Shigella* and enteroinvasive *Escherichia coli* strains are derived from distinct ancestral strains of *E. coli

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The differentiation between *Shigella* subspecies, and the phylogenetic position of *Shigella* clones within *Escherichia coli* clones was determined by analysis of restriction fragment length polymorphisms of rDNA (ribotyping). Seventy-five *Shigella* strains belonging to the four subspecies and 13 enteroinvasive *E. coli* (EIEC) strains were compared with the 72 *E. coli* strains of the ECOR collection, which have been classified into four phylogenetic groups (A, B1, B2 and D). Seventeen *Shigella dysenteriae* ribotypes, 12 *Shigella flexneri* ribotypes, 23 *Shigella boydii* ribotypes, 12 *Shigella sonnei* ribotypes and 13 EIEC ribotypes were identified following digestion with *Hind*III and *Eco*RI. Correspondence analysis of the data showed that *S. boydii* serotype 13 strains were distantly related to the other *Shigella* strains, and that *S. sonnei* and *S. flexneri* were distinct from *S. boydii* and *S. dysenteriae*. The ribotypes of *Shigella* and ECOR strains were indistinguishable, and *S. sonnei*, *S. flexneri* and most *S. dysenteriae* strains were closely related to phylogenetic group D, whereas *S. dysenteriae* serotype 1 strains belonged to phylogenetic group B1, and *S. boydii* strains were evenly distributed between the two groups. The *Shigella* strains were distantly related to group B2, which contains *E. coli* strains frequently implicated in extra-intestinal infections in humans. In contrast, the 13 EIEC strains were more widely distributed between phylogenetic groups B1, A and B2. Thus, there was no primordial *Shigella* species and *Shigella* and EIEC strains are derived from different ancestral strains.

Keywords: *Shigella*, *Escherichia coli*, enteroinvasive *Escherichia coli* (EIEC), phylogeny

INTRODUCTION

DNA hybridization studies have shown that *Shigella* (with the exception of *Shigella boydii* serotype 13) and *Escherichia coli* belong to the same genetic species (Brenner *et al.*, 1972, 1973, 1982). However, nutritional characterization, numerical taxonomy and enzyme electrophoresis show that there are differences between these organisms (Johnson *et al.*, 1975; Veron & Le Minor, 1975; Ochman *et al.*, 1983) and within *Shigella*, between the four taxospecies, *Shigella dysenteriae*, *Shigella flexneri*, *S. boydii* and *Shigella sonnei* (Goullet 1980; Dodd & Jones, 1982). A previous analysis of

esterase electrophoretic polymorphisms distinguished five clusters among the *Shigella*: (1) *S. dysenteriae* serotype 1; (2) *S. flexneri* serotypes 1–5; (3) *S. flexneri* serotype 6 and *S. boydii* serotypes 2 and 4; (4) *S. sonnei*; and (5) *S. boydii* serotype 13. These clusters are more closely related to *E. coli* than to each other (Goullet & Picard, 1987).

The concept of a clonal structure was developed by Selander & Levin (1980) for *E. coli* and subsequent studies have shown that the linkage disequilibrium in this species is very close to the theoretical maximum, indicating that recombination events are rare. Moreover, the clonal structure of the species was demonstrated by the similarity obtained within the ECOR collection of 72 *E. coli* strains for phylogenetic trees based on three kinds of data (Ochman & Selander, 1984; Desjardins *et al.*, 1995). The four phylogenetic

Abbreviations: EIEC, enteroinvasive *Escherichia coli*; MLEE, multilocus enzyme electrophoresis; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

groups A, B1, B2 and D, described by Herzer *et al.* (1990) on the basis of multilocus enzyme electrophoresis (MLEE), are also distinguishable by random amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP) analysis of rDNA genes (ribotyping). Thus ribotyping can be useful for classification of the *E. coli* and *Shigella* clones.

We first investigated the differentiation between the *Shigella* clusters by rDNA gene RFLP analysis of *Shigella* strains belonging to the four taxospecies. We then determined the phylogenetic position of the *Shigella* clones and of a group of enteroinvasive *E. coli* (EIEC) strains within the ECOR collection. This allowed us to assign the *Shigella* and EIEC strains to the previously described *E. coli* phylogenetic groups A, B1, B2 and D (Herzer *et al.*, 1990).

METHODS

Bacterial strains

Shigella strains. The strains tested are listed in Table 1. We used 22 *S. dysenteriae* strains, 13 *S. flexneri* strains, 24 *S. boydii* strains (including two serotype 13 strains) and 16 *S. sonnei* strains.

E. coli strains. The 72 ECOR strains were obtained from R. K. Selander, Pennsylvania State University, USA and the 13 EIEC strains were obtained from Dr P. Bouvet, Institut Pasteur, France (strains CNRSS 84-293, 85-90, 85-200, 85-206, 85-209, 85-242, 85-255, 94-8778, 95-7511 and 95-11794) and Dr Sansonetti, Institut Pasteur, France (strains 4608-0124, 1-72 and 13-80). The EIEC were shown to be enteroinvasive by the Sereny test (Sereny, 1955) for three strains or by hybridization using DNA probes (Bohnert *et al.*, 1988) for the others. Among the EIEC, two were motile.

RFLP of rDNA regions. Bacterial DNA was prepared as previously described (Picard-Pasquier *et al.*, 1989). DNA was digested with *EcoRI* and *HindIII* restriction endonucleases (Boehringer) according to the manufacturer's instructions and was analysed by electrophoresis on submerged 0.8% agarose gels containing ethidium bromide. DNA fragment size markers (marker II; Boehringer) were used. Separated DNA restriction fragments were transferred to a nylon membrane (Amersham) by Southern blotting (Southern, 1975). Ribosomal 16S and 23S RNA from *E. coli* (Boehringer) was labelled by random oligopriming using a chemiluminescence system (Boehringer) as previously described (Bingen *et al.*, 1992). Hybridization, washing and detection procedures were as previously described (Bingen *et al.*, 1992).

Statistical analysis. The data were summarized in two two-way tables containing 72 rows for the 72 ECOR strains and 88 rows for the 75 *Shigella* strains and the 13 EIEC strains. The number of columns corresponded to the number of rDNA fragments produced by *EcoRI* and *HindIII* endonuclease digestion. For each column, the rDNA fragment was coded using a binary code, present = 2 or absent = 1, for each strain.

Correspondence analysis (Greenacre, 1984; Lebart *et al.*, 1984; Tenenhaus & Young, 1985) was performed for *Shigella* strains using one table. The other table was used for correspondence analysis of ECOR strains, in which the strains of *Shigella* and EIEC were treated as supplementary observations and were projected onto the factorial plane F1/F2 (see

Fig. 3) obtained from the ECOR strains. Calculations were carried out using SPAD.N software.

RESULTS

RFLP analysis of rDNA regions

S. dysenteriae. rDNA was digested with *EcoRI* and 20 rDNA fragments 2–24 kb in size (Fig. 1a) were obtained from the 22 strains. We distinguished 17 rDNA RFLP patterns. When rDNA was digested with *HindIII*, fragments of 1.9–23.1 kb (Fig. 1b) were obtained and 10 rDNA RFLP patterns were identified. A combination of patterns resulting from both digestions was used to define ribotypes. Seventeen ribotypes were identified among the strains.

S. flexneri. *EcoRI* digestion produced 19 rDNA fragments 2–24 kb in size (Fig. 1a). There were 12 rDNA RFLP patterns in the 13 strains. *HindIII* digestion produced 18 rDNA fragments 1.9–23.1 kb in size which distinguished nine rDNA RFLP patterns (Fig. 1b). Twelve ribotypes were identified among the strains.

S. boydii. *EcoRI* digestion gave 23 rDNA fragments 2–24 kb in size (Fig. 1a), which distinguished 22 rDNA RFLP patterns among the 24 strains. *HindIII* digestion gave 21 rDNA fragments 1.9–23.1 kb in size (Fig. 1b), which distinguished 16 rDNA RFLP patterns. Twenty-three ribotypes were identified among the strains.

S. sonnei. *EcoRI* digestion produced 19 rDNA fragments with sizes of 2–24 kb (Fig. 1a), distinguishing 11 rDNA RFLP patterns. *HindIII* digestion produced 12 rDNA fragments 1.2–23.1 kb in size (Fig. 1b), which distinguished three rDNA RFLP patterns. Twelve ribotypes were identified among the strains.

EIEC. *EcoRI* digestion produced 16 rDNA fragments with sizes of 2–24 kb. Thirteen rDNA RFLP patterns were distinguished. *HindIII* digestion gave eight rDNA fragments of 1.9–23.1 kb in size, which distinguished nine rDNA RFLP patterns. Thirteen ribotypes were identified among the 13 strains.

Statistical analysis

Correspondence analysis was carried out with the ribotyping data obtained by the combination of the two endonucleases for the 75 *Shigella* strains. The plane F1/F2, which accounted for 25.5% of the total variance, showed that *S. sonnei* and *S. flexneri* strains were clearly distinguished from *S. boydii* (except serotype 13) and *S. dysenteriae* strains by the negative values of the first axis (Fig. 2). The second axis differentiated *S. sonnei* strains from *S. flexneri* strains, and *S. boydii* strains from *S. dysenteriae* strains. *S. boydii* serotype 13 strains were clearly distinguished from the other *Shigella* strains by the negative values of the first axis and the positive values of the second axis.

Correspondence analysis was carried out with the ribotyping data obtained by combination of the two endonucleases for the 72 ECOR strains and the plane

Table 1. Strains of *Shigella* examined

<i>S. dysenteriae</i>					
Strain no.		Serotype	Strain no.		Serotype
D1	8.76	3	D12	67.81	2
D2	15.77	1	D13	85.99	2
D3	53.1	2	D14	93.46	7
D4	30.73	5	D15	93.45	4
D5	2.64	2	D16	93.51	10
D6	5.77	2	D17	52.27	1
D7	31.73	7	D18	53.136	1
D8	12.77	1	D19	54.95	1
D9	3.76	2	D20	55.90	1
D10	6.77	4	D21	56.33	1
D11	48.37	5	D22	62.17	1
<i>S. flexneri</i>					
Strain no.		Serotype	Strain no.		Serotype
F1	10.80	6	F8	82.48T	2a
F2	14.79	6	F9	52.24	4
F3	18.70	2	F10	52.36	1a
F4	6.74	6	F11	52.39	2b
F5	66.79	4	F12	52.40	3
F6	302.78	6	F13	52.44	5
F7	3.77	6			
<i>S. boydii</i>					
Strain no.		Serotype	Strain no.		Serotype
B1	52.23	1	B13	E.20	2
B2	42.80	1	B14	E.24	5
B3	41.80	11	B15	E.25	9
B4	12.80	10	B16	E.33	9
B5	19.80	5	B17	E.35	1
B6	54.73	1	B18	E.36	5
B7	56.36	5	B19	93.21	11
B8	12.77	10	B20	85.92	14
B9	2.77	9	B21	5.16	11
B10	16.76	2	B22	56.18	11
B11	57.43	9	B23	76.114	13
B12	5.99	10	B24	76.115	13
<i>S. sonnei</i>					
Strain no.		Biotype*	Strain no.		Biotype*
S1	217.77	a	S9	13.77	a
S2	2.77	a	S10	1.78	g
S3	110.77	a	S11	82.49T	g
S4	4.76	d	S12	51.1	g
S5	46.70	a	S13	52.55	d
S6	5.75	d	S14	55.56	d
S7	241.77	d	S15	66.4	a
S8	15.77	d	S16	67.63	a

* Biotypes were defined according to Szturm-Rubinstein (1964).

Fig. 1. Representative RFLP patterns of *Shigella* and ECOR rDNA regions obtained with (a) *Hind*III and (b) *Eco*RI digestion enzymes. Lanes: 1, strain ECOR (E) 1; 2, strain E29; 3, strain E50; 4, strain E63; 5, *Shigella* strain D2; 6, strain D10; 7, strain F3; 8, strain F5; 9, strain B6; 10, strain B10; 11, strain S5; 12, strain S8. The strains are numbered as in Table 1. Positions of size markers are indicated on the right in kb.

F1/F2 was obtained (Fig. 3). The 75 *Shigella* strains and the 13 EIEC strains, treated as supplementary observations, were projected onto this plane, which accounted for 34.85% of the total variance. The majority of the ECOR strains were classified within the four phylogenetic groups A, B1, B2 and D previously distinguished by Herzer *et al.* (1990) using MLEE and by Desjardins *et al.* (1995) using ribotyping and RAPD analysis. The *Shigella* strains were projected into the same area as the ECOR strains. This shows that their ribotypes are very close to those of *E. coli* strains. However, no *Shigella* strains were projected within the area of phylogenetic groups A and B2, and all *Shigella* strains were projected within the area of phylogenetic groups B1 and D. Seven of the eight *S. dysenteriae* serotype 1 strains were

Fig. 2. Correspondence analysis of the 75 *Shigella* strains using RFLP analysis of the rRNA genes. Projection of the strains, numbered as in Table 1, onto the plane F1/F2. For clarity, when several strains are projected onto the same point, only one is indicated.

projected into the area of group B1. *S. sonnei*, *S. flexneri* and the other *S. dysenteriae* strains were mostly projected into the area of phylogenetic group D. *S. boydii* strains were more widely distributed within these two ECOR areas. Eight of the 13 EIEC strains studied belonged to group B1, 4 to group A and 1 to group B2.

DISCUSSION

As with previous studies based on biochemical and serological characters (Dodd & Jones, 1982) and electrophoretic polymorphism of esterases (Goullet, 1980; Goullet & Picard, 1987), clusters of *Shigella* were distinguished by ribotyping (Figs 2, 3). Thus, *S. sonnei* and *S. flexneri* were clearly distinguished from *S. boydii* and *S. dysenteriae*, whereas the distinction between *S. boydii* and *S. dysenteriae* was less clear (Fig. 2). The strains of *S. boydii* serotype 13, which belong to a distinct DNA hybridization group (Brenner *et al.*, 1982), were clearly distinguished from the other *Shigella* strains. Our rDNA RFLP data did not demonstrate the close taxonomic relationship between *S. flexneri* serotype 6 strains and *S. boydii* strains previously shown on the basis of several characters (Timakov *et al.*, 1972; Dodd & Jones, 1982; Goullet *et al.*, 1983).

Ribotyping was highly discriminatory in the *Shigella* subspecies as 17 ribotypes were identified in *S. dysenteriae*, 12 in *S. flexneri*, 23 in *S. boydii* and 12 in *S. sonnei*. Serotyping (Orskov *et al.*, 1977), biotyping (Marranzano *et al.*, 1985) and isoenzyme analysis (Ochman *et al.*, 1983) suggest that *S. sonnei* is genetically homogeneous. Our study is consistent with previous work showing that ribotyping can discriminate between *S. sonnei* clones (Karaolis *et al.*, 1994). RAPD analysis of

Fig. 3. Correspondence analysis of the 72 ECOR strains with the 75 *Shigella* and 13 EIEC strains treated as supplementary observations, using RFLP analysis of rRNA gene data. (a) The 72 ECOR strains (●), the 13 EIEC strains (△) and the 75 *Shigella* strains (○) are projected onto the plane F1/F2. The phylogenetic groups of ECOR strains, A, B1, B2 and D (Herzer *et al.*, 1990), are indicated by dotted lines. (b) Enlargement of the area of groups B1 and D. The *Shigella* strains are numbered as in Table 1. e, ECOR.

the 75 *Shigella* strains using the 18 δ and 338 δ primers (Desjardins *et al.*, 1995) was less able than ribotyping to discriminate the four taxospecies (data not shown).

Previous studies have shown sufficient similarity between *E. coli* and *Shigella* to place both in the same species (Brenner *et al.*, 1972, 1973; Goullet, 1980; Hartl & Dykhuizen, 1984; Ochman *et al.*, 1983; Whittam *et al.*, 1983). The correspondence analysis comparing ECOR ribotypes and *Shigella* ribotypes supports this (Fig. 3). However, *Shigella* clones were more closely related to certain phylogenetic groups of the ECOR collection as they were unambiguously projected within the B1 and D groups even though some strains were

projected within the B1 group area bordering the A group area. Moreover, *S. sonnei*, *S. flexneri* and most *S. dysenteriae* strains were closely related to group D, whereas *S. dysenteriae* serotype 1 strains were closely related to group B1 and *S. boydii* strains were widely distributed among the B1 and D groups. The *Shigella* strains were unrelated to group A. There have been several studies of the relationship between a limited sample of *Shigella* isolates and *E. coli* strains. These studies have produced conflicting results. MLEE analysis was used to classify most *Shigella* electrophoretic types to *E. coli* group II (corresponding to phylogenetic groups D and B1 of Herzer *et al.*, 1990) (Whittam *et al.*, 1983; Ochman *et al.*, 1983; Ochman & Selander, 1984). Pupo *et al.* (1997) used the same technique to group most *Shigella* strains in a single cluster in phylogenetic group A, with *S. flexneri* serotype 2 and 4 strains in group D. However, the same authors showed that *mdh* sequencing groups all *Shigella* strains except *S. sonnei* within the *E. coli* B1 group strains. Such differences may be due to a higher rate of recombination in *Shigella* than in *E. coli* strains (Desjardins *et al.*, 1995; Guttman, 1997) or to convergent evolution resulting from selective pressure linked to their strict human intestinal biotype (Maynard Smith, 1996). Overall, the data support the notion put forward by Goullet & Picard (1987) in a study of esterase electrophoretic polymorphism that there was no single primordial *Shigella* species and *Shigella* strains are derived from different ancestral strains of *E. coli*.

The *Shigella* clones were found to be distantly related to the B2 phylogenetic group. This is consistent with their virulence in humans. Thus, *Shigella* cause bacillary dysentery whereas phylogenetic group B2 consists of highly pathogenic strains, frequently implicated in extra-intestinal infections and rarely isolated from the intestinal microflora (Goullet & Picard, 1986a, b; Bingen *et al.*, 1998). In contrast, despite having comparable virulence to *Shigella* and a homologous virulence plasmid in common (Ménard *et al.*, 1996), the EIEC were found to belong to three phylogenetic groups of *E. coli*, consistent with the results of Pupo *et al.* (1997). Most strains were closely related to group B1, four strains belonged to group A and one to group B2. The proportion of EIEC belonging to the B2 group was similar to the proportion of *E. coli* strains belonging to the phylogenetic B2 group in the normal intestinal microflora of healthy individuals (Goullet & Picard, 1986b).

It has been suggested that *Shigella* plasmid virulence genes were acquired from another genus, based on the fact that the A+T content of the plasmid genes (70–73%) is higher than that of the chromosomal genes (50%) (Hale, 1991). Endonuclease digestion and Southern hybridization showed that the virulence plasmids of *Shigella* and EIEC are essentially homologous but have different endonuclease patterns (Sansonetti *et al.*, 1983). This suggests that these may have been a common ancestor molecule which has been subject to evolution. Phylogenetic plasmid analysis

should help to determine how the virulence plasmid was acquired by *Shigella* and EIEC strains.

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