Phylogenetic Relationships of *Salmonella typhi* and *Salmonella typhimurium* Based on 16S rRNA Sequence Analysis

HERNAN R. CHANG,^{1*} L. H. LOO,¹ K. JEYASEELAN,² L. EARNEST,² AND E. STACHEBRANDT³

Department of Microbiology¹ and Department of Biochemistry,² Faculty of Medicine, National University of Singapore, Singapore 0511, Republic of Singapore, and DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 38124 Braunschweig, Germany³

The 16S rRNA gene sequences of *Salmonella typhi* and *Salmonella typhimurium* were amplified by PCR, cloned, and sequenced. These sequences were analyzed by comparison with reference organisms from the family Enterobacteriaceae. Both *S. typhi* and *S. typhimurium* belong to the gamma subdivision of the class Proteobacteria.

The genus *Salmonella* is a genus of the family Enterobacteriaceae. The following two different classification systems have been validly proposed for species of the genus *Salmonella*: (i) the two-species system, with *Salmonella enterica* and *Salmonella bongori*; and (ii) the five-species system, with *S. bongori*, *Salmonella choleraesuis*, *Salmonella enteritidis*, *Salmonella typhi*, and *Salmonella typhimurium*. In this communication we follow the five-species classification. All species share a high degree of DNA homology (2). By using cross-absorption and antiserum reactions to differentiate O (somatic or polysaccharide) and H (flagellar) antigens, workers have defined over 2,000 *Salmonella* serotypes (18). Some serotypes have a marked host specificity, and some others can infect many animal species besides humans and can produce invasive disease, such as enteric fever. For instance, *S. typhi* causes enteric (typhoid) fever and is only pathogenic in humans, whereas *S. typhimurium* causes gastroenteritis and is pathogenic for several mammalian species. Bacteriophage typing has been used to differentiate *S. typhi* within serotypes (6). Despite their different sensitivities to specific bacteriophages, these bacteria appear to be clonal, as determined by multilocus enzyme electrophoresis and DNA hybridization analysis (11). In order to gain insight into the phylogenetic relationships of both *S. typhi* and *S. typhimurium*, we cloned and sequenced their 16S ribosomal DNAs (rDNAs) (5) by using a PCR approach.

Cloning and sequencing of 16S rRNA genes. *S. typhi* ATCC 19430 was obtained from the American Type Culture Collection (Rockville, Md.), and *S. typhimurium* NCTC 8391 was obtained from the Culture Collection of the Department of Microbiology, National University of Singapore. Single colonies of these strains were grown in liquid media, and their genomic DNAs were extracted by lysis with lysozyme and sodium dodecyl sulfate followed by isolation with *N*-cetyl-*N,N,N*-trimethylammonium bromide (Merck, Darmstadt, Germany) and precipitation with ethanol (16). Each purified DNA was subjected to PCR with universal primers containing flanking *EcoRI* sites (17). The amplified 16S rDNAs were purified and ligated to the pT7Blue T-vector (Novagen, Inc., Madison, Wis.) according to the manufacturer's instructions and were transformed by electroporation into competent *Escherichia coli*. After selection of recombinant colonies, isolated colonies

were grown, and their plasmid DNAs were extracted and purified (14). Each purified plasmid DNA was amplified by using a *Taq* DyeDeoxy terminator cycle sequencing kit in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) and was analyzed by using a model 373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) and a set of sequencing primers. In addition, following digestion of clones with *EcoRI* (New England Biolabs, Inc., Beverly, Mass.) and subcloning into pUC19 plasmids, we sequenced both strands of three *S. typhi* subclones and two *S. typhimurium* subclones.

Phylogenetic analysis. Pairwise evolutionary distances were computed from the 16S rDNA similarity values (data not shown) by using the correction of Jukes and Cantor (7). The distance matrix method of De Soete (3) and neighbor-joining analysis (4, 13) were used to reconstruct phylogenetic trees from the distance matrix. Bootstrap values, calculated for 300 trees, were generated by using the algorithms Nboot and NJFind. Almost complete 16S rDNA sequences were determined for *S. typhi* ATCC 19430 and *S. typhimurium* NCTC 8391; these sequences comprised 99.2% of the *Escherichia coli* 16S rDNA sequences (1). The two sequences were aligned with the homologous sequences of members of the family Enterobacteriaceae for which almost complete 16S rDNA sequences had been deposited in the EMBL or Ribosomal Database Project (10) (Fig. 1), and similarity values were determined. The sequences of the two *Salmonella* strains show 99.7% similarity. The five differences are found at positions 572, 609, 1136, 1137, and 1269. The similarity values for the two *Salmonella* strains and other members of the Enterobacteriaceae range between 97.6 and 91.7%. The phylogenetic trees, generated on the basis of corrected similarity values (7), were identical in that the two *Salmonella* strains were placed in a phylogenetic cluster that contains *Enterobacter cloacae*, *Escherichia coli* and *Citrobacter freundii* (96.3 to 97.6% similarity), *Pantoea agglomerans* (about 95% similarity), *Serratia marcescens* (95.5 to 96% similarity), and *Erwinia carotovora* (95.2 to 95.7% similarity) were moderately related to the *Escherichia coli* cluster, while the other strains belonging to the Enterobacteriaceae included in this study formed a separate subline of descent. A phylogenetic dendrogram is shown in Fig. 1.

Analysis of the two *Salmonella* sequences with unpublished short 16S rDNA stretches of about 300 nucleotides (between positions 300 and 600) from *S. typhimurium* K-12, *S. typhi*, *S. choleraesuis*, *S. enteritidis*, and "*Salmonella dublin*" which are deposited in the EMBL revealed 100% sequence similarity (data not shown). The nucleotide sequence of this stretch is so conserved that even the 16S rDNAs of *Salmonella* species and

* Corresponding author. Present address: Laboratory of Nutrition/Infection, CRI-3, Department of Medicine, Beth Israel Deaconess Medical Center, West Campus/Harvard Medical School, 194 Pilgrim Road, Boston, MA 02115. Phone: (617) 632-0132. Fax: (617) 632-0204. E-mail: rochang@west.bidmc.harvard.edu.

FIG. 1. Phylogenetic dendrogram based on distance matrix analysis of 16S rRNA gene sequences from *S. typhi* and *S. typhimurium* strains sequenced in this work and reference strains. Reference sequences of the following organisms were obtained from EMBL: *Escherichia coli* ATCC 25922 (accession no. X80724), *Photobacter luminescens* DSM 3368 (X82248), *Proteus vulgaris* IFAM 1731 (X07652), *Xenorhabdus nematophilus* DSM 3370 (X82251), *Yersinia intermedia* ER-3854 (X75279), and *Yersinia ruckeri* ATCC 29473 (X75275). Reference sequences of the following organisms were obtained from the Ribosomal Database Project (10), in which no strain designations are indicated for the species: *Citrobacter freundii* (accession no. M59291), *Erwinia carotovora* (M59149), *Serratia marcescens* (M59160), *Pantoea agglomerans*, *Hafnia alvei* (M59155), and *Plesiomonas shigelloides* (M59159). The sequence of *Enterobacter cloacae* DSM 30054 was kindly provided by F. A. Rainey.

those of the *Escherichia coli* strains are either absolutely identical or differ only in one or two nucleotides. Consequently, analysis of this stretch does not allow discrimination of phylogenetic relationships.

Previous DNA-DNA reassociation data (2, 8, 9, 15) and the 16S rRNA gene sequence similarities correlate very well: (i) both methods place the genus *Salmonella* in the family *Enterobacteriaceae*; (ii) the close 16S rDNA relationship of *Salmonella* species to members of the genera *Escherichia* and *Citrobacter*, shown previously by DNA-DNA reassociation to be about 45% (2), is confirmed; and (iii) the results of both methods indicate that the genera *Erwinia*, *Pantoea*, and *Serratia* are less closely related to the genera *Salmonella*, *Escherichia*, and *Citrobacter* and that the genera *Yersinia*, *Hafnia*, *Proteus*, *Xenorhabdus*, and *Photorhabdus* are even more remotely related. The genus *Salmonella* contains five validly described species, which are highly related as determined by DNA-DNA reassociation. The high degree of DNA hybridization similarity for *S. typhi* and *S. typhimurium* (84%) (2) was confirmed by a high 16S rDNA similarity value (99.7%).

Probes developed for the 23S rRNA of *Salmonella* species could be used to discriminate between two groups of *Salmo-*

nella species, one containing *S. typhimurium*, *S. typhi*, *S. enteritidis*, and *S. choleraesuis* and the other containing *S. bongor* (12). These results confirmed those obtained by Reeves et al. (11) by multilocus enzyme electrophoresis. The 16S rDNA sequences obtained in this work may be used to improve diagnostic tools for members of the genus *Salmonella* based on molecular genetics.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the EMBL database (Cambridge, United Kingdom) under the following accession numbers: *S. typhi* ATCC 19430^T, Z47544; and *S. typhimurium* NCTC 8391, Z49264.

This work was supported by grant RP390373 from the National University of Singapore.

We are grateful to S. H. Chan for his interest and encouragement.

REFERENCES

1. Brosius, J., M. L. Palmer, J. P. Kennedy, and H. P. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 75:4801-4805.
2. Crossa, J. H., D. J. Brenner, W. H. Ewing, and S. Falkow. 1973. Molecular relationships among the *Salmonellae*. *J. Bacteriol.* 115:307-315.
3. De Soete, G. 1983. A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* 48:621-626.
4. Felsenstein, J. 1993. PHYLIP (phylogeny inference package), version 3.5c. J. Felsenstein, University of Washington, Seattle.
5. Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. Tanner, L. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrs, K. N. Chen, and C. R. Woese. 1980. The phylogeny of prokaryotes. *Science* 209:457-463.
6. Gershman, M. 1977. Single phage-typing set for differentiating salmonellae. *J. Clin. Microbiol.* 5:302-314.
7. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21-132. In H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, Inc., New York, N.Y.
8. Le Minor, L., M. Y. Popoff, B. Laurent, and D. Hermant. 1986. Individualisation d'une septième sous-espèce de *Salmonella*: *S. choleraesuis* subsp. *indica* subsp. nov. *Ann. Inst. Pasteur (Paris)* 137B:211-217.
9. Le Minor, L., R. Rohde, and J. Taylor. 1970. Nomenclature des *Salmonella*. *Ann. Inst. Pasteur* 119:206-210.
10. Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese. 1994. The Ribosomal Database Project. *Nucleic Acids Res.* 22:3485-3487.
11. Reeves, M. W., G. M. Evins, A. A. Heiba, B. D. Plikaytis, and J. J. Farmer III. 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. *J. Clin. Microbiol.* 27:313-320.
12. Rönnner, S. G. E., and E. Stackebrandt. 1994. Development of 23S rDNA-oligonucleotide probes for the identification of *Salmonella* species. *Syst. Appl. Microbiol.* 17:257-264.
13. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
14. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. Stoleru, G. H., L. Le Minor, and A. M. Lheritier. 1993. Polynucleotide sequence divergence among strains of *Salmonella* sub-genus IV and closely related organisms. *Ann. Microbiol. (Paris)* 127A:477-486.
16. Townner, P. 1992. Purification of DNA, p. 47-55. In T. A. Brown (ed.), *Essential molecular biology, a practical approach*, vol. 1. IRL Press at Oxford University Press, Oxford, United Kingdom.
17. Weisberg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic analysis. *J. Bacteriol.* 173:697-703.
18. World Health Organization. 1980. Center for Reference and Research on *Salmonella* antigenic formulae of the *Salmonella*. WHO International *Salmonella* Center, Pasteur Institute, Paris, France.