Molecular and evolutionary relationships among enteric bacteria

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Classification of bacterial species into genera has traditionally relied upon variation in phenotypic characteristics. However, these phenotypes often have a multifactorial genetic basis, making unambiguous taxonomic placement of new species difficult. By designing evolutionarily conserved oligonucleotide primers, it is possible to amplify homologous regions of genes in diverse taxa using the polymerase chain reaction and determine their nucleotide sequences. We have constructed a phylogeny of some enteric bacteria, including five species classified as members of the genus *Escherichia*, based on nucleotide sequence variation at the loci encoding glyceraldehyde-3-phosphate dehydrogenase and outer membrane protein 3A, and compared this genealogy with the relationships inferred by biotyping. The DNA sequences of these genes defined congruent and robust phylogenetic trees indicating that they are an accurate reflection of the evolutionary history of the bacterial species. The five species of *Escherichia* were found to be distantly related and, contrary to their placement in the same genus, do not form a monophyletic group. These data provide a framework which allows the relationships of additional species of enteric bacteria to be inferred. These procedures have general applicability for analysis of the classification, evolution, and epidemiology of bacterial taxa.

Introduction

Classification of bacteria into species, genera and higher categories has been a historically enigmatic issue. While an abundance of phenotypic characters allows rigorous taxonomic treatment of many eukaryotic phyla, few readily available characters aid in the unambiguous classification of bacterial strains. Accurate classification of bacterial species is necessary for clinical and epidemiological studies, and a taxonomy which accurately reflects the evolutionary relationships among species provides a framework for elucidating the evolution of genes, gene families and metabolic pathways, for interpreting the distribution of extrachromosomal elements, and for inferring potential transfer of genetic material between species.

Traditionally, metabolic characteristics, such as the ability to metabolize certain carbon sources, or the production or catabolism of various biochemical compounds, have been used to classify bacterial strains into

species and species groups on purely phenetic grounds. More recently, total DNA relatedness has been proposed as a more reliable method for grouping conspecific bacterial strains (e.g. Brenner, 1978). While either method can define species boundaries, criteria for grouping species into genera are less clear. Biochemical and metabolic phenotypes often have a multifactorial genetic basis, that is, multiple genes are responsible for their expression. Owing to convergent evolutionary events in distantly related lineages, these characters cannot be used reliably for a hierarchical grouping of species in a cladistic manner (one based on the identification of shared derived characteristics). DNA hybridization methods, while sufficient to define the range of variation expected for a bacterial species, do not provide an accurate assessment of relatedness allowing the grouping of congeneric species, and the placement of species into genera has relied upon characteristic metabolic features which are neither rigorously defined nor statistically testable (Brenner *et al.*, 1978, 1980).

To eliminate such problems in the classification of bacterial species, their genetic relationships must be assessed beyond the accuracy allowed by total DNA relatedness. The DNA sequences of slowly evolving loci can provide the information necessary to unambiguously classify taxa above the species level and avoid the

Abbreviations: CAI, codon adaptation index; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers M63343–M63374.

inconsistencies intrinsic to epistatic phenotypic traits. The necessary DNA sequences are readily obtainable by the polymerase chain reaction (PCR) (Saiki *et al.*, 1985, 1988). Classification based on patterns of nucleotide substitutions allows statistical testing of the inferred evolutionary relationships, while statistical tests of phylogenies based on weighted phenotypic characters are either unavailable or unreliable. *Escherichia coli* is one of the most intensively studied organisms in terms of its genetics, physiology and molecular biology. Since 1973, an additional four species of *Escherichia* have been described: *Escherichia blattae* (Burgess *et al.*, 1973), *Escherichia fergusonii* (Farmer *et al.*, 1985), *Escherichia hermannii* (Brenner *et al.*, 1982a) and *Escherichia vulneris* (Brenner *et al.*, 1982b). With the exception of *E. blattae*, DNA relatedness was used to classify each group as a distinct species. The classification of all four species in this genus was based upon overall phenotypic similarity, and each exhibited a combination of metabolic characters atypical for other genera.

By designing oligonucleotide primers homologous to evolutionarily conserved sequences, it is possible to amplify homologous regions of ubiquitous genes in diverse taxa using PCR and to determine their nucleotide sequences (Kocher *et al.*, 1989; Taylor & Kreitman, 1989). We have determined the DNA sequences of major portions of the *gap* and *ompA* genes, which encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and outer membrane protein 3A, respectively, of strains representative of eleven species of enteric bacteria, including the five species of *Escherichia*. The inferred genetic relationships were used to test the correlation of the phenotypic characters defining the genus *Escherichia*.

Methods

Bacterial strains and genetic loci. Chromosomal DNA was isolated from the eighteen strains of enteric bacteria listed in Table 1, by methods described previously (Sawyer *et al.*, 1987). The *gap* locus encodes GAPDH and is located at 39.3 min on the *Escherichia coli* linkage map (Bachmann, 1990). The *ompA* locus encodes outer membrane protein 3A and is located at 21.8 min on the linkage map (Bachmann, 1990). Sequences homologous to the *E. coli gap* and *ompA* loci were verified in each of the strains listed in Table 1 by Southern blot genomic DNA hybridization analysis (data not shown). Using the published nucleotide sequences of the *gap* locus from *E. coli*, *Bacillus stearothermophilus* and eukaryotes (Branlant & Branlant, 1985; Branlant *et al.*, 1989; Stone *et al.*, 1985), and of the *ompA* locus from *E. coli* and *Serratia marcescens* (Beck & Bremer, 1980; Braun & Cole, 1984), oligonucleotide primers were synthesized complementary to highly conserved regions of these genes containing low codon redundancy. These primers allow the PCR amplification of a 906 bp fragment encompassing 91% of the *gap* locus and a 711 bp fragment spanning 68% of the *ompA* locus. The *gap* oligonucleotides were: dACTATCAAAGTAGGTATCAA, forward strand at nucleotide 4; dGGATTGGCCGCATCGGCCG, forward strand at nucleotide 25;

Table 1. Strains analysed for *gap* and *ompA* sequences

Strain	<i>ompA</i>		<i>gap</i>	
	CAI*	Mol% G+C	CAI*	Mol% G+C
<i>Citrobacter freundii</i> OS60†	0.76	51.8	0.82	49.6
<i>Enterobacter aerogenes</i> E482‡	0.73	53.6	0.75	50.2
<i>Escherichia blattae</i> ATCC 29907 ^T	0.81	54.5	0.80	52.4
<i>E. blattae</i> ATCC 33429				
<i>E. blattae</i> ATCC 33430				
<i>Escherichia coli</i> K12§	0.79	54.7	0.83	50.1
<i>Escherichia fergusonii</i> ATCC 35469 ^T	0.82	53.5	0.83	49.4
<i>E. fergusonii</i> ATCC 35471				
<i>E. fergusonii</i> ATCC 35472				
<i>Escherichia hermannii</i> ATCC 33650 ^(T)	0.81	53.1	0.83	50.5
<i>E. hermannii</i> ATCC 33652				
<i>Escherichia vulneris</i> ATCC 29943 ^T	0.81	55.6	0.83	51.7
<i>E. vulneris</i> ATCC 33821	0.77	53.9	0.82	51.2
<i>E. vulneris</i> ATCC 33822				
<i>Klebsiella pneumoniae</i> LD119	0.82	56.5	0.82	52.6
<i>Salmonella typhimurium</i> LT2§	0.74	54.2	0.78	50.8
<i>Serratia marcescens</i> ATCC 13880 ^T	0.73	53.5	0.69	53.3
<i>Serratia odorifera</i> ATCC 3307 ^T	0.73	53.7	0.69	52.4

* Codon adaptation index (Sharp & Li, 1987b).

† Clinical isolate (Bergström *et al.*, 1984).

‡ Clinical isolate from B. Wiedermann, Bonn, Germany.

§ Laboratory collection.

|| Clinical isolate from S. Normark, Östersund, Sweden.

^T Type strain.

dCCGAAGCTCGTTGTCGTACCA, reverse strand at nucleotide 910. The *ompA* oligonucleotides were: dAAAGCTCAGGGCGTTCA, forward strand at nucleotide 280; dGCGGCTGAGTTACAACGTCTTT, reverse strand at nucleotide 1031.

Template preparation. PCR (Saiki *et al.*, 1985, 1988) was performed in a buffer containing 50 mM-KCl, 10 mM-Tris (pH 8.4), 2.5 mM-MgCl₂, 0.01% gelatin, 800 µM-dNTP, 25 U *Taq* polymerase ml⁻¹ (Cetus), 2 ng of each oligonucleotide primer µl⁻¹ and 10–50 ng chromosomal DNA. The amplified sample was size-fractionated by agarose gel electrophoresis and DNA fragments of the appropriate size were isolated on Schleicher and Schuell NA45 DEAE paper. The paper-bound DNA was briefly washed in a buffer [150 mM-NaCl, 50 mM-Tris (pH 8.0), 10 mM-EDTA] at room temperature and eluted in a solution of 1 M-NaCl, 50 mM-Tris (pH 8.0), 10 mM-EDTA at 68 °C for 30 min. The DNA was serially extracted with buffer-saturated phenol and chloroform/isoamyl alcohol (24:1, v/v), and precipitated with 2.5 vols 100% ethanol in the presence of a linear polyacrylamide carrier (Galliard & Strauss, 1990). DNA was recovered by centrifugation, rinsed with 70% (v/v) ethanol to remove salts and dried in a vacuum desiccator prior to resuspension in 10 mM-Tris, 1 mM-EDTA.

DNA sequencing. Each DNA template (100 ng) was denatured in 0.2 M-NaOH for 5 min, neutralized by the addition of one-third vol. 3 M-potassium acetate (pH 4.6) and precipitated with 2.5 vols of 100% ethanol in the presence of additional polyacrylamide carrier. Following incubation at –20 °C for 30 min, DNA was recovered by centrifugation as described above and sequenced with Sequenase 2.0 (United States Biochemicals) according to the manufacturer's instructions. The sequences of both strands of each DNA template were determined using internal oligonucleotide primers (sequences determined by these methods yield over 99.9% accuracy). DNA sequence analysis was facilitated by the PAUP (version 2.4, D. Swofford) and MacClade

(version 2.1, W. Maddison & D. Maddison) program packages. Statistics applied in creating and testing phylogenies are detailed below.

Results and Discussion

Ribosomal RNA sequences have proved useful in discerning the relationships between distantly related taxa of eubacteria, archaeobacteria and eukaryotes (e.g. Woese, 1987). However, the slow rates of evolution of these molecules preclude their use in the fine-scale phylogenetic positioning of taxa; the 16S rRNA sequence differs by only 2–3% between *E. coli* and *Salmonella typhimurium* (C. Woese, personal communication). While substitutions at this locus provide a powerful tool for discerning the relationships between very distantly related taxa, they do not supply adequate information for the elucidation of relationships between more closely related taxa, e.g. members of the same genus.

In contrast, typical protein-encoding genes evolve at a relatively rapid rate. The synonymous sites (positions at which substitutions would not alter the amino acid composition of the protein) differ by 50–60% between *E. coli* and *Sal. typhimurium* (Ochman & Wilson, 1988; Sharp & Li, 1987a). Assuming a random accumulation of substitutions at these sites, this level of divergence limits their utility in phylogenetic reconstruction of higher taxa because most sites are assumed to have mutated at least once and would therefore contain ambiguous phylogenetic information.

The bacterial genome contains a class of highly expressed genes which experience selective pressures on codon choice, reflected in high codon usage bias (Sharp & Li, 1987b). Not surprisingly these genes evolve more slowly at synonymous sites (Sharp & Li, 1987a). The *gap* and *ompA* loci are highly expressed genes which exhibit a high degree of codon bias (Table 1). Between *E. coli* and *Sal. typhimurium*, these genes differ by 16 and 20% at synonymous sites, respectively. This level of nucleotide divergence allows an adequate estimation of the genetic relationships between closely related species, while minimizing convergent evolutionary events. Our data indicate that DNA sequences of slowly evolving genes such as *gap* and *ompA* may be useful in determining the taxonomic placement of novel isolates, as well as in resolving the phylogenetic relationships amongst established taxa.

The nucleotide sequences of *gap* and *ompA* are the first to be obtained from all the representative enteric species subsequent to the divergence of the genus *Serratia*. The aligned sequences of portions of the *gap* and *ompA* loci from eighteen strains of enteric bacteria are presented in

Table 2. Nucleotide sequence differences between strains within species

Strain*	gap	ompA	
	Changes	Changes	Deletions
<i>Escherichia blattae</i>			
29907/33429	3	12	0
29907/33430	2	12	1
33429/33430	3	8	1
<i>Escherichia fergusonii</i>			
35469/35471	2	1	0
35469/35472	1	1	0
35471/35472	1	0	0
<i>Escherichia hermannii</i>			
33650/33652	3	1	0
<i>Escherichia vulneris</i>			
29943/33821	71	94	3
29943/33822	73	95	3
33821/33822	5	7	0

* ATCC numbers from Table 1.

Fig. 1. The *ompA* sequences from *E. coli*, *Sal. typhimurium* and *Serr. marcescens* and the *gap* sequence from *E. coli* agree well with previously determined sequences (Beck & Bremer, 1980; Branlant & Branlant, 1985; Braun & Cole, 1984; Freudl & Cole, 1983). The G + C contents of the sequences range from 49 to 56 mol% (Table 1) and, for the most part, reflect the overall genomic G + C content of the species (Normore & Brown, 1970). However, the G + C contents of these loci from *Serratia* do not reflect the overall G + C content of the *Serratia* genome (59 mol%; Normore & Brown, 1970), indicating that the nucleotide content of these genes is highly conserved across enteric species and, as noted by Sharp (1990), the selective forces reflected in codon usage bias are not constrained by mutational pressures towards high G + C content. The sequences from each species display high levels of codon usage bias (Table 1), as calculated by the codon adaptation index (CAI) of Sharp & Li (1987b). Although the CAI is based on the pools of preferred codons estimated from the *E. coli* genome, these data suggest that preferred codons are probably similar among divergent enteric species, and are not significantly altered by selective constraints on overall genomic nucleotide content.

Table 2 shows that there is little sequence variation among conspecific strains. Only *E. vulneris* ATCC 29943 is very divergent from other strains typed to this species. This result is not unexpected, as total DNA relatedness is reported as being significantly lower between this and other putative *E. vulneris* isolates (Brenner *et al.*, 1982b). Therefore, with the exception of two representative strains of *E. vulneris* (ATCC 29943 and ATCC 33821), DNA sequences from single strains of each species were

Nucleotide sequences at the gap locus

	72				99				126				153	
Eco	ATTGTTTTC	CGTGCTGCT	CAGAAACGT	TCTGACATC	GAGATCGTT	GCAATCAAC	GACCTGTTA	GACGCTGAT	TACATGGCA	TACATGCTG	AAATATGAC	TCCAATCAC		
Ebl1	-----	-----	-G-----	-----	-----	-----	-----	-A-----	-T-----	-----	-----	-----		
Ebl2	-----	-----	-G-----	-----	-----	-----	-----	-A-----	-T-----	-----	-----	-----		
Ebl3	-----	-----	-G-----	-----	-----	-----	-----	-A-----	-T-----	-----	-----	-----		
Ehe1	-----	-----	-A-CT-----	-----	-A-----	-T-----	-T-----	-G-----	-T-----	-----	-----	-----		
Ehe2	-----	-----	-A-CT-----	-----	-A-----	-----	-T-----	-G-----	-T-----	-----	-----	-----		
Evu1	-----	-----	-----	-----	-----	-----	-----	-A-G-----	-T-----	-----	-G-----	-----		
Evu2	-----	-----	-G-----	-----	-A-----	-GC-----	-T-----	-C-----	-G-----	-----	-G-----	-A-----		
Evu3	-----	-----	-G-----	-----	-A-----	-GC-----	-T-----	-C-----	-G-----	-----	-G-----	-A-----		
Efe1	-----	-G-----	-----	-T-----	-T-----	-----	-T-----	-C-----	-T-----	-----	-----	-----		
Efe2	-----	-G-----	-----	-T-----	-T-----	-----	-T-----	-C-----	-T-----	-----	-----	-----		
Efe3	-----	-G-----	-----	-T-----	-T-----	-----	-T-----	-C-----	-T-----	-----	-----	-----		
Sty	-----	-----	-----	-T-----	-----	-----	-----	-A-G-----	-----	-----	-----	-----		
Cfr	-----	-----	-AG-----	-----	-----	-----	-----	-C-----	-T-----	-----	-----	-----		
Kpn	-----	-----	-----	-----	-----	-----	-----	-A-G-----	-T-----	-----	-G-----	-----		
Eae	-----	-----	-----	-----	-A-----	-GT-----	-T-CC-G-----	-A-----	-G-----	-----	-G-C-----	-A-----		
Sma	-----	-----	-AG-G-----	-----	-----	-----	-----	-A-G-----	-T-----	-----	-G-----	-T-----		
Sod	-----	-----	-G-----	-C-----	-T-----	-A-----	-T-----	-G-----	-A-----	-T-----	-----	-T-G-----		
	180				207				234				261	
Eco	GGCCGTTTC	GACGGTACC	GTTGAAGTG	AAAGACGGT	CATCTGATC	GTTAACGGT	AAAAAAATC	CGTGTTACC	GCTGAACGT	GATCCGGCT	AACCTGAAA	TGGGACGAA		
Ebl1	-----	A-----	-----	-----	-----	-----	-----	-----	-----	-C-----	-----	-A-----		
Ebl2	-----	A-----	-----	-----	-----	-----	-----	-----	-----	-C-----	-----	-A-----		
Ebl3	-----	A-----	-----	-----	-----	-----	-----	-----	-----	-C-----	-----	-A-----		
Ehe1	-T-----	-C-----	-----	-----	-C-G-----	-C-----	-----	-----	AAA-----	-----	-----	-A-----		
Ehe2	-T-----	-C-----	-----	-----	-C-G-----	-C-----	-----	-----	AAA-----	-----	-----	-A-----		
Evu1	-T-----	-----	-----	-----	-G-----	-----	-----	-----	-----	-C-----	-----	-T-----		
Evu2	-T-----	A-C-----	-----	-C-----	-G-T-----	-C-----	-CC-----	-----	AAA-----	-C-----	-----	-----		
Evu3	-T-----	A-C-----	-----	-C-----	-G-T-----	-C-----	-CC-----	-----	AAA-----	-----	-----	-----		
Efe1	-T-C-----	-C-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----		
Efe2	-T-C-----	-C-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----		
Efe3	-T-C-----	-C-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----		
Sty	-----	-----	-T-----	-----	-----	-----	-----	-----	-----	-----	-----	-----		
Cfr	-----	A-----	-----	-----	-----	-A-----	-----	-----	-----	-----	-----	-----		
Kpn	-T-----	-----	-----	-----	-G-----	-----	-----	-----	-----	-C-----	-----	-G-----		
Eae	-T-----	-C-----	-----	-----	-C-G-T-----	-T-C-----	-CC-----	-C-----	-T-----	-GAAA-----	-C-A-----	-----	-A-----	
Sma	-----	A-----	-G-----	-A-----	-T-----	-C-----	-G-T-----	-C-----	-CC-----	-A-GAAA-----	-C-----	-G-----	-A-----	
Sod	-----	A-----	-G-----	-A-----	-T-----	-C-----	-G-----	-C-----	-CC-----	-A-GAAA-----	-C-----	-G-----	-A-----	
	288				315				342				369	
Eco	GTGTTGTTT	GACGTTGTC	GCTGAAGCA	ACTGTTCTG	TTCCTGACT	GACGAAACT	GCTCGTAAA	CACATCACC	GCTGGTGCG	AAAAAGGTG	GTTATGACT	GGTCCGTCT		
Ebl1	-CA-C-----	-A-----	-T-----	-----	-GC-----	-C-----	-C-----	-----	-C-----	-A-----	-----	-----		
Ebl2	-CA-C-----	-A-----	-T-----	-----	-GC-----	-C-----	-C-----	-----	-C-----	-A-----	-----	-----		
Ebl3	-CA-C-----	-A-----	-T-----	-----	-GC-----	-C-----	-C-----	-----	-C-----	-A-----	-----	-----		
Ehe1	-G-----	-----	-T-----	-----	-C-G-A-C-----	-----	-C-----	-A-----	-----	-C-----	-A-T-----	-GC-----	-C-----	
Ehe2	-G-----	-----	-T-----	-----	-C-G-A-C-----	-----	-C-----	-A-----	-----	-C-----	-A-T-----	-GC-----	-C-----	
Evu1	-----	-----	-T-----	-----	-C-G-A-C-----	-----	-C-----	-----	-----	-C-----	-A-C-----	-C-----	-C-----	
Evu2	A-C-C-----	-----	-T-----	-----	-C-G-A-C-----	-----	-G-----	-----	-T-----	-C-----	-A-T-----	-C-----	-C-----	
Evu3	A-C-C-----	-----	-T-----	-----	-C-G-A-C-----	-----	-G-----	-----	-T-----	-C-----	-A-T-----	-C-----	-C-----	
Efe1	-----	-A-T-----	-----	-----	-G-A-C-----	-----	-C-----	-----	-----	-A-T-----	-C-----	-----	-T-C-----	
Efe2	-----	-A-T-----	-----	-----	-G-A-C-----	-----	-C-----	-----	-----	-A-T-----	-C-----	-----	-T-C-----	
Efe3	-----	-A-T-----	-----	-----	-G-A-C-----	-----	-C-----	-----	-----	-A-T-----	-C-----	-----	-T-C-----	
Sty	-----	-A-G-----	-T-C-----	-----	-A-C-----	-----	-G-----	-----	-----	-A-----	-C-----	-G-----	-----	
Cfr	-----	-A-A-----	-T-----	-----	-GC-----	-----	-A-----	-T-----	-G-----	-C-----	-A-----	-C-----	-----	
Kpn	-----	-----	-T-----	-----	-C-G-A-C-----	-----	-C-----	-----	-----	-C-----	-A-C-----	-C-----	-----	
Eae	A-C-----	-----	-A-----	-----	-T-----	-C-G-A-C-----	-----	-A-----	-----	-T-----	-G-----	-G-A-T-----	-C-----	-T-C-----
Sma	-G-C-----	-T-G-----	-T-----	-----	-G-----	-C-G-A-C-----	-----	-A-----	-----	-C-C-T-----	-A-----	-C-----	-C-A-C-----	
Sod	-G-C-----	-G-T-----	-----	-----	-C-G-A-C-----	-----	-C-----	-A-----	-----	-G-A-----	-C-C-----	-G-A-T-----	-C-----	-C-A-C-----
	396				423				450				477	
Eco	AAAGACAAC	ACTCCGATG	TTCGTTAAA	GGCGCTAAC	TTTGACAAA	TATGCTGGC	CAGGACATC	GTTTCCAAC	GCTTCCTGC	ACCACCAAC	TGCCTGGCT	CCGCTGGCT		
Ebl1	-----	-G-----	-----	-CGC-----	-----	-C-CC-----	-C-A-----	-----	-G-----	-----	-----	-----	-A-----	
Ebl2	-----	-G-----	-----	-CGC-----	-----	-C-CT-----	-C-A-----	-----	-G-----	-----	-----	-----	-A-----	
Ebl3	-----	-G-----	-----	-CGC-----	-----	-C-CC-----	-C-A-----	-----	-G-----	-----	-----	-----	-A-----	
Ehe1	-----	-TG-----	-C-----	-T-----	-----	-----	-CAAC-----	-----	-A-----	-----	-----	-----	-----	
Ehe2	-----	-TG-----	-C-----	-T-----	-----	-----	-CAAC-----	-----	-A-----	-----	-----	-----	-----	
Evu1	-----	-----	-----	-CGC-----	-----	-C-CT-----	-C-A-----	-----	-T-----	-A-----	-T-----	-----	-----	
Evu2	-----	-----	-T-----	-----	-----	-C-AA-----	-T-----	-----	-T-----	-A-----	-----	-A-----	-G-----	
Evu3	-----	-----	-T-----	-----	-----	-C-AA-----	-T-----	-----	-T-----	-A-----	-----	-A-----	-G-----	
Efe1	-----	-----	-C-----	-----	-----	-----	-----	-----	-T-----	-----	-----	-----	-----	
Efe2	-----	-----	-C-----	-----	-----	-----	-----	-----	-T-----	-----	-----	-----	-----	
Efe3	-----	-----	-C-----	-----	-----	-----	-----	-----	-T-----	-----	-----	-----	-----	
Sty	-----	-C-A-----	-T-----	-----	-----	-C-AA-----	-----	-----	-----	-----	-T-----	-G-----	-----	
Cfr	-----	-T-----	-----	-T-----	-----	-C-AA-----	-T-----	-----	-----	-----	-T-----	-----	-----	
Kpn	-----	-----	-----	-CGC-----	-----	-C-GCT-----	-----	-----	-----	-----	-----	-G-----	-----	
Eae	-----	-C-A-----	-----	-CGC-----	-T-A-----	-C-A-CT-----	-C-----	-----	CGA-----	-----	-----	-A-----	-----	
Sma	-----	-TGGC-----	-C-A-----	-CGC-----	-----	-G-----	-----	-----	-G-----	-C-T-----	-----	-G-----	-A-----	
Sod	-----	-TG-----	-C-A-----	-TG-----	-T-TG-----	-C-CA-GTCC-----	-C-G-T-----	-A-----	-----	-----	-T-----	-----	-A-----	

	504				531				558				585	
Eco	AAAGTTATC	AACGATAAC	TTCGGCATC	ATCGAAGGT	CTGATGACC	ACCGTTCAC	GCTACTACC	GCTACTCAG	AAAACCGTT	GATGGCCCG	TCTCACAAA	GACTGGCGC		
Ebl1	---GG-T	-----	---T---	G-T---CG	-----	---A---	---A-C	-----	---A-C	-----	---A-C	-----		
Ebl2	---GG-T	-----	---T---	G-T---CG	-----	---A---	---A-C	-----	---A-C	-----	---A-C	-----		
Ebl3	---GG-T	-----	---T---	G-T---CG	-----	---A---	---A-C	-----	---A-C	-----	---A-C	-----		
Ehe1	-----	---C---	-----	-----	-----	-----	---G-C-T	---A---	---T---	---C---	-----	-----		
Ehe2	-----	---C---	-----	-----	-----	-----	---G-C-T	---A---	---T---	---C---	-----	-----		
Evu1	-----	---C---	---T---	G-T---C	-----	-----	---C---	---A---	-----	-----	-----	-----		
Evu2	-----	---C---	---T---	---C---	---T---	---T---	---G---	---C---	-----	-----	-----	-----		
Evu3	-----	---C---	-----	---C---	---T---	---T---	---G---	---C---	-----	-----	-----	-----		
Efe1	-----	-----	-----	---C---	-----	-----	---A---	-----	---T---	---C---	-----	-----		
Efe2	-----	-----	---G---	---C---	-----	-----	---A---	-----	---T---	---C---	-----	-----		
Efe3	-----	-----	-----	---C---	-----	-----	---A---	-----	---T---	---C---	-----	-----		
Sty	-----	---C---	-----	-----	---T---	---T---	-----	---A-C---	-----	---C---	-----	-----		
Cfr	-----	---C---	-----	-----	---T---	---T---	---G-C---	-----	-----	-----	-----	-----		
Kpn	-----	---C---	---T---	G-T---C	-----	---C---	---C---	-----	-----	-----	-----	-----		
Eae	-----	---C---	-----	-----	---T---	-----	---A-C---	-----	-----	-----	-----	-----		
Sma	---C---	---C---	---T---	G-T---C	-----	-----	---C-C---	---G---	-----	-----	-----	-----		
Sod	---C---	---C-A	---T---	G-T---CG	-----	-----	---G---	---C---	-----	---C---	---T---	-----		
	612				639				666				693	
Eco	GGCGGCCGC	GGCGCTTCC	CAGAACATC	ATCCCGTCC	TCTACCGGT	GCTGCTAAA	GCTGTAGGT	AAAGTACTG	CCGGAAGTG	AATGGCAAA	CTGACTGGT	ATGGCGTTC		
Ebl1	-----	---A---	-----	-----	-----	-----	---A---	---G---	-----	---C---	-----	-----		
Ebl2	-----	---A---	-----	-----	-----	-----	---A---	---G---	-----	---C---	-----	-----		
Ebl3	-----	---A---	-----	-----	-----	-----	---A---	---G---	-----	---C---	-----	-----		
Ehe1	-----	---GG-T	-----	-----	-----	-----	---A---	---T---	-----	---C---	-----	-----		
Ehe2	-----	---GG-T	-----	-----	-----	-----	---A---	---T---	-----	---C---	-----	-----		
Evu1	-----	---AG-T	-----	-----	-----	---C---	-----	-----	---A---	---C---	---C---	-----		
Evu2	-----	---A---	-----	-----	-----	-----	---A---	-----	---A---	---C---	-----	-----		
Evu3	-----	---A---	-----	-----	-----	-----	---A---	-----	---A---	---C---	-----	-----		
Efe1	-----	---T---	---T---	-----	-----	-----	---A---	-----	---A---	---C---	-----	-----		
Efe2	-----	---T---	---T---	-----	-----	-----	---A---	-----	---A---	---C---	-----	-----		
Efe3	-----	---T---	---T---	-----	-----	-----	---A---	-----	---A---	---C---	-----	-----		
Sty	-----	---A-T	-----	-----	---T-C	-----	---G---	-----	---A---	-----	-----	-----		
Cfr	-----	---A---	-----	-----	---A---	-----	-----	-----	---A---	---C---	-----	-----		
Kpn	-----	---AG-T	-----	-----	---C---	-----	---A---	-----	---A---	---C---	---C---	-----		
Eae	-----	---GG-T	-----	---A-T	-----	-----	---A---	-----	---A---	-----	-----	-----		
Sma	-----	---A---	-----	---T-T	---C---	---A---	---A---	---TA-C	---A-G---	---A---	---A-C	-----		
Sod	-----	---A-T	-----	---T-T	-----	---C-G	---A---	---TA-C	---T-G---	---C---	-----	-----		
	720				747				774				801	
Eco	CGCGTTCGG	ACCCGGAAC	GTATCTGTA	GTTGACCTG	ACCGTTCGT	CTGGAAAAA	GCTGCAACT	TACGAGCAG	ATCAAAAGCT	GCCGTTAAA	GCTGCTGCT	GAAGGCGAA		
Ebl1	-----	---T---	-----	---T---	---T-A---	---CT---	C-A---	---AG-A	---GAAA	---A-G---	---C-T---	---C---		
Ebl2	-----	---T---	-----	---T---	---T-A---	---CT---	C-A---	---AG-A	---GAAA	---A-G---	---C-T---	---C---		
Ebl3	-----	---T---	-----	---T---	---T-A---	---CT---	C-A---	---AG-A	---GAAA	---A-G---	---C-T---	---C---		
Ehe1	-----	---T---	---C-T	-----	-----	-----	---A-TT-	---AG-C	---GAAA	---GA-C---	-----	-----		
Ehe2	-----	---T---	---C-T	-----	-----	-----	---A-TT-	---AG-C	---GAAA	---GA-C---	-----	-----		
Evu1	-----	---T---	---C-T	-----	-----	-----	---A-GT-C	---AG-A	---GAAA	---AA-C---	---G-T---	---C---		
Evu2	-----	---T---	---C-T	-----	---G---	-----	---A-GT-	---AG-	---GAAA	---TA-C---	---C-T---	---C---		
Evu3	-----	---T---	---C-T	-----	---A---	-----	---A-GT-	---AG-	---GAAA	---TA-C---	---C-T---	---C---		
Efe1	-----	---T---	-----	-----	-----	-----	C-G-T-	-----	-----	---T---	---C---	---T---		
Efe2	-----	---T---	-----	-----	-----	-----	C-G-T-	-----	-----	---T---	---C---	-----		
Efe3	-----	---T---	-----	-----	-----	-----	C-G-T-	-----	-----	---T---	---C---	-----		
Sty	-----	---T-T---	---C-T	-----	-----	-----	---G-T-C	-----	-----	---T---	---C---	-----		
Cfr	-----	---T---	---C-T	-----	-----	-----	---TT-	---AG-A	---T-GAAA	---AA-C---	---T-C---	---CCG		
Kpn	-----	---T---	---T---	-----	-----	-----	---A-GT-C	---AG-A	---GAAA	---A-C---	---C-T---	---CT		
Eae	-----	---A-T-T---	---C-T	-----	---G---	-----	---TT-	---T-AG-A	---T-GAAA	---AA---	---T-C---	---CC-		
Sma	-----	---T-T---	---G-C-T	-----	---G---	-----	C-G-T-C	---AG-A	---GAAA	---A-G---	---AC-C---	---TCT		
Sod	-----	---T-G-A---	---G---	---G---	---CG---	-----	C-GT-	---A-AG-A	G-TGT-G	---GA-C-G	---AA-G---	-----		
	828				855				882				909	
Eco	ATGAAAGGC	GTTCTGGGC	TACACCGAA	GATGACGTA	GTATCTACC	GATTTC AAC	GGCGAAGTT	TGCACTTCC	GTGTTTCAT	GCTAAAGCT	GGTATCGCT	CTGAACGAC		
Ebl1	-----	---T---	-----	---C-T	-----	-----	---ACC	-----	-----	---C-C---	---A---	-----		
Ebl2	-----	---T---	-----	---C-T	-----	-----	---ACC	-----	-----	---C-C---	---A---	-----		
Ebl3	-----	---T---	-----	---C-T	-----	-----	---ACC	-----	-----	---C-C---	---A---	-----		
Ehe1	-----	---T---	---C-T-T	---T---	---C---	-----	-----	-----	-----	---C-A---	---A---	-----		
Ehe2	-----	---T---	---C-T	---T---	---C---	-----	-----	-----	-----	---C-A---	---A---	-----		
Evu1	-----	---T---	---C-T	-----	-----	-----	---A-C---	-----	-----	---C-A---	---A---	-----		
Evu2	-----	---T---	---C-T	---G-C---	---C---	-----	---A-C---	-----	-----	---C-A---	---A---	-----		
Evu3	-----	---T---	---C-T	---G-C---	---C---	-----	---A-C---	-----	-----	---C-A---	---A---	-----		
Efe1	-----	---T---	---C---	-----	-----	-----	-----	-----	-----	-----	---A---	-----		
Efe2	-----	---T---	---C---	-----	-----	-----	-----	-----	-----	-----	---A---	-----		
Efe3	-----	---T---	---C---	-----	-----	-----	-----	-----	-----	-----	---A---	-----		
Sty	-----	---G-T---	---C-T	-----	-----	---T---	---A---	-----	-----	---A---	---C-G---	-----		
Cfr	-----	---T---	---C---	-----	-----	-----	---T---	-----	-----	-----	---A---	-----		
Kpn	-----	---T---	---C-T	---T---	---C---	-----	-----	-----	-----	---G---	---A---	-----		
Eae	-----	---T---	---C-T	-----	-----	-----	---G---	-----	-----	---A---	---A---	-----		
Sma	-----	---TGTT---	---C-G	---T-C---	-----	-----	---G---	CTG	-----	---C-C---	---A---	-----		
Sod	C-----	---G---	-----	---C-T	---T---	-----	---T-GAAA	CTG	-----	---C-A---	---A---	-----		

927		
Eco	AACTTCGTG	AAACTGGTA
Ebl1	-----	-----T
Ebl2	-----	-----T
Ebl3	-----	-----T
Ehe1	-----T	-----T
Ehe2	-----T	-----T
Evu1	-----T	-----T
Evu2	-----T	-----T
Evu3	-----T	-----T
Efe1	-----	-----
Efe2	-----	-----
Efe3	-----	-----
Sty	-----	-----C
Cfr	-----	-----
Kpn	-----	-----T
Eae	-----	-----T
Sma	-----	-----T
Sod	-----T	-----T

Nucleotide sequences at the *ompA* locus

324											
Eco	CTGACCGCT	AAACTGGGT	TACCCAATC	ACTGACGAC	CTGGACATC	TACACTCGT	CTGGGTGGC	ATGGTATGG	CGTGCAGAC	ACTAAATCC	AACGTTTAT
Ebl1	-----T	-----CA	-----G	TA-----	-----T	-----T	-----T	-----	-----C	-----AA	-----AGT
Ebl2	-----T	-----CA	-----G	TA-----	-----T	-----T	-----T	-----	-----C	-----AA	-----GG
Ebl3	-----T	-----CA	-----G	TA-----	-----T	-----T	-----T	-----	-----C	-----AA	-----AGT
Ehe1	-----T	-----	-----	-----A	-----T	-----	-----T	-----T	-----T	-----G	-----AT
Ehe2	-----T	-----	-----	-----A	-----T	-----	-----T	-----T	-----T	-----G	-----AT
Evu1	-----	-----	-----G	-----T	-----	-----C	-----C	-----	-----C	-----T	-----G
Evu2	-----T	-----C	-----	-----G	-----T	-----C	-----C	-----T	-----T	-----G	-----T
Evu3	-----	-----C	-----	-----	-----T	-----C	-----C	-----T	-----T	-----G	-----T
Efe1	-----	-----	-----	-----	-----	-----T	-----T	-----T	-----	-----C	-----G
Efe2	-----	-----	-----	-----	-----	-----T	-----T	-----T	-----	-----C	-----G
Efe3	-----	-----	-----	-----	-----	-----T	-----T	-----T	-----	-----C	-----G
Sty	T-----	-----	-----T	-----T	-----T	-----C	-----	-----T	-----	-----C	-----G
Cfr	-----	-----	-----	-----T	-----T	-----T	-----T	-----T	-----	-----G	-----AA
Kpn	-----	-----	-----G	-----T	-----	-----C	-----C	-----	-----C	-----T	-----T
Eae	-----	-----	-----G	-----T	-----G	-----C	-----C	-----A	-----	-----C	-----G
Sma	-----G	-----A	-----C	-----T	-----G	-----T	-----	-----T	-----	-----T	-----G
Sod	-----G	-----A	-----C	-----T	-----G	-----T	-----	-----T	-----	-----T	-----G
351											
378											
399											
Eco	CTGACCGCT	AAACTGGGT	TACCCAATC	ACTGACGAC	CTGGACATC	TACACTCGT	CTGGGTGGC	ATGGTATGG	CGTGCAGAC	ACTAAATCC	AACGTTTAT
Ebl1	-----T	-----CA	-----G	TA-----	-----T	-----T	-----T	-----	-----C	-----AA	-----AGT
Ebl2	-----T	-----CA	-----G	TA-----	-----T	-----T	-----T	-----	-----C	-----AA	-----GG
Ebl3	-----T	-----CA	-----G	TA-----	-----T	-----T	-----T	-----	-----C	-----AA	-----AGT
Ehe1	-----T	-----	-----	-----A	-----T	-----	-----T	-----T	-----T	-----G	-----AT
Ehe2	-----T	-----	-----	-----A	-----T	-----	-----T	-----T	-----T	-----G	-----AT
Evu1	-----	-----	-----G	-----T	-----	-----C	-----C	-----	-----C	-----T	-----G
Evu2	-----T	-----C	-----	-----G	-----T	-----C	-----C	-----T	-----T	-----G	-----T
Evu3	-----	-----C	-----	-----	-----T	-----C	-----C	-----T	-----T	-----G	-----T
Efe1	-----	-----	-----	-----	-----	-----T	-----T	-----T	-----	-----C	-----G
Efe2	-----	-----	-----	-----	-----	-----T	-----T	-----T	-----	-----C	-----G
Efe3	-----	-----	-----	-----	-----	-----T	-----T	-----T	-----	-----C	-----G
Sty	T-----	-----	-----T	-----T	-----T	-----C	-----	-----T	-----	-----C	-----G
Cfr	-----	-----	-----	-----T	-----T	-----T	-----T	-----T	-----	-----G	-----AA
Kpn	-----	-----	-----G	-----T	-----	-----C	-----C	-----	-----C	-----T	-----T
Eae	-----	-----	-----G	-----T	-----G	-----C	-----C	-----A	-----	-----C	-----G
Sma	-----G	-----A	-----C	-----T	-----G	-----T	-----	-----T	-----	-----T	-----G
Sod	-----G	-----A	-----C	-----T	-----G	-----T	-----	-----T	-----	-----T	-----G
417											
444											
471											
498											
Eco	000000000	AAAAACCAC	GACACCGGC	GTTTCTCCG	GTCTTCGCT	GCGCGTGTT	GAGTACGCG	ATCACTCCT	GAAATCGCT	ACCCGCTCG	GAATACCAG
Ebl1	GGCGATCGT	-GC-----	-T-----	-----	-----A	-----A	-----A	TGG--C--G	TCC-----	-----	-----TC
Ebl2	GGCGACCGT	-GC-----	-T-----	-----	-----A	-----A	-----A	TGG--C--G	TCC-----	-----	-----TC
Ebl3	GGCGACCGT	-GC-----	-T-----	-----	-----A	-----A	-----A	TGG--C--G	TCC-----	-----	-----TC
Ehe1	GGCTCTTTC	-G-T--T	-----T	-----C--A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Ehe2	GGCTCTTTC	-G-T--T	-----T	-----C--A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Evu1	GGCGAC	000-----	-----	-----C--A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Evu2	GCATCTTTC	-G-----	-T-----T	-----C--A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Evu3	GCATCTTTC	-G-----	-T-----T	-----C--A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Efe1	GAATCTGAC	-----	-----T	-----A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Efe2	GAATCTGAG	-----	-----T	-----A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Efe3	GAATCTGAG	-----	-----T	-----A	-----A	-----CC-G	-----GG-A	G-T--C--G	-C-----	-----	-----C
Sty	CGTCTACT	-----	-----C	-----A	-----G	-----A	-----C	-----T	-----C	-----A	-----T
Cfr	-----TTC	-G-----	-T-----T	-A--C--A	-A-----	-T--C--A	-T--A	-T-----	-----	-----	-----C
Kpn	GTTTCCCGT	-GCG-A---	-----T	-----C--A	-A--T---	-----C--A	-----GG--T	G--T--G--	-C-----	-----	-----GTT
Eae	GGTGAC	000G----	-----T	-----C--A	-A-----	-T--C--	-----GG--CA	-G--C--G--	-C-----	-----	-----GTT
Sma	CAGCGTCTG	-GCG-----	-----	-----C--GGCT	-TT-----	-A-----	C--G--CAAA	A--CTGG--	-----	-----T	-----TCGTA-G
Sod	GAGCGTCTG	-GC-----	-----	-----C--GGCT	-TT-----	-A-----	C--G--CAAA	A--CTGG--	-----T	-----C	-----TCGTT-G
525											
552											
579											
603											
Eco	AACATCGGT	GACGCACAC	ACCATCGGC	ACTCGTCCG	GACAACGGC	ATGCTGAGC	CTGGGTGTT	TCCTACCGT	TTGCGTCAG	GGCGAGGCA	000GCTCCA
Ebl1	-----T	-----TGGT	-G-T--T	-----	-----	-----	G-----C	-----	-----	-A--A--T	000--A--G
Ebl2	-----T	-----TGGT	-G-T--T	-----	-----	-----	G-----C	-----	-----	-A--A--T	000--A--G
Ebl3	-----T	-----TGGT	-G-T--T	-----	-----	-----	G-----C	-----	-----	-A--A--T	000000--G
Ehe1	-----TA	-----TA	-G-T--T	-C-----	-----T--T	C-----	G--A-----	-----	-----	CAA--A--	GCA--G--
Ehe2	-----TA	-----TA	-G-T--T	-C-----	-----T--T	C-----	G--A-----	-----	-----	CAA--A--	GCA--G--
Evu1	-----T	-----TGG	-TG-A--	GT-----	-----	-----	G--T-----	-----	-----	-AA--AAT	000--A--G
Evu2	-----T	-----TGG	-TG-A--	GT-----	-----	-----	G--T-----	-----	-----	-AA--AAT	000--A--G
Evu3	-----T	-----TGG	-TG-A--	GT-----	-----	-----	G--T-----	-----	-----	-AA--AAT	000--A--G
Efe1	-----TA	-----TA	-G-T--T	-C-----	-----T--T	C-----	G--A-----	-----	-----	CAA--A--	GCA--G--
Efe2	-----TA	-----TA	-G-T--T	-C-----	-----T--T	C-----	G--A-----	-----	-----	CAA--A--	GCA--G--
Efe3	-----TA	-----TA	-G-T--T	-C-----	-----T--T	C-----	G--A-----	-----	-----	CAA--A--	GCA--G--
Sty	-----T	-----TGG	-TG-A--	GT-----	-----	-----	G--T-----	-----	-----	-AA--AAT	000--A--G
Cfr	-----C	-----GGG	-TG-G--T	-C-----T	-T-----	C-----	G--T-----	-T-----	-C-----	CAA--A--T	000--G--
Kpn	-----C	-----GGG	-TG-G--T	-C-----T	-T-----	C-----	G--T-----	-T-----	-C-----	CAA--A--T	000--G--
Eae	-----G	-----GGT	-G-T--T	G--A-----	-----AC	-----	-C-----	-T-----C	-----	-AT--C--T	GTT-----
Sma	-----G	-----GGT	-G-T--T	G--A-----	-----AC	-----	-C-----	-T-----C	-----	-AT--C--T	GTT-----
Sod	-----C	-----GGT	-G-T--T	G--A-----	-----AC	-----	-C-----	-T-----C	-----	-AT--C--T	GTT-----

	630												657												684												711																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
Eco	CCGGCTCCA	GCTCCGGCA	CCGGAAGTA	CAGACCAAG	CAC TTCACT	CTGAAGTCT	GACGTTCTG	TTCAACTTC	AACAAAGCA	ACCCTGAAA	CCGGAAGGT	CAGGCTGCT																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																

analysed further. The phylogenetic relationships among isolates were analysed for each gene using a maximum parsimony algorithm (PAUP). In both cases, a single most parsimonious tree was found. Moreover, the branching orders for the *gap* and *ompA* loci were congruent. This common tree is presented in Fig. 2. The relative branching order of the species was tested using the Felsenstein test on four-taxon subgroups, assuming a stochastic molecular clock (Felsenstein, 1985). This test allows a statistical comparison of the three possible relationships between four taxa, and confirmed all branch points (the resolution of relationships between *E. vulneris*, *E. hermannii* and *Enterobacter aerogenes* required the elimination of synonymous changes from the data set).

Since the sequences of *gap* and *ompA* both define a single phylogenetic tree, it is likely that this genealogy accurately reflects the evolutionary relationships among bacterial lineages. More specifically, it is unlikely that horizontal transfer of both genes between enteric species (requiring independent transfer of the two loci or conjugative transfer of a portion of the bacterial chromosomes exceeding 1000 kb) has occurred. In addition, the sequences from independent isolates typed as the same species are quite similar, suggesting that the original classification of these strains within a species, based on total DNA relatedness and overall phenotypic similarity, is valid. The overall branching order and pattern of divergence among taxa (*E. coli*, *Sal. typhimurium*, *Klebsiella pneumoniae*, *Ent. aerogenes*, and *Serr. marcescens*) reflected by these genes is similar to earlier assessments of their genetic relationships based on a combination of phenotypic and genetic characters (Brenner & Falkow, 1971; Cocks & Wilson, 1972; Ochman & Wilson, 1987).

Species of *Salmonella* are traditionally considered to be those most closely related to *E. coli*. If *Escherichia* were considered to represent a monophyletic group, additional species of *Escherichia* should be more closely related to *E. coli* than is *Salmonella*. From the phylogeny presented in Fig. 2, it is apparent that the genus *Escherichia* is not a monophyletic group. That is, neither the ancestor of all *Escherichia* species, nor all its descendent taxa, belong to that genus. The Felsenstein statistics presented in Table 3 (Felsenstein, 1985) support the hypothesis that only

Fig. 2. Phylogeny of selected enteric bacteria based on DNA sequences from the *gap* and *ompA* loci, generated by a maximum parsimony algorithm. Taxon designations refer to strains listed in Table 1. The phylogeny was rooted using the nucleotide sequence of the *gap* locus from a strain of *Morganella morganii* (H. Ochman, unpublished results).

E. coli and *E. fergusonii* are congeneric species and diverged following the split of *Escherichia* from *Salmonella* ($P < 0.05$). However, this hypothesis is rejected for *E. blattae*, *E. hermannii*, and both genotypes of *E. vulneris* ($P < 0.05$). The data suggest that these *Escherichia* species are more distantly related to *E. coli* than is *Salmonella* and should be placed in alternative genera. Since bacterial genera should reflect monophyletic groups, the putative species of *Escherichia* must be reclassified or this genus would encompass species of *Salmonella*, *Citrobacter*, *Enterobacter* and *Klebsiella*. In a similar manner, *Serr. odorifera* was classified according to phenotypic characters (Grimont *et al.*, 1978). As is evident from Fig. 2, such classification does not allow definition of the genus *Serratia* as a monophyletic group.

The DNA hybridization data of Brenner and co-workers (Brenner *et al.*, 1982a, b) also indicate that both *E. hermannii* and *E. vulneris* are distantly related to *E. coli*. However, neither species could be unambiguously classified into any one genus based on DNA hybridization studies. The DNA hybridization data are summarized in Table 4. In the cases of *E. hermannii* and *E. vulneris*, metabolic characteristics implied close relationships to *E. coli* and to one another. These species were classified in the genus *Escherichia* on the basis of metabolic profiles, despite the data from DNA hybridiz-

Fig. 1 (on preceding pages). Nucleotide sequences of the *gap* and *ompA* loci from enteric bacteria. Taxa designations are: Eco, *Escherichia coli*; Ebl1, *E. blattae* ATCC 29907; Ebl2, *E. blattae* ATCC 33429; Ebl3, *E. blattae* ATCC 33430; Ehe1, *E. hermannii* ATCC 33650; Ehe2, *E. hermannii* ATCC 33652; Evu1, *E. vulneris* ATCC 29943; Evu2, *E. vulneris* ATCC 33821; Evu3, *E. vulneris* ATCC 33822; Efe1, *E. fergusonii* ATCC 35469; Efe2, *E. fergusonii* ATCC 35471; Efe3, *E. fergusonii* ATCC 35472; Sty, *Salmonella typhimurium*; Cfr, *Citrobacter freundii*; Kpn, *Klebsiella pneumoniae*; Eae, *Enterobacter aerogenes*; Sma, *Serratia marcescens*; Sod, *Serr. odorifera*. Nucleotide numbering corresponds to positions in the published *E. coli* sequence. Nucleotides unchanged from the *E. coli* sequence are designated as '-', and nucleotide deletions are designated as '0'.

Table 3. Summary of statistics from Felsenstein's test

Taxon designations for potential phylogenies are: Eco, *Escherichia coli* K12; Sty, *Salmonella typhimurium* LT2; OUT, outgroup specified for each test; **, test species specified. Statistic 'C' is the number of informative characters supporting each tree; statistic 'S' corresponds to the number of steps separating the best tree from the next-best tree. *, Measures which are statistically significant at $P < 0.05$. *E. vulneris* 'a' and 'b' are strains ATCC 29943 and ATCC 33821, respectively.

Test	Potential phylogeny:					
	Eco ** Sty OUT		Eco ** Sty OUT		Eco ** Sty OUT	
	C	S	C	S	C	S
<i>E. blattae</i> , gap OUT: <i>K. pneumoniae</i>	11	—	2	—	26*	15*
<i>E. blattae</i> , ompA OUT: <i>Serr. marcescens</i>	7	—	9	—	21*	12*
<i>E. fergusonii</i> , gap OUT: <i>E. vulneris</i> a	14*	7*	5	—	7	—
<i>E. fergusonii</i> , ompA OUT: <i>K. pneumoniae</i>	26*	20*	6	—	4	—
<i>E. hermannii</i> , gap OUT: <i>Ent. aerogenes</i>	4	—	6	—	26*	20*
<i>E. hermannii</i> , ompA OUT: <i>C. freundii</i>	9	—	10	—	19*	9*
<i>E. vulneris</i> a, gap OUT: <i>Ent. aerogenes</i>	3	—	6	—	36*	30*
<i>E. vulneris</i> a, ompA OUT: <i>E. hermannii</i>	8	—	10	—	18*	8*
<i>E. vulneris</i> b, gap OUT: <i>K. pneumoniae</i>	1	—	3	—	36*	33*
<i>E. vulneris</i> b, ompA OUT: <i>Ent. aerogenes</i>	6	—	2	—	30*	24*

ation studies. Our data support the original DNA hybridization data (Brenner *et al.*, 1982*a, b*) and suggest a reconsideration of the generic placement of the taxa.

The DNA sequence data summarized in Table 2 show that *E. vulneris* ATCC 29943 is not closely related to other strains typed as *E. vulneris* and the phylogenetic placement of *E. vulneris* strains indicate that *E. vulneris* ATCC 29943 and other *E. vulneris* strains are not conspecific. The Felsenstein statistics support the hypothesis that *E. vulneris* ATCC 29943 is closely related to *K. pneumoniae* (for gap and ompA, 36/40 and 30/38 sites, respectively, support that grouping; $P < 0.05$). Therefore, while biochemical characteristics and total DNA relatedness suggest that these strains may be conspecific, the genetic evidence presented here does not support this hypothesis. Rather, the DNA data suggest that *E. vulneris* ATCC 29943 be placed in the genus *Klebsiella*, and not be considered a strain of the taxon *E. vulneris*, as defined by the type strain ATCC 33821.

Attempts to discern the evolutionary relationships among enteric bacteria have utilized a wide range

of characters, including immunological relationships (Cocks & Wilson, 1972), metabolic pathways (Ahmad *et al.*, 1990; Jensen, 1985), DNA hybridization (Brenner, 1978; Brenner & Falkow, 1971) and standard biochemical and enzymic profiles (Brenner, 1981, 1984; Edwards & Ewing, 1962). An underlying weakness of these methods is the inability to directly measure the genetic relationships between species. Alternatively, an assessment of homologous DNA sequences among species not only resolves fine-scale taxonomic relationships but the data allow an analysis of gene evolution within the context of the evolutionary history of the organisms. Although it has been argued that constraints on the evolution of protein and ribosomal RNA molecules preclude their use as taxonomic tools (Meyer *et al.*, 1986), one can statistically test phylogenies inferred from these macromolecules.

From published sequence data, it is possible to design oligonucleotide primers that will allow PCR amplification of homologous genes from distantly related taxa. This approach has been applied to examine the

Table 4. Comparison of DNA hybridization and nucleotide divergences of *Escherichia coli* and other enteric bacteria

Strain	DNA hybridization*	Total changes†	
		gap	ompA
<i>Escherichia coli</i> K12	100	0	0
<i>Escherichia fergusonii</i> ATCC 35469	60	43	25
<i>Salmonella typhimurium</i> LT2	45	55	68
<i>Citrobacter freundii</i> OS60	50	62	78
<i>Escherichia hermannii</i> ATCC 33650	31	81	77
<i>Escherichia vulneris</i> ATCC 33821	35	94	92
<i>Klebsiella pneumoniae</i> LD199	38	71	95
<i>Enterobacter aerogenes</i> E482	37	107	93
<i>Escherichia blattae</i> ATCC 29907	29	75	104
<i>Serratia marcescens</i> ATCC 13880	24	128	145
S‡		22§	20§

* Percentage association of genomic DNA at 60 °C (Brenner & Falkow, 1971; Brenner *et al.*, 1982a, b; Farmer *et al.*, 1983).

† Total number of substitutions between *E. coli* and the given taxon at the respective locus.

‡ The Kendall rank test *S* statistic testing an inverse correlation between the total number of substitutions between *E. coli* and the other enteric strains at each locus and their percentage reassociation during DNA hybridization. § denotes a significant negative correlation ($P < 0.05$).

evolutionary relationships among metazoans based on cytochrome *b* oxidase genes of mitochondrial DNA (Kocher *et al.*, 1989) and could be extended to include any group of organisms. The only requirement in using macromolecules to infer accurate phylogenies is that their sequences have sufficient variation to discern evolutionary relationships but are minimally affected by convergent events. We have shown that genetic loci experiencing selection reflected in codon usage bias satisfy these requirements.

Owing to its ubiquitous distribution and conservative evolution, GAPDH would serve as an ideal molecule for inferring fine-scale phylogenetic relationships among organisms not readily distinguished by rDNA sequences. Aside from the eighteen *gap* genes from enteric bacteria reported in this paper, the nucleotide sequences of GAPDH-encoding genes have been determined for some 40 taxa, including representatives from each urkingdom (Doolittle *et al.*, 1990). Using a large set of GAPDH-encoding sequences, Doolittle *et al.* (1990) postulated that the high degree of similarity between the *E. coli gap* gene and eukaryotic GAPDH-encoding sequences resulted from a horizontal transfer event introducing a eukaryotic *gap* homologue into the bacterial genome (see also Martin & Cerff, 1986). Other eubacterial lineages, notably *Bacillus stearothermophilus*, *Thermus aquaticus*, *Zymomonas mobilis* and the cyanobacterial-derived chloroplasts, harbour *gap* genes distinct from those of

E. coli and eukaryotes. The *gap* sequences presented in this paper clearly establish that any horizontal transfer event must have occurred prior to the divergence of the enteric bacteria. Phylogenies based on the *gap* and *ompA* sequences are congruent (Fig. 2), indicating that these genes were not subject to horizontal transfer processes. Moreover, a second potential GAPDH-encoding locus has been identified in *E. coli* (Alefounder & Perham, 1989) which is similar to the non-enteric prokaryotic *gap* genes (Doolittle *et al.*, 1990). To identify the lineage accommodating the initial transfer event, one must determine the sequences of GAPDH-encoding genes from species having diverged from the *E. coli* lineage prior to *Serr. odorifera*, yet following the divergence of the purple bacterium *Z. mobilis*.

While DNA sequence data may be useful in the generic placement of novel species, a revision of the taxonomy of extant enteric species would cause considerable confusion. The long-standing practice of classifying aberrant bacterial strains as novel species without regard to evolutionary history (e.g. the classification of non-lactose-fermenting strains of *E. coli* in *Shigella*) has constrained the ability of classification to reflect phylogeny. The advent of PCR and rapid DNA sequencing has allowed the elucidation of fine-scale phylogenetic relationships among bacterial species, which is necessary to examine the evolution of their genomes.

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