested chromosomal DNA from all *S. typhi* strains was hybridized with DNA probes for the enterochelin synthesis genes and the enterochelin receptor gene *fepA* (Fig. 3); in each case, only a single hybridization band was evident. Similar results were obtained with 16 other Peruvian strains of *S. typhi* (data not shown).

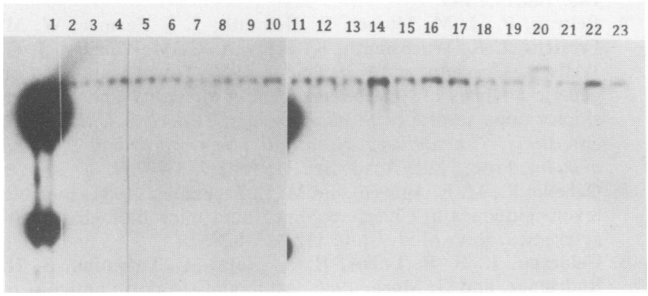

FIG. 2. Chromosomal DNA from different *S. typhi* strains digested with *Pst*I and hybridized with the 36-kDa porin probe. Lanes: 1, pST13 DNA; 2, Ty2; 3, TyIN1; 4, 803; 5, 295; 6, 26; 7, 349; 8, D1176; 9, D1067; 10, H39; 11, 309; 12, 132; 13, 610; 14, 351; 15, H34; 16, 1334; 17, 255; 18, 4693; 19, H47; 20, D1163; 21, 2047; 22, 2531; 23, 4224.

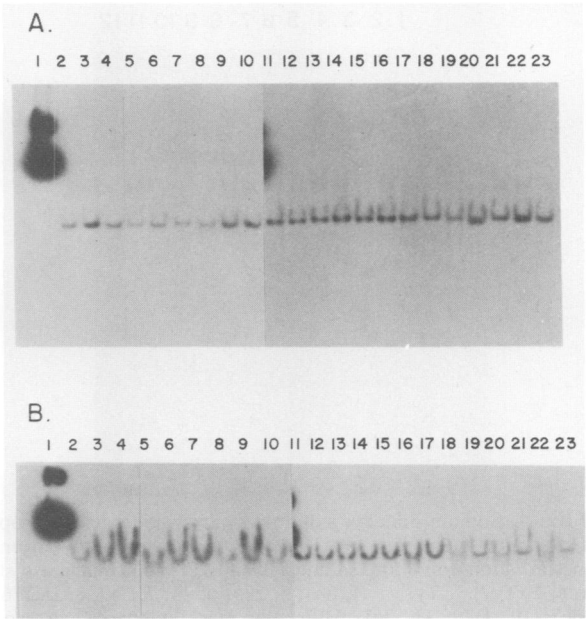

FIG. 3. Chromosomal DNA from different *S. typhi* strains digested with *Pst*I and *Eco*RI and hybridized with the enterochelin synthesis DNA probe. (A) Lanes: 1, pITS12 DNA; 2, Ty2; 3, TyIN1; 4, 803; 5, 295; 6, 26; 7, 349; 8, D1176; 9, D1067; 10, H39; 11, 309; 12, 132; 13, 610; 14, 351; 15, H34; 16, 1334; 17, 255; 18, 4693; 19, H47; 20, D1163; 21, 2047; 22, 2531; 23, 4224. All *S. typhi* chromosomal DNAs were digested with *Pst*I. (B) As in panel A except that all *S. typhi* chromosomal DNAs were digested with *Eco*RI.

Iron-transport systems. All *S. typhi* isolates studied produced bioactive enterochelin but not aerobactin. All the *S. typhi* isolates were resistant to colicin B and cloacin DF13. All *S. typhi* strains grown under conditions of iron starvation expressed the three previously identified iron starvation-induced proteins in their outer membranes (Fig. 4) (11). These proteins have M_rs of 69,000, 78,000, and 83,000 (Fig. 4). Moreover, all the strains hybridized with the enterochelin synthesis and enterochelin receptor gene probes but not with the aerobactin synthesis or aerobactin receptor DNA probes in colony hybridization assays.

Outer membrane proteins. The patterns of *S. typhi* outer membrane proteins from cell isolates were identical, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). This conserved pattern includes proteins that, as has been shown, are strongly immunogenic in typhoid fever (6, 11), such as the high-molecular-weight iron starvation-induced proteins, the porins, and a 14.5-kDa protein (11) (L. Aron, G. Faundez, C. Gonzalez, and F. Cabello, unpublished results).

LT production. None of the *S. typhi* strains produced immunoreactive LT. Moreover, the supernatant of *S. typhi* laboratory strain Ty2 failed to produce cytopathic changes in CHO, Vero, and Y1 cell monolayers, providing independent evidence for a lack of production of bioactive LT by *S. typhi*. Finally, a DNA probe for the *E. coli* LT gene did not hybridize with *S. typhi* colonies or with *S. typhi* chromosomal DNA digested with *Hin*dIII when hybridizations were performed at 37 and 42°C in 25% formamide and at 65°C in 30% formamide.

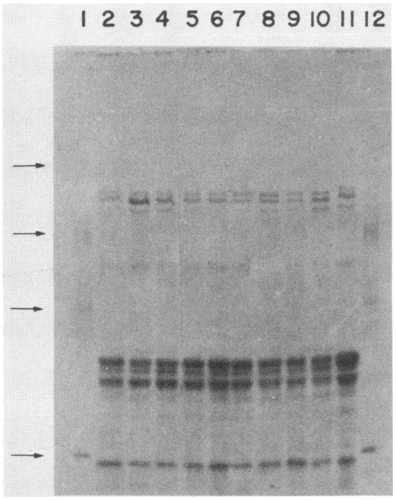

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of *S. typhi* strains grown under iron starvation conditions. Lanes: 1 and 12, molecular mass standards; 2, Ty2; 3, TyIN1; 4, 803; 5, 295; 6, 26; 7, 349; 8, D1776; 9, D1067; 10, H39; 11, 351. Molecular mass standards correspond (arrows, from top to bottom) to 97.4, 66.2, 42.7, and 21.5 kDa.

DISCUSSION

The clonal distribution of several pathogenic bacteria has been suggested by use of a variety of methodologies that include determination of antigenic and biochemical properties, analysis of outer membrane proteins, multilocus enzyme electrophoresis, and restriction enzyme analysis of plasmid and chromosomal DNAs (4). The analysis of the clonal nature of different bacterial species has been used to identify bacterial clones in *E. coli*, *Haemophilus influenzae* type b, and *Salmonella* spp. that show some preferential pathogenic potential (4). The conserved antigenic characteristics of geographically and temporally diverse isolates of *S. typhi*, their identical isoenzyme patterns, and the similar patterns of restriction endonuclease digests of the chromosomal DNA indicate that *S. typhi* strains can be considered one clone (14, 17). Nonetheless, this type of analysis does not generate information regarding the evolution of specific virulence properties or the role of horizontally transmitted plasmid and chromosomal genes in virulence.

The restriction fragment length polymorphism analysis of *S. typhi* chromosomal DNA indicates a highly conserved and stable genome in *S. typhi* and underlines the correlation between conserved DNA sequences and stable phenotypes in *S. typhi*. This relationship is also illustrated by the almost absolute conservation of the pattern of outer membrane proteins among *S. typhi* strains. The conservation of the DNA sequences studied indicates that it may be possible to develop DNA probes from unique segments in these genes to detect *S. typhi* in the environment and in fluids and tissues from patients. The identical patterns of immunogenic outer membrane proteins of all the *S. typhi* strains studied also suggest that these proteins could be used as reagents in diagnostic assays and as antigens in the preparation of vaccines.

The presence of the genes and gene products involved in enterochelin iron uptake and the conservation of their sequences among *S. typhi* strains suggest that this system may play a crucial role as a virulence factor in the natural history of *S. typhi* infections in humans. This hypothesis is supported by the induction of the enterochelin system under iron starvation conditions in vitro as well as in vivo, the latter demonstrated by the immunogenicity of the iron starvation-induced proteins in patients with typhoid fever (11). Likewise, the lack of direct involvement in pathogenesis may allow rRNA genes to escape selective pressure and thus may explain the variability and the usefulness of rRNA gene restriction patterns in differentiating *S. typhi* strains (3).

Contrary to a previous report (10), we failed to demonstrate in any of the *S. typhi* strains assayed the production of LT in an enzyme-linked immunosorbent assay. In addition, tissue culture assays failed to detect the production of this toxin in *S. typhi* Ty2. We also could not detect hybridization of the *S. typhi* chromosomal DNA, under low- or high-stringency conditions, with an LT probe corresponding mainly to the B-subunit gene of LT. Our findings might differ from published results because of differences in the methodology, such as the use of supernatants instead of cell lysates for the detection of LT activity, or because of the use of epidemiologically different *S. typhi* strains (10).

S. typhi strains have adapted to a reduced host range, infecting only humans and primates. This reduced host range may be the result of its highly conserved genetic and phenotypic characteristics. Conversely, adaptation to infect human beings and primates may select for *S. typhi* displaying a conserved genotype and phenotype.

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