

Identification and Phylogeny of *Enterobacter sakazakii* Relative to *Enterobacter* and *Citrobacter* Species

Carol Iversen,¹ Michael Waddington,² Stephen L. W. On,³ and Stephen Forsythe^{1*}

Division of Applied Microbiology and Environmental Biology, School of Biomedical and Natural Sciences, The Nottingham Trent University, Nottingham, United Kingdom¹; Accugenix, Newark, Delaware²; and Danish Institute of Food and Veterinary Research, Copenhagen, Denmark³

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The phylogenetic relationships of *Enterobacter sakazakii* strains were investigated using 16S ribosomal DNA (rDNA) and *hsp60* sequencing. Each analysis distributed *E. sakazakii* strains among four clusters, indicating substantial taxonomic heterogeneity. The *E. sakazakii* type strain 16S rDNA sequence was 97.8% similar to that of *Citrobacter koseri* but 97.0% similar to that of *Enterobacter cloacae*.

In recent years, *Enterobacter sakazakii* has been associated with necrotizing enterocolitis, bacteremia, and infant meningitis through the ingestion of contaminated powdered infant formula milk (5). Since *E. sakazakii* has been reported to be 50% related to *Enterobacter cloacae* and *Citrobacter koseri* by DNA-DNA hybridization (3) and the latter organism produces brain abscesses similar to those caused by *E. sakazakii* (9), the 16S ribosomal DNA (rDNA) and *hsp60* housekeeping gene sequences from these organisms were compared.

A total of 126 strains identified as *E. sakazakii* by biochemical test kits (API20E and ID32E; bioMérieux) were sequenced. These included the type strain, NCTC 11467, and strains NCIMB 5920, NCIMB 8272, ATCC 51329, and ATCC 29004. Seven *E. sakazakii* strains were from European hospitals; seven strains were supplied by Jeff Farber, Health Canada; six strains were supplied by Nestlé-CRN, Vers-chez-les-Blanc, Lausanne, Switzerland; six strains were from J. Hoogkamp-Korstanje, St Radboud, Nijmegen, The Netherlands; and a further five strains were from the Centers for Disease Control and Prevention, Atlanta, Ga. All other strains were food and environmental isolates from the culture collection at Nottingham Trent University, Nottingham, United Kingdom (6). Nine *C. koseri* strains were also sequenced, including the type strain, ATCC 25408, and strains ATCC 25409, ATCC 25410, and ATCC 27026. Four additional *C. koseri* strains were from S. Townsend, Children's Hospital, Los Angeles, Calif., and one strain was obtained from a European hospital.

Comparative 16S rDNA sequence analysis using the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems) was performed according to standard procedures (2) at Accugenix (Newark, Del.). Sequencing of the *hsp60* gene was performed by AGOWA (Berlin, Germany) using the primer sequences GGT AGA AGA AGG CGT GGT TGC (forward) and ATG CAT TCG GTG GTG ATC ATC AG (reverse) (4) to generate a 341-nucleotide PCR product. Additional 16S and

hsp60 DNA sequences from type strains of *Enterobacter* species and *Citrobacter freundii* were obtained from GenBank. Sequences were aligned, corrected for multiple base changes (Jukes-Cantor method), and compared by using Bionumerics version 3.0 (Applied Maths, Kortrijk, Belgium). Relationships between strains were depicted in dendrogram form using the neighbor-joining method. Gaps and unknown bases were not considered in the analysis. The statistical significance of the branch points was evaluated by use of bootstrap analysis using 1,000 replicates. The trees were rooted to *Pantoea agglomerans*, and the scale bars (see Fig. 1) represent percentage sequence divergence as measured by the length of horizontal lines connecting any two strains. Bootstrap values derived from 1,000 replicates indicating the statistical significance of the branch points are also shown.

According to 16S rDNA sequence comparisons, the type strain of *E. sakazakii* was closer to *C. koseri* (97.8% similar) than to any other *Enterobacter* species, although *E. cloacae* was 97.0% similar and *C. freundii* was 96.0% related by this method. Our findings are consistent with the polyphyletic nature of *Enterobacter* spp. revealed by both 16S rDNA and *gyrB* sequence comparisons (1). In the absence of defined phylogenetic criteria for delineating genera, the results observed with *Citrobacter* and *Enterobacter* species suggest that further studies are warranted to clarify their relationship. The 16S rDNA-based analysis divided the *E. sakazakii* strains into four clusters (Fig. 1). The majority (110 strains) were in cluster 1, with 0.1 to 1.2% difference from the type strain. These included 17 clinical strains and 3 nonpigmented strains. Nine strains (including one clinical strain) exhibited 1.6 to 1.9% sequence divergence from the type strain and formed a second cluster closely related to *E. sakazakii* (Fig. 1). The third cluster contained five strains (including one clinical strain), which despite being identified as *E. sakazakii* by the API20E and ID32E kits were more closely related (97.5 to 97.8% similar) to taxa including *Enterobacter pyrinus*, *Enterobacter hormaechei*, and *C. koseri* (Fig. 1). This cluster included the Preceptrol strain ATCC 51329, which was 3.0% divergent from the *E. sakazakii* type strain. The fourth cluster contained two strains, one of which was a clinical isolate. These strains were also identified as *E. sakazakii* by API20E and ID32E, but their 16S rDNA sequences were just

* Corresponding author. Mailing address: Division of Applied Microbiology and Environmental Biology, School of Biomedical and Natural Sciences, The Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, United Kingdom. Phone: (44) 115 8483529. Fax: (44) 115 8486636. E-mail: Stephen.forsythe@ntu.ac.uk.

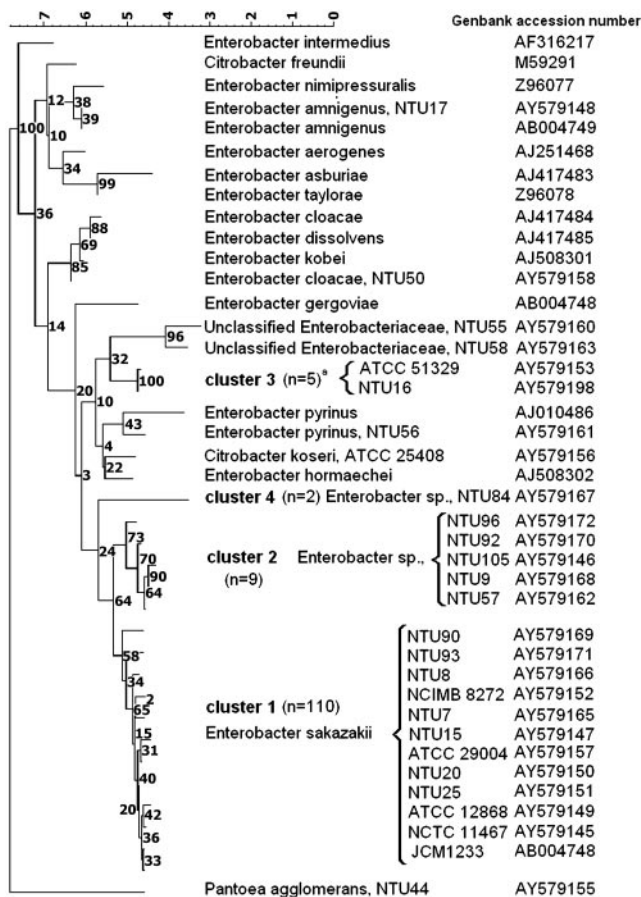


FIG. 1. Neighbor-joining tree of *E. sakazakii* and related organisms based on partial 16S rDNA sequences. Strains presented are representative of the total strains sequenced. Numbers in parentheses indicate the number of strains in a given cluster.

96.5% similar to the type strain of that species. Our 16S rDNA sequence analysis also confirmed that several strains identified as *E. sakazakii* by commercial biochemical kits belonged to distinct species, including *Enterobacter amnigenus* and *E. cloacae* (7), as well as potentially novel taxa (Fig. 1).

hsp60 sequences were obtained for 47 representative strains, including 41 strains of *E. sakazakii* that were representative of the different strain origins and of the four different 16S rDNA clusters (Fig. 2). Although the bootstrap values for the 16S rDNA-defined *E. sakazakii*-like clusters were somewhat low (Fig. 1), strains assigned to each of the 16S rDNA-based clusters also formed distinct clades in the *hsp60* analysis (Fig. 2), although the tree topologies differed, reflecting the different similarity values obtained. Cluster 1 strains remained closely related (97.6 to 99.4% similar) to the *E. sakazakii* type strain, but cluster 2 (89.2 to 90.4% similar) and cluster 4 (88.8% similar) strains were more distinct. By contrast, cluster 3 isolates shared 95.2% *hsp60* sequence homology with the *E. sakazakii* type strain, and their position in the *hsp60*-derived dendrogram supports our classification of these strains as *E. sakazakii*-like strains. In contrast, the distinct positions of strains NTU55 and NTU58 suggest that these strains should

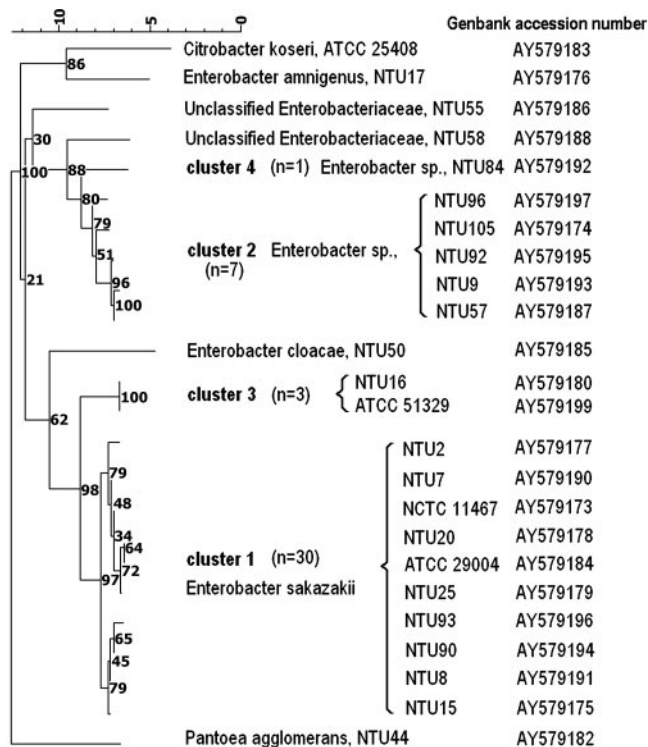


FIG. 2. Neighbor-joining tree of *E. sakazakii* and related organisms based on partial *hsp60* sequences. Strains presented are representative of the total strains sequenced. Numbers in parentheses indicate the number of strains in a given cluster.

not be considered to be *E. sakazakii* despite their identification as this species by some biochemical test kits.

This investigation has demonstrated that *E. sakazakii* is genetically diverse and potentially more taxonomically complex than hitherto realized. Clusters 2, 3, and 4 described here may represent novel species closely related to *E. sakazakii*; further study is necessary to confirm their taxonomic positions. This need is due to the nonlinear relationship often observed between 16S rDNA sequence similarities and the “gold standard” for species delineation, i.e., quantitative DNA-DNA hybridization data (8). Nonetheless, the implications of our study for accurate identification of *E. sakazakii* are self evident and relevant to clinicians, epidemiologists, and infant formula manufacturers.

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