Polynucleotide Sequence Relatedness Among *Shigella* Species

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Polynucleotide sequence relatedness in strains of *Shigella* species was assessed by determining the extent of reassociation in heterologous deoxyribonucleic acid preparations. Thermal elution chromatography on hydroxyapatite was used to separate reassociated nucleotide sequences from nonreassociated sequences and to determine the degree of unpaired bases within related nucleotide sequences. Almost all *Shigella* strains share 80% or more of their nucleotide sequences. Less than 3% of unpaired bases are present in these related sequences. The same extent of relatedness is present between *Shigella* and *Escherichia coli* strains. Strains of *S. boydii* C13 are highly interrelated. These strains average only about 65% relatedness to other *Escherichiae*. We were unable to detect preferentially high relatedness between those *Shigella* and *E. coli* strains that contain identical or related O antigens.

The tribe *Escherichieae* Bergey et al. (9) contains two genera, *Escherichia* Castellani and Chalmers and *Shigella* Castellani and Chalmers. These genera are highly related based on biochemical reactions (8), serological cross-reactions (8), amino acid sequence similarity in proteins (13), and total deoxyribonucleic acid (DNA) sequence similarity (4).

The four *Shigella* species, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, are quite similar biochemically (10). Each species is divided into several serotypes, and many of these cross-react with *Escherichia coli* serotypes (8).

Shigella species show 80 to 89% DNA relatedness to *E. coli* K-12. The thermal stability of reassociated DNA duplexes held in common between these organisms indicates less than 2% unpaired bases or divergence (4). One objective of this study was to determine the level of DNA relatedness among *Shigella* species compared to that between *Shigella* species and *E. coli* strains.

It was recently demonstrated that *E. coli* strains grouped by pathogenicity and immunoelectrophoretic mobility patterns show preferentially high intragroup DNA relatedness. A second objective of the present study was to determine whether preferentially high overall DNA relatedness is present in reactions between shigellae and *E. coli* strains that contain identical or similar O antigens.

MATERIALS AND METHODS

Organisms and media. The strains used in this study are listed in Table 1. Cultures of these organisms were maintained on brain heart infusion agar slants and were propagated on brain heart infusion broth. The medium employed for labeling cells has been described previously (3).

Preparation of DNA and DNA reassociation. Both unlabeled and labeled DNA were prepared, purified, and sheared as described previously (3, 4). Conditions for DNA reassociation and separation of single-stranded from reassociated DNA on hydroxyapatite were presented elsewhere (5). These conditions were used in this study with the following modifications.

The buffer concentration employed during incubation was increased from 0.14 M PB to 0.28 M PB (PB = phosphate buffer, an equimolar mixture of NaH_2PO_4 and Na_2HPO_4 , pH 6.8). DNA reassociates approximately 2.7 times as fast in 0.28 M PB as in 0.14 M PB (7). The amount of unlabeled DNA in the reassociation reaction was decreased from 400 $\mu\text{g}/\text{ml}$ to 150 $\mu\text{g}/\text{ml}$, and the reaction was carried to 100 Cots (DNA concentration $\times$ time units; see reference 7). The incubation mixture was diluted in distilled water to 0.14 M PB before being applied to hydroxyapatite. The result of these modifications is to economize on the amount of unlabeled DNA needed for each test. Control reactions indicate that completeness of reassociation is not affected by these modifications. The concentration of labeled DNA (0.1 $\mu\text{g}/\text{ml}$; specific activity 50,000 to 150,000 counts per min per μg) was not changed. In control experiments the reassociation of labeled DNA in 0.28 M PB was 2.5% or less.

TABLE 1. *Strains employed*

Strain	Source ^a
Bethesda 6	CDC
<i>Edwardsiella tarda</i> 1795-62	CDC
<i>Enterobacter aerogenes</i> 1494-70	CDC
<i>Enterobacter liquefaciens</i> 6136-66	CDC
<i>Escherichia coli</i> B	WRAIR
<i>Escherichia coli</i> K-12	Univ. of Washington
<i>Escherichia coli</i> 028	WRAIR
<i>Escherichia coli</i> 0115	SSI
<i>Escherichia coli</i> 0124	WRAIR
<i>Escherichia coli</i> 0124	SSI
<i>Escherichia coli</i> 0136	WRAIR
<i>Escherichia coli</i> 0136	SSI
<i>Escherichia coli</i> 0143	WRAIR
<i>Escherichia coli</i> 0144	WRAIR
<i>Escherichia coli</i> 0147	WRAIR
<i>Serratia marcescens</i>	WRAIR
<i>Salmonella typhimurium</i> LT2	NIH
<i>Shigella boydii</i> C1	WRAIR
<i>Shigella boydii</i> C7	WRAIR
<i>Shigella boydii</i> C8	WRAIR
<i>Shigella boydii</i> C10	WRAIR
<i>Shigella boydii</i> C13	WRAIR
<i>Shigella boydii</i> C13 1610-55	CDC
<i>Shigella boydii</i> C13 2045-54	CDC
<i>Shigella boydii</i> C13 2406-51	CDC
<i>Shigella dysenteriae</i> A1	WRAIR
<i>Shigella dysenteriae</i> A2	WRAIR
<i>Shigella dysenteriae</i> A3	WRAIR
<i>Shigella dysenteriae</i> A10	WRAIR
<i>Shigella flexneri</i> 2a 24570	S. Falkow
<i>Shigella flexneri</i> 6	WRAIR
<i>Shigella sonnei</i>	WRAIR
<i>Shigella sonnei</i> avirulent	WRAIR
<i>Shigella sonnei</i> virulent	WRAIR

^a Abbreviations: CDC, Center for Disease Control, Atlanta, Ga., from W. H. Ewing, G. J. Hermann, and W. J. Martin; WRAIR, Walter Reed Army Institute of Research, Washington, D.C.; SSI, Statens Serum Institute, Copenhagen, Denmark, from F. Ørskov; NIH, National Institutes of Health, Bethesda, Md., from T. Theodore.

Relative binding values were not corrected for the background reassociation of labeled DNA.

Spectrophotometric determination of genome size. The molecular complexity or genome size of bacterial DNA was determined using the technique of Gillis et al. (11) as previously described (4).

RESULTS

Relatedness data from DNA reassociation reactions followed by thermal elution chromatography on hydroxyapatite are shown in Table 2, and reactions among *Escherichiae* are summarized in Table 3. With the exception of *S. boydii* C13, to be discussed below, the

Shigella strains tested showed at least 77% relatedness. We assume that each one percent of unpaired bases within a reassociated heterologous DNA duplex causes a one degree decrease in duplex thermal stability (1, 12). Therefore the thermal elution midpoint ($\Delta T_{m(e)}$) values in Tables 2 and 3 indicate less than 3% unpaired bases, or divergence, present in related sequences in shigellae. In general, relatedness between species of *Shigella* is between 80 and 90% with 2% or less divergence. In reactions carried out at the more stringent 75 C incubation temperature (not shown), relative binding is only slightly lower than that obtained at 60 C. This result confirms the high degree of similarity between DNA from *Shigella* strains. In most cases strains from one *Shigella* species are no more related to each other than to other species of *Shigella*. *S. sonnei* strains may be an exception based on the virtual identity of the three strains tested.

The *E. coli* strains tested here and in a previous study (4) exhibit the same high degree of relatedness to a given *Shigella* species as is seen between species of *Shigella*. Furthermore, the extent of divergence in DNA sequences shared by *E. coli* and *Shigella* strains is no greater than that observed in *Shigella* DNA heteroduplexes. It may be noted that the two separate subcultures of *E. coli* strains 0124 and 0136 gave essentially identical results with all *Shigella* strains. These results in effect serve to control the variability in repeated DNA isolations from a given strain.

Reactions involving *Shigella* species and representatives of other genera of enterobacteria are also shown in Table 2. The degree of binding observed and the thermal lability of the related sequences indicate that extensive divergence has occurred between *Escherichiae* and members of the tribes *Edwardsiellae*, *Salmonelleae*, and *Klebsielleae*. These data confirm results obtained using *E. coli* reference strains (2).

Spectrophotometric determinations of genome size obtained by comparing initial rates of DNA reassociation (11) were performed on *E. coli* K-12, *S. flexneri*, and *S. dysenteriae* strains A3 and A10. *E. coli* K-12 has a genome size of 2.56×10^9 daltons (4). *S. flexneri* has the same genome size as *E. coli* K-12. The genomes in *S. dysenteriae* strains are about 5% (strain A10) to 10% (strain A3) larger than that of *E. coli* K-12. It appears from the reciprocal-binding data that the *S. boydii* C10 genome is about 10% smaller than the genomes of *E. coli* K-12 and *S. flexneri*.

The strains initially labeled in this study were

S. boydii C10, *S. flexneri* 2a 24570, and *S. sonnei* virulent. The reactions of DNA from these organisms with DNA from *S. boydii* C13 seemed abnormally low (68-75%), especially since the $\Delta T_{m(e)}$ values were 6.8 C and 7.7 C. When DNA from strain C13 was labeled, the same comparatively low extent of relatedness and comparatively low thermal stability was seen in reactions with DNA from all tested shigellae and *E. coli* strains (Table 2). Three additional C13 strains were obtained from the Center for Disease Control. DNA from the first C13 strain averaged 96% relatedness with these new C13 strains, whereas DNA from *E. coli* B showed an average 65% relatedness to the C13 strains (Table 2).

These data, together with the fact that homologous C13 reference DNA averaged 87% reassociation with a $\Delta T_{m(e)}$ of 90 C or higher, rule against the possibility of a poor C13 DNA preparation being responsible for the low binding and thermal stability of heterologous C13 reactions.

Except for *S. sonnei* strains, virtually all shigellae share identical or reciprocal O antigens with one or more strains of *E. coli* (reference 8, p. 138). The average relatedness of *E. coli* strains to shigellae is shown in Table 3. Reactions with *S. sonnei* virulent are just as high as with *S. boydii* C10 and *S. flexneri* 2a 24570. To test possible preferential relatedness between *E. coli* and *Shigella* strains with common or related antigens, individual strains were compared as shown in Table 4. In no case was the reaction of an *E. coli* strain with the antigenically related *Shigella* strain higher than its reaction with other shigellae. In addition, the reaction of the three *Shigella* strains with antigenically related *E. coli* strains was no greater than reactions with *E. coli* strains with which they do not share O antigens.

DISCUSSION

C13 is a rarely encountered serotype of *S. boydii* (W. H. Ewing, personal communication). DNA from the four C13 strains used in this study is almost totally related. Both in terms of total reaction and thermal stability of the related sequence, the reaction of C13 DNA with all other *Escherichiae* is strikingly low. C13 reference strain DNA formed extensive and stable DNA duplexes in both homologous reactions and reactions involving other C13 strains. These facts essentially rule out any possibility that the low relatedness of C13 DNA to that of other shigellae was due to a poor C13

DNA preparation. Neither can one explain these data on the assumption that C13 strains contain abnormally large genomes, since (i) reciprocal binding data indicate that the C13 genome is at most 4 to 10% larger than the genomes of the other *Shigella* reference strains, and (ii) a difference in genome size cannot account for the substantially decreased thermal stability of the heterologous C13 DNA duplexes.

Based on our limited survey, the C13 strains seem to have conserved their gross DNA sequences while having diverged from all other *Escherichiae*. It is of great interest to determine whether some unknown ecological niche is responsible for this unusual pattern of evolutionary divergence and to determine whether any of the other rarely encountered *Shigella* serotypes show a similar pattern.

DNAs from almost all other shigellae were 80% or more related with evidence of less than 3% divergence in related sequences. These data and selected genome size determinations fall into the same range as previously found in a much larger sampling of *E. coli* strains (4).

The three *S. sonnei* strains tested are indistinguishable based on DNA reassociation. Strains of *S. boydii*, *S. dysenteriae*, and *S. flexneri* do not exhibit preferentially high intraspecies binding as compared to interspecies binding. Furthermore, average relatedness between *Shigella* species and *E. coli* strains is just as high as relatedness between species of *Shigella*.

It must again be emphasized that these data were derived from only a few strains of each *Shigella* species. Within this framework, however, it is clear that, excepting *S. sonnei*, 20 to 25% of the unrelated DNA exists in strains of a given species of *Shigella* and that the same extent of divergence is present between species of *Shigella* and between shigellae and *E. coli* strains.

Preferentially high binding was not observed between two pairs of *Shigella* and pathogenic *E. coli* strains that not only have identical or related O antigens but also cause disease by similar mechanisms. This result is apparently in contrast to previous results (6) in which preferentially high binding was present among groups of *E. coli* strains prevalent in certain types of infection and whose O antigens have a common pattern of electrophoretic mobility. If these two pairs of strains are representative of all shigellae and *E. coli* strains with related O antigens, the explanation for this result is not known. However, one must consider that the O antigen and pathogenicity account for a smaller

TABLE 2. DNA reassociation reactions at 60 °C^a

Source of unlabeled DNA	<i>S. boydii</i> C10		<i>S. boydii</i> C13		<i>S. flexneri</i> 2a		<i>S. sonnei</i> vir.		<i>E. coli</i> B		<i>E. coli</i> K-12 ^b	
	% relative binding	$\Delta T_m(e)$	% relative binding	$\Delta T_m(e)$	% relative binding	$\Delta T_m(e)$	% relative binding	$\Delta T_m(e)$	% relative binding	$\Delta T_m(e)$	% relative binding	$\Delta T_m(e)$
<i>S. boydii</i> C1	83	7.4	63	7.4	82	2.8	84	1.8			81	
<i>S. boydii</i> C7	84	7.4	68	7.4	87	1.0	77	1.5			89	1.3
<i>S. boydii</i> C8	94	6.3	65	6.3	79	1.0	77	1.5	76	1.1		
<i>S. boydii</i> C10	100	6.7	72	6.7	81	1.3	78	1.5	74	1.2		
<i>S. boydii</i> C13	75		100		71	6.8	68	7.7	65	6.8		
<i>S. boydii</i> C13 1610-55		0.2	98						63	6.0		
<i>S. boydii</i> C13 2045-54		0.9	92				65	6.4				
<i>S. boydii</i> C13 2406-51		0.3	97				64	6.9				
<i>S. dysenteriae</i> A1	83	6.3	65	6.3	83	1.9	76	2.5			82	1.3
<i>S. dysenteriae</i> A2	84	6.2	68	6.2	80	0.9	83	1.5			87	1.7
<i>S. dysenteriae</i> A3	97	6.9	62	6.9	81	0.6	83	1.0	77	1.1	80	
<i>S. dysenteriae</i> A10	86	7.5	66	7.5	79	2.2	78	2.7				
<i>S. flexneri</i> 2a 24570	90	7.6	66	7.6	100		83	1.9	83	1.6	84	1.0
<i>S. flexneri</i> 6	97	6.9	68	6.9	83	1.6	84	1.4			85	1.0
<i>S. sonnei</i>	89	5.3	69	5.3	87	1.2	100	0.1			86	1.4
<i>S. sonnei</i> avirulent	92	7.1	64	7.1	83	0.9	101	0.5			84	0.9
<i>S. sonnei</i> virulent	88	7.6	61	7.6	78	0.5	100				87	0.5
<i>E. coli</i> B							86		100		94	0.8
<i>E. coli</i> K-12		7.9	71	7.9			87	0.9			100	
<i>E. coli</i> 028	93		63		85	0.5	87	1.3				
<i>E. coli</i> 0115	78		65		78	1.2	80	2.0				
<i>E. coli</i> 0124 (SSI)	88		64		88	1.3	85	1.7				
<i>E. coli</i> 0124 (WRAIR)	89		63		89	0.8	85	2.4				
<i>E. coli</i> 0136 (SSI)	86	6.7	60	6.7	83	1.5	86	1.1				
<i>E. coli</i> 0136 (WRAIR)	86		64		87	1.2	87	1.5				
<i>E. coli</i> 0143	87		64		91	1.5	85	2.9				
<i>E. coli</i> 0144	86		68		85	1.3	86	1.5				
<i>E. coli</i> 0147	88		64		89	0.7	86	1.4				
Bethesda 6					49	13.2	52	13.6			47	

[illegible]

^a Relative binding at 60 °C and $\Delta T_{m(e)}$ values are an average of three or more determinations. Average homologous binding percentages are: *E. coli* B, 91; *E. coli* K-12, 88; *S. boydii* C10, 83; *S. boydii* C13, 87; *S. flexneri* 2a 24570, 91; *S. sonnei* virulent, 87. The average $T_{m(e)}$ value for these strains was 90 °C.

Data taken from Bremer, unpublished results, and from reference 1.

proportion of the genome in these strains than in previously tested *E. coli* strains. Therefore, these similarities cannot be detected against the background of 80% or greater total DNA relatedness. Alternatively, the preferentially high relatedness among groups of pathogenic *E. coli* strains may be due to conservation of genes other than those for O antigens and pathogenicity.

It has been suggested (4) that the parameters of DNA relatedness, guanine plus cytosine content, and genome size form the basis of a molecular definition of a species. *E. coli* strains form a group whose DNAs contain 48 to 52% guanine plus cytosine, have a molecular weight of 2.3×10^9 to 3.0×10^9 , and show 80% or higher relative reassociation. On these bases it is difficult to distinguish between *E. coli* and *Shigella* species. It certainly seems that these organisms are sufficiently related to be included in the same genus. Alternatively, C13 strains (and possibly other strains that have not been looked at) have deviated from the mainstream of *Shigella-E. coli* DNA evolution while retaining the biochemical identification of the species *S. boydii*.

We are strongly of the opinion that polynucleotide sequence relatedness is the most reliable basis for classification. However, one cannot ignore metabolic and serological criteria for taxonomy. It is true that certain antigens are rather nonspecific, that a given antigen or biochemical reaction assays less than 0.1% of the genetic capacity of the average bacterial genome, that a negative biochemical reaction may result from just one base change in otherwise identical genes, and that widely different enzymes can catalyze the same reaction. Nevertheless, with relatively few exceptions, taxonomic groupings based on biochemical reactions and serology agree very well with DNA relatedness data.

Whether one likes it or not, practical and logical factors are quite important in considering changes in existing systems of taxonomy and nomenclature. For example, if a culture is routinely keyed out as belonging to the genus *Shigella*, it is both confusing and unrealistic to rename or reclassify the organism by using data not useful in the clinical laboratory. Such action would probably not change laboratory usage of the familiar name of classification. This is not to imply that changes in classification should not be made. Our intent is to urge caution and consultation with both clinical and basic researchers before recommending changes in established taxonomic groups. One alternative (or perhaps an inevitable consequence of

TABLE 3. Summary of reassociation data

Source of unlabeled DNA	Average % relative binding, 60 C ^a				$\Delta T_{m(e)}$, 60 C ^b		
	<i>S. boydii</i> C10	<i>S. boydii</i> C13	<i>S. flexneri</i> 2a 24570	<i>S. sonnei</i> virulent	<i>S. boydii</i> C13	<i>S. flexneri</i> 2a 24570	<i>S. sonnei</i> virulent
<i>S. boydii</i> C1, C7, C8 and C10 strains	87	67	82	80	7.0	1.5	1.6
<i>S. boydii</i> C13 strains	75	96	71	68	0.5	6.8	7.7
<i>S. dysentery</i> strains	88	65	81	80	6.7	1.4	1.9
<i>S. flexneri</i> strains	94	67	83	84	7.3	1.6	1.7
<i>S. sonnei</i> strains	90	65	83	100	6.7	1.2	0.3
<i>E. coli</i> strains ^c	87	65	86	85	7.3	1.1	1.7

^a Homologous reactions are not included in average.

^b See Table 2 for definition of ΔT_{me} .

^c The average values for the two 0124 strains and the two 0136 strains were used rather than including the separate values for each of these strains.

TABLE 4. Relatedness among strains of *Shigella* species and *E. coli* strains with identical or reciprocal *O* antigens

Source of unlabeled DNA	% relative binding, 60 C				
	<i>S. boydii</i> C8	<i>S. boydii</i> C10	<i>S. boydii</i> C13	<i>S. flexneri</i> 2a 24570	<i>S. sonnei</i> virulent
<i>E. coli</i> 028 (reciprocal to C13)	82	93	63	85	87
<i>E. coli</i> 0115		78	65	78	80
<i>E. coli</i> 0124 ^a	83	89	64	89	85
<i>E. coli</i> 0136 ^a		86	62	85	87
<i>E. coli</i> 0143 (identical to C8)	85	87	64	91	85
<i>E. coli</i> 0144	83	86	68	85	86
<i>E. coli</i> 0147	86	88	64	89	86

^a Two strains tested; the average value from reactions with both strains is shown.

current taxonomic trends) is one taxonomy for the purpose of defining genotypic relatedness and another for use in the laboratory. An additional alternative is to use pathotypes or other infraspecific designations for organisms that are closely related, yet require separation for clinical or other specific purposes.

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