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Polynucleotide Sequence Relationships among Members of *Enterobacteriaceae*

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Polynucleotide relationships were examined among many representatives of the *Enterobacteriaceae* by means of agar, membrane filter, and hydroxyapatite procedures. The amount of deoxyribonucleic acid (DNA) that reassociated was dependent, especially in interspecific reactions, on the annealing temperature. In only three cases: *Escherichia coli-Shigella flexneri*, *Salmonella typhimurium-S. typhi*, and *Proteus mirabilis-P. vulgaris*, was relative interspecific duplex formation 80% or higher. In most cases interspecies DNA duplex formation was 40% or less of that obtained from intraspecies DNA reassociation reactions. The stability of *E. coli-S. flexneri* DNA duplexes formed at either 60 or 75 C was virtually identical to that of homologous *E. coli* DNA duplexes, and the degree of interspecies duplex formation was minimally affected by the temperature increase (86% at 60 C; 77% at 75 C). The thermal stability of DNA duplexes formed at 60 C between DNA from *E. coli* and DNA from strains of *Aerobacter aerogenes*, *S. typhimurium*, *S. typhi*, and *P. mirabilis* was about 12 to 14 C below that of reassociated *E. coli* DNA. At 75 C, the formation of the interspecific DNA duplexes was markedly decreased, but the stability of the DNA able to reassociate at this temperature approximated that of reassociated *E. coli* DNA. The degree of reassociation and the thermal stability of *E. coli-S. flexneri* DNA duplexes suggests relatively little evolutionary divergence in these organisms. The other enterobacteria tested, however, have diverged to a point where less than one-half of their DNA can reanneal with *E. coli* DNA at 60 C and less than 10% reacts at 75 C. The degree of divergence between various enterobacteria does not appear to be uniform along the DNA molecule. Ribosomal ribonucleic acid (RNA)-specific sequences are conserved among most enterobacteria. An examination of messenger RNA relatively specific for the lactose operon suggests that specific chromosomal genes may diverge more or less than the genome as a whole.

Ideally, microbial taxonomy should be based on phylogenetic relationships. This type of taxonomy has, until recently, been impossible, largely owing to the lack of a fossil record and to the relatively few morphological features available for study. Recent advances in protein and nucleic acid biochemistry, and in microbial genetics, allow a preliminary appraisal of relatedness at the molecular level.

Several years ago, it was noted that there was virtually no information concerning the genetic organization of most enteric organisms (38). This remains essentially the case. It still is not known whether the close identity of gross chromosome organization in strains of *Escherichia coli* (49) to that in strains of *Salmonella* (18, 25, 26, 45) and *Shigella* (20, 48) species extends to strains of *Aerobacter*, *Serratia*, or *Proteus* species. In addition, there is little information about the pattern of nucleotide sequence divergence among enteric bacteria. Several factors which are barriers to recombination make comparative genetic studies difficult among many enteric bacteria. These include restriction and modification, surface incompatibility, the presence of independent fertility groups, different specificity of recombination enzymes (10, 16, 51), and dissimilarity in deoxyribonucleic acid (DNA) base sequence.

Nucleic acid reassociation experiments, however, allow one to circumvent the problems of mating incompatibility, and to assess both overall interspecific relatedness and relatedness within specific portions of the genome. The nucleic acid reassociation experiments reported here examine quantitative relationships among many species of enteric organisms. In several cases, the thermal stability of reassociated DNA was determined for reactions carried out at different incubation temperatures to allow assessment of different levels of interspecies relationships.

MATERIALS AND METHODS

Organisms and media. The strains used in the present investigation are listed in Table 1. Antibiotic medium no. 3 (Penassay Broth, Difco), meat extract-agar, and Brain Heart Infusion (Difco) were used for routine cultivation of organisms. For the purpose of labeling DNA, log-phase cells were suspended in a tris(hydroxymethyl)aminomethane (Tris)-glucose salt medium lacking phosphate salts and containing 0.5 to 1.0% Brain Heart Infusion. Carrier-free $H_2{}^{32}PO_4$ (New England Nuclear Corp., Boston, Mass.) was added to this medium, and the cultures were incubated at 37 C overnight. Media used in labeling of ribonucleic acid (RNA) are listed below.

Preparation of DNA. Cells were harvested from broth by centrifugation. DNA used in some of the earlier DNA-agar experiments and in the RNA-DNA reactions carried out on membrane filters was extracted from bacterial strains as described by Marmur (36). These DNA preparations were sheared, when desired, by sonic treatment at 4 C in a Branson Sonifier (Heat Systems Co., Melville, N. Y.) to an average molecular weight of 2.5×10^5 to 3.5×10^5 daltons (17).

In many of the DNA-agar experiments and in all of the experiments utilizing hydroxyapatite, DNA was prepared by a modification of the method of Berns and Thomas (4). Bacterial cells were suspended at an approximate 1:50 dilution (w/v) in a solution containing 0.1 M sodium chloride, 0.1 M ethylenediaminetetraacetate (EDTA; 0.05 M EDTA was used in later experiments), 0.05 M Tris hydrochloride buffer, *p*H 8.2 (Tris buffer), and self-digested (37 C, 2 hr) Pronase (Calbiochem, Los Angeles, Calif.) at 50 μg/ml. Sodium lauryl sulfate (SLS) was added to a final concentration of 0.5%, and the suspension was incubated at 37 C overnight to maximize cell lysis and Pronase action. The SLS concentration was increased to 1%, and an equal volume of phenol was added to the suspension. The phenol and aqueous layers were well mixed and then gently shaken for several minutes. The mixture was centrifuged to separate the phase. The aqueous phase was collected, and sodium perchlorate was added to a 1 M concentration. An equal volume of chloroform was added to the aqueous

TABLE 1. *Bacterial strains employed*

Organism	Source
Escherichia coli K-12-1	University of Washington culture collection
E. coli K-12 200 μ *lac*$^-(z^+y^-)$	WRAIR[a] (25)
E. coli K12-1485	(17)
E. coli K12 *lac*Δ	A. D. Pardee
Salmonella typhi 643	WRAIR (25)
S. typhi 643 lac_3	WRAIR (25)
S. typhi 643 X30T	WRAIR (25)
S. typhi 643 X30W	WRAIR (25)
S. typhimurium LT2	T. Theodore
S. typhimurium 7823	ATCC
Salmonella sp. (ser.) *ballerup*	Brown University culture collection
Salmonella sp. (ser.) *arizonae*	W. H. Ewing
Shigella flexneri 2a 24570	WRAIR (48)
Serratia marcescens	(17)
Aerobacter aerogenes	University of Washington culture collection
Proteus mirabilis-1	(21)
P. mirabilis-1 F-*lac*$^+$	(21)
P. morganii	C. A. Stuart (19)
P. rettgeri	C. A. Stuart (19)
P. vulgaris	C. A. Stuart (19)
P. inconstans (Providence 29911)	C. A. Stuart (19)
Bethesda-10	W. H. Ewing
Neisseria perflava	D. Kingsbury
N. gonorrhoeae	D. Kingsbury
N. catarrhalis	D. Kingsbury
Pasteurella pestis	WRAIR
Pseudomonas aeruginosa	University of Washington culture collection

[a] Walter Reed Army Institute of Research.

phase, and the mixture was shaken and centrifuged. The aqueous phase was recovered, and the chloroform treatment was repeated once or twice until the interphase material was largely removed. Two volumes of cold 95% ethyl alcohol were added to the aqueous phase and the resultant DNA precipitate was loosely spooled around a glass rod, washed in ethyl alcohol, and suspended in SSC/100 (SSC = 0.15 M sodium chloride + 0.015 M sodium citrate). The DNA was repeatedly (three or four times) precipitated (after the addition of NaCl to 0.1 M final concentration) with 95% ethyl alcohol and suspended in SSC/100 until the precipitate was translucent. The DNA solution was made 0.1 M to NaCl and 0.05 M to EDTA and Tris buffer, and was incubated with 25 μg of pancreatic ribonuclease per ml (crystallized; Worthington Biochemical Corp., Freehold, N.J.) at 37 C or, in more recent preparations, at 60 C for 1 hr. SLS was added to 0.5%, and the DNA was incubated overnight at 37 C in the presence of 50 μg of Pronase per ml. The SLS concentration was then increased to 1%, and the DNA was again treated once with phenol and twice with chloroform. The DNA was then repeatedly (three or four times) precipitated with 2-ethoxyethanol and suspended in SSC/100. It should be noted that dilute DNA solutions are poorly precipitated by 2-ethoxyethanol, and the majority of the DNA may be lost (29). We try to have DNA solutions at a concentration of 1 mg/ml or higher for precipitation by 2-ethoxyethanol. When desired, the DNA was fragmented by mechanical shear at 50,000 psi to a molecular weight of approximately 2×10^6 (9) and was filtered through Metricel filter discs (0.45 μm pore size, Gelman Instrument Co., Ann Arbor, Mich.). Labeled DNA fragments were denatured by heating; they were further purified (for hydroxyapatite experiments) by passing them through a hydroxyapatite column equilibrated with 0.12 M PB (PB = phosphate buffer, an equimolar mixture of NaH_2PO_4 and Na_2HPO_4, *p*H 6.8) and were held at 60 C. Material that bound to the column under these conditions was discarded. This procedure decreases the "zero time" binding (label bound to the column immediately after the DNA has been denatured) from about 2 to 2.5% to about 0.5 to 0.7% (Kohne and Britton, *unpublished data*).

Preparation of DNA-agar and DNA-agar reactions. DNA was denatured and embedded in Oxoid Ionagar No. 2 as described by Bolton and McCarthy (5). It was then pressed through a stainless-steel screen (35 mesh), extensively washed with 2× SSC at 66 C, and assayed optically (260 nm) for DNA content by dissolving the agar in 5 M sodium perchlorate. Agar in amounts of 0.2 to 0.5 g, containing between 300 and 1,000 μg of DNA per g, was incubated for 15 to 18 hr at 60 or 66 C with denatured, ^{32}P-labeled DNA fragments contained in a volume of 2× SSC equal to the weight of agar employed. The ratio of DNA in agar to DNA fragments was 200:1,000. The DNA-agar was then transferred to a glass tube fitted with a Saran screen bottom (3) and was washed with ten 15-ml portions of 2× SSC at the incubation temperature to remove unbound DNA. Labeled DNA fragments bound to the agar were recovered by washing the agar with five 15-ml portions of water at 75 C. All wash fluids were assayed for trichloroacetic acid-precipitable radioactivity. The percentage of DNA fragments bound to the DNA-agar was calculated as bound counts/total counts × 100.

Preparation of labeled 23S RNA. Cells were grown [by the method of McCarthy and Bolton (33)] in glucose-salts medium containing ^3H-uridine (2 μc/ml), extracted, and chromatographed on methylated albumin-Kieselguhr columns (35). The peak containing 23*S* RNA was pooled and rechromatographed before use.

Preparation of pulse-labeled RNA. Pulse-labeled RNA was prepared by growing *E. coli* K-12 W1485 F^- in 500 ml of TCG (50) medium to a cell density of about 2.5×10^8 cells/ml and then adding 1 μc of ^3H-uridine per ml (2.5 c/mmole). After 90 sec of incorporation, the culture was poured over an equal volume of crushed, frozen medium. The cells were collected by centrifugation at 4 C, washed with TM-buffer (10^{-2} M Tris, *p*H 7.4, containing 10^{-3} M Mg^{++}). RNA was extracted by freezing and thawing the cells three times in the presence of 200 μg of lysozyme per ml and 50 μg of deoxyribonuclease per ml (both from Worthington Biochemical Corp.). The lysate was brought to room temperature, and lysis completed with 0.4% SLS for 5 min. The lysate was chilled and shaken three times with water-saturated phenol. The aqueous phase was then extracted three times with cold, anhydrous ether to remove the phenol. Traces of ether remaining in the aqueous phase were removed by bubbling nitrogen through the solution. The RNA was precipitated with three volumes of cold ethyl alcohol. The precipitate was dissolved in TM buffer and again precipitated.

Preparation of lactose messenger RNA (mRNA). The method used was similar to that described by Naono, Rouviere, and Gros (43), which exploits the preferential transcription of the lactose operon during the diauxic growth of *E. coli*. A strain of *E. coli* K-12 200 μ *lac*$^-$ (z^+y^-) was infected with the F merogenote F-*lac*$^+$ (z^+y^+) and grown overnight in minimal salts medium containing 0.2% glucose. The culture was centrifuged in the cold and suspended to an optical density (OD) at 420 nm of 0.500 in minimal medium containing 0.03% glucose and 0.2% lactose. The culture was incubated at 37 C with shaking for 120 min, at which time growth from glucose stopped (OD increased from 0.50 to 1.05). After a stationary phase of approximately 20 min, the second phase of growth on lactose started, and at this time 1 μc of ^3H-uridine per ml (13 c/mmole; Schwarz BioResearch Inc., Orangeburg, N.Y.) was added to the culture for 60 sec. The labeled cells were washed with TM buffer, and the RNA was extracted as described for pulse-labeled RNA. Approximately 2 mg of the RNA (6.5×10^5 counts per min per mg) was chromatographed on a methylated albumin-Kieselguhr column (35) with the use of a linear gradient of 0.3 to 0.8 M NaCl. Pilot experiments were carried out by use of a mixture of ^{14}C-labeled RNA pulsed just before adaptation to lactose under diauxie growth conditions and ^3H-RNA prepared as lactose adaption took place. The ^3H-RNA contained a significant percentage (10% or more) eluting at about 0.7 M NaCl (about 27*S*)

that could duplex with DNA derived from *P. mirabilis* F-*lac*$^+$ DNA, whereas only a negligible fraction (about 1%) of the ^{14}C-labeled RNA reacted with this DNA. Approximately 1.3 $\times$ 10^5 counts/min of ^{3}H-RNA in 2$\times$ SSC, containing most of the hybridizable F-*lac*$^+$ material, was incubated repeatedly with 100-μg samples of denatured DNA from *E. coli lac* Δ, an Hfr strain carrying a deletion of the lactose operon. The remaining RNA contained about 3 $\times$ 10^4 counts/min, and was filtered through a type A (coarse) filter (Schleicher & Schuell Co., Keene, N.H.). Samples of this RNA were incubated overnight at 67 C with membrane filters containing 100 μg of appropriate DNA preparations. The filters were treated with 30 μg of ribonuclease per ml (60 min in 2$\times$ SSC at 26 C), washed, dried, and counted.

Immobilization of DNA on filters. DNA was denatured just before use by heating at 100 C for 10 min in SSC/10 at a concentration not exceeding 50 μg/ml. Alternatively, DNA was denatured with alkali as described by Gillespie and Spiegelman (22). Denatured DNA solutions were diluted with 6$\times$ SSC to approximately five times their original volume, and the DNA was immobilized on type B6 nitrocellulose filters (Schleicher & Schuell Co.) according to the method of Gillespie and Spiegelman (22). The amount of DNA retained by the filters was determined by monitoring the OD at 260 nm before and after filtration of the denatured DNA solution through the filters. The amount of DNA fixed to a single 25-mm filter was 100 μg $\pm$ 5 μg.

Binding of mRNA to DNA immobilized on membrane filters. Membrane filters to which 100 μg of denatured DNA were fixed by heat (22) in vacuo were incubated with an appropriate quantity of mRNA (usually 12 to 50 μg) in 5 ml of 2$\times$ SSC for 20 hr at 60 or 67 C in a glass vial. The membrane filters were washed with 2$\times$ SSC, 50 ml per side, and were incubated for 60 min at 26 C in 5 ml of 2$\times$ SSC containing 20 μg of pancreatic ribonuclease per ml (Worthington Biochemical Corp.). The filters were washed with cold 2$\times$ SSC, 50 ml per side, dried in an oven, and counted. Some experiments were also performed with 10 μg of T1 ribonuclease per ml (Miles Laboratories, Inc., Elkhart, Ind.) and a combination of T1 and pancreatic ribonuclease. There was, however, no significant difference in the degree of relative binding compared with those obtained by use of pancreatic ribonuclease alone.

Separation of single- and double-stranded DNA on hydroxyapatite. The effectiveness of hydroxyapatite for fractionating native and denatured DNA was first demonstrated by Bernardi (3). Miyazawa and Thomas (41) developed a technique for fractionating double-stranded DNA bound to hydroxyapatite. A modification of this procedure (9) was used to determine the thermal stability of reassociated bacterial DNA. In this procedure, thermally denatured, ^{32}P-labeled *E. coli* DNA fragments were incubated at 60, 66, or 75 C with a 4,000- to 7,500-fold excess of denatured unlabeled DNA fragments in 1 ml of 0.12 M PB. The length of incubation was chosen so that unlabeled DNA fragments would be at least 80% reassociated, whereas the reassociation of labeled DNA fragments with one another would be only 1 to 2%. After incubation, the samples were quickly cooled in an ice bath and frozen until use. Each sample was subsequently thawed and passed through a 10-ml hydroxyapatite (BioRad Laboratories, Richmond, Calif.) column held at the temperature at which the fragments had been incubated. In some later experiments, a batch procedure (7) which allowed six or more samples to be processed simultaneously was employed. The columns were then washed with 15-ml portions of 0.12 M PB at temperatures increasing in increments of 2.5 C to 100 C. As the column temperature exceeded the dissociation temperature of DNA duplexes bound to the hydroxyapatite, the resultant single-stranded DNA was eluted from the column. The column was finally washed with two or three 15-ml portions of 0.4 M PB to elute any remaining material bound to the hydroxyapatite. It should be noted that the capacity of hydroxyapatite to adsorb double-stranded DNA varies from lot to lot. A typical lot of hydroxyapatite, in our hands, bound approximately 100 to 250 μg of double-stranded DNA/ml. It is desirable to use an amount of hydroxyapatite two to three times higher than that necessary to adsorb the amount of DNA employed, to insure against exceeding the column capacity. It has also been noted that zero-time binding of single-stranded DNA often increases markedly (up to 10 to 15%) in old batches of hydroxyapatite (after 4 months or longer). This difficulty can be overcome by boiling the hydroxyapatite before use.

When only the percentage of reassociated DNA fragments, rather than the thermal stability of the reassociated product, was of interest, the column was washed with 15-ml portions of 0.12 M PB as above, then with one 15-ml 0.12 M PB wash at 95 C and one at 100 C, and finally with three washes with 0.4 M PB.

Radioactivity assay. Samples from agar experiments, as well as many of the samples from hydroxyapatite experiments, were precipitated in 5% trichloroacetic acid in the presence of approximately 100 μg of yeast RNA carrier. The precipitates were collected on membrane filters, dried, and placed in counting vials. A 15-ml amount of scintillation fluid was added, and the samples were counted in either a Packard or a Nuclear-Chicago liquid scintillation spectrometer. Alternatively, eluates from hydroxyapatite experiments were placed directly into counting vials and assayed by Cerenkov counting (11).

RESULTS

Quantitative nucleic acid relationships among the Enterobacteriaceae. An expanding body of data has accumulated which attempts to define the relationships between bacteria by a study of heteroduplex DNA molecules—or mRNA-DNA hybrid molecules. In a reassociation experiment, nucleic acids are incubated under a set of conditions such that single-stranded DNA fragments (or mRNA molecules) may collide with unlabeled DNA in free solution (hydroxyapatite method), immobilized in agar (5), or on filters

(13, 22). Double-stranded duplexes are formed as bases in one strand pair with their complementary bases in the other strand. The reassociation of DNA is affected by several different parameters among which are the following.

(i) The base composition (39): guanine and cytosine (G + C) base pairs exhibit greater thermal stability than adenine + thymine (A + T) base pairs. The greater the fraction of G + C base pairs within a given DNA duplex, the higher is the thermal stability of that duplex.

(ii) The size of nucleic acid fragment (9, 32, 34): with very small DNA fragments, no specificity is to be expected since any particular short base sequence may be found in all DNA molecules. The minimal specific DNA fragment for bacteria is about 15 nucleotides (34). The length of DNA fragments affects DNA reassociation in free solution (9). The fragments used in this study were about 500 to 600 and 1,000 nucleotides in length. The larger fragments reassociate about 25% faster than the smaller ones (9).

(iii) The ionic strength (9): both the rate of DNA reassociation and the thermal stability of reassociated DNA increase as the ionic strength is increased. For any given set of experiments reported below, the ionic strength was held as a constant parameter.

(iv) The temperature of incubation (6, 27, 33): the optimal temperature for reassociation is some 30 C below the temperature at which a DNA becomes denatured (37, 39). Temperatures significantly lower than the optimum permit distantly related (6, 32) and nonspecific sequences to reassociate (27, 32). Incubation temperatures employed in this study were never less than 30 C below the melting temperature of the DNA under study.

(v) DNA concentration and the time of incubation (9, 33): the concentration of labeled DNA (or mRNA) and unlabeled DNA employed, as well as the time of incubation, must be carefully chosen to obtain meaningful reassociation data. Generally, in agar and filter reactions, the ratio of unlabeled to labeled DNA in a reaction was at least 100:1. This large ratio provided an excess of available sites with which any related labeled nucleic acid sequence could reassociate. The incubation time was chosen so that reassociation between labeled DNA (or mRNA) and unlabeled DNA was maximal. DNA concentrations and incubation time are particularly critical in free solution reactions, since labeled fragments reassociated with one another cannot be distinguished from the desired reassociation product of labeled with unlabeled DNA fragments. In hydroxyapatite experiments, therefore, the labeled DNA concentration was small enough to insure little or no label-label reassociation during the course of incubation. Concomitantly, the unlabeled DNA concentration was large enough to permit the reassociation of labeled with unlabeled DNA fragments to proceed to virtual completion. The term "duplex" is used interchangeably with "fragments bound," as we assume that bound fragments are in a duplex arrangement.

Table 2 presents a compilation of reassociation studies performed according to several different criteria, done by agar, filter, and hydroxyapatite techniques, and with measurement of both DNA-DNA and mRNA-DNA duplex formation. The nature of Table 2 precludes detailed comments; however, several general features of these data are evident. Essentially the same results were obtained whether a filter, agar, or hydroxyapatite method was employed (compare columns 1 and 4 and columns 2, 3, and 5). Greater than 80% relative reassociation was obtained in only three interspecific reactions: *E. coli-S. flexneri*, *S. typhi-S. typhimurium*, and *P. mirabilis-P. vulgaris*. Where tested, this high level of relative binding proved to be essentially temperature-insensitive. The vast majority of DNA-DNA and mRNA-DNA reactions showed 40% or less relative binding according the most relaxed criteria (60 C in 2× SSC or 0.12 M PB), less than 30% relative binding with intermediate criteria (66 or 67 C in 2× SSC or 66 C in 0.12 M PB), and 10% or less relative binding with the most stringent criterion employed (75 C in 0.12 M PB). Even DNA from so-called intermediate forms, such as the classical *Salmonella* sp. (ser.) *ballerup*, a strain of the Bethesda group, and *Salmonella* sp. (ser.) *arizonae* showed comparatively little binding with both *S. typhimurium* mRNA and *E. coli* mRNA. DNA from several organisms, presumably unrelated to enterobacteria, was included in these studies as a control for reaction specificity. No DNA from the *Neisseria* species tested showed appreciable reaction with *E. coli* DNA. Marginal reassociation was observed between DNA of *Pasteurella pestis* or *Pseudomonas aeruginosa* and that of *E. coli* (Table 2).

These experiments were designed to examine possible nucleic acid sequence similarities between broad, general groups of organisms. Quantitative reassociation was studied in most detail in the genus *Proteus*. Aside from the high relative relatedness between DNA preparations from strains of *P. mirabilis* and *P. vulgaris*, *P. mirabilis* DNA showed 16% or less reaction with DNA from any other *Proteus* species, or

TABLE 2. *Relative nucleic acid binding relationships among enterobacteria*[a]

Nucleic acid reaction	DNA-agar, 60 C	DNA-agar, 66 C	mRNA*/DNA filters, 67 C	Hydroxyapatite		
				60 C	66 C	75 C
	%	%	%	%	%	%
E. coli/S. typhimurium* LT2	38 ± 2 (8)	26 ± 4 (6)	25 ± 4 (6)	39 ± 6 (10)	30 ± 4 (4)	9 ± 2 (5)
E. coli/S. typhimurium* 7823	—	—	—	38 (1)	—	9 (1)
E. coli/A. aerogenes*	41 ± 3 (3)	—	22 ± 2 (6)	37 ± 4 (3)	23 (1)	8 ± 2 (4)
E. coli/S. typhi* 643	—	—	27 ± 1 (3)	43 ± 4 (5)	—	9 ± 3 (4)
E. coli/P. mirabilis* 1	5 (1)	—	5 ± 1 (5)	6 ± 2 (3)	—	1 ± 1 (6)
E. coli/S. flexneri* 24570	82 (1)	—	82 ± 7 (6)	86 ± 7 (5)	—	80 ± 3 (3)
E. coli/Bethesda-10	—	—	24 ± 1 (3)	—	—	—
E. coli/Salmonella* sp. (ser.) *ballerup*	—	—	29 ± 2 (3)	—	—	—
E. coli/Salmonella* sp. (ser.) *arizonae*	—	—	23 ± 0 (3)	—	—	—
E. coli/S. marcescens*	—	—	9 ± 3 (6)	—	—	—
E. coli/P. morganii*	—	—	6 ± 1 (3)	—	—	—
E. coli/P. aeruginosa*	—	—	—	4 (1)	—	0 (2)
E. coli/P. pestis*	4 (1)	—	—	—	—	—
E. coli/N. perflava*	2 (1)	—	—	—	—	—
E. coli/N. gonorrhoeae*	—	—	—	0 (3)	—	0 (3)
E. coli/N. catarrhalis*	—	—	—	1 (1)	—	0 (1)
S. typhimurium/S. typhi*	—	—	87 ± 3 (3)	—	—	—
S. typhimurium/E. coli*	—	—	30 ± 3 (3)	—	—	—
S. typhimurium/A. aerogenes*	—	—	34 ± 3 (3)	—	—	—
S. typhimurium/Sh. flexneri*	—	—	26 ± 4 (3)	—	—	—
S. typhimurium/ Bethesda-10*	—	—	26 ± 2 (3)	—	—	—
S. typhimurium/Salmonella* sp. (ser.) *ballerup*	—	—	34 ± 1 (3)	—	—	—
S. typhimurium/Salmonella* sp. (ser.) *arizonae*	—	—	42 ± 2 (3)	—	—	—
S. typhimurium/S. marcescens*	—	—	14 ± 4 (3)	—	—	—
S. typhimurium/P. mirabilis*	—	—	8 ± 1 (3)	—	—	—
S. typhimurium/P. morganii*	—	—	12 ± 2 (3)	—	—	—
P. mirabilis/P. vulgaris*	92 (1)	—	—	—	—	—

[a] DNA-agar reactions and mRNA*/DNA filter reactions were carried out in 2 × SSC. Hydroxyapatite reactions were carried out in 0.12 M PB. In all cases the intraspecies reaction (e.g., *E. coli*/E. coli*) was arbitrarily designated as 100%. All other reactions percentages are relative values. The amount of reassociation (binding) observed for intraspecies reactions was as follows: DNA-agar, 60 C = 30 to 45%; DNA-agar, 66 C = 20 to 30%; RNA*/DNA filters, 67 C = 20 to 30% of total RNA bound to homologous DNA; hydroxyapatite, 60 C = 82 to 97%; hydroxyapatite, 66 C = 80%, hydroxyapatite, 75 C = 75 to 92%. Asterisks designate the source of radioactive fragments. Numbers in parentheses show the number of experiments.

TABLE 2.—*Continued*

Nucleic acid reaction	DNA-agar, 60 C	DNA-agar, 66 C	mRNA*/DNA filters, 67 C	Hydroxyapatite		
				60 C	66 C	75 C
	%	%	%	%	%	%
*P. mirabilis**/*P. rettgeri*	6 (1)	—	—	—	—	—
*P. mirabilis**/*P. inconstans*	16 (1)	—	—	—	—	—
*P. mirabilis**/*E. coli* K-12	5 (1)	—	—	7 ± 1 (2)	—	1 ± 0.5 (2)
*P. mirabilis**/*S. typhimurium* LT2	—	—	—	5 (1)	—	—
*P. mirabilis**/*S. typhimurium* 7823	—	—	—	4 (1)	—	0.5 (1)
*P. mirabilis**/*S. typhi* 643	—	—	—	6 (1)	—	0.7 (1)
*P. mirabilis**/*A. aerogenes*	—	—	—	5 (1)	—	0.3 (1)
*Sh. flexneri**/*E. coli* K-12	—	—	77 ± 6 (5)	—	—	—
*Sh. flexneri**/*S. typhimurium*	—	—	20 ± 0 (2)	—	—	—
*Sh. flexneri**/*S. typhi* 643	—	—	21 ± 2 (5)	—	—	—
*S. typhi**/*S. typhimurium*	—	—	89 ± 1 (2)	—	—	—
*S. typhi**/*Sh. flexneri*	—	—	30 ± 1 (2)	—	—	—
*S. typhi**/*E. coli* K-12	—	—	21 ± 1 (2)	—	—	—

indeed with any other enteric organism. These observations, coupled with the generally low values obtained for interspecies DNA reassociation with other genera, are evidence for marked nucleotide sequence divergence in the *Enterobacteriaceae*.

Thermal stability of interspecies DNA duplexes formed at increasing incubation temperatures. Variation in the degree of reassociation with increased incubation temperatures is assumed to provide information as to the relative amounts of closely and partially related base sequences between the DNA of one organism and that of another. A corollary to this assumption is that the thermal stability of DNA duplexes formed at various incubation temperatures will be an index of the extent of base pairing. It was recently reported that the thermal stability of *E. coli-S. typhimurium* DNA duplexes increased despite decreased binding when the reactions were incubated at 66 C instead of at 60 C (6).

The thermal stability of interspecies enteric DNA duplexes was studied in 60 and 75 C reactions by measuring the thermal release of reassociated DNA fragments bound to hydroxyapatite. It should be noted that the method of thermal elution is a process of dissociating strand pairs with steps of increasing temperature and assaying (by radioactivity) the eluted fractions. The melting temperature ($T_{m(e)}$) obtained by the release of DNA fragments due to complete strand separation is not theoretically nor experimentally equivalent to the T_m measured optically by following the disruption of the helical content of DNA (9, 37). As a practical matter, however, the optical T_m (89 C ± 1 C, in 0.12 M PB) agrees rather closely with the elution $T_{m(e)}$ (87.5 C ± 1 C) for *E. coli* DNA.

Table 3 shows the stability of some *E. coli*-enterobacterial DNA duplexes. In the intraspecies *E. coli* reassociation experiments, the absolute binding was approximately 90% (as high as 97% in certain experiments) for a 60 C reaction and some 5 to 7% less in 75 C reactions. This binding was arbitrarily designated 100%. The reaction between the DNA of *E. coli* and the DNA of *S. flexneri* exhibited some 85% relative reassociation at the 60 C incubation temperature and about 80% at the 75 C incubation temperature. Similarly, the thermal stability of the *E. coli-S. flexneri* DNA reassociation products was the same at both temperatures and was very similar to the stability of the *E. coli-E. coli* DNA. reassociation products. The reaction between *E. coli* DNA and *S. typhimurium* showed about 40% relative reassociation at the 60 C

TABLE 3. *Thermal elution midpoints of enterobacterial DNA duplexes formed at 60 or 75 C*[a]

Nucleic acid reaction	$T_{m(e)}$ from 60 C incubation	Relative binding	$T_{m(e)}$ from 75 C incubation	Relative binding
	C	%	C	%
E. coli/E. coli*	87.5 ± 1.0	100	87.5 ± 1.5	100
E. coli/S. typhimurium* LT2	74.0 ± 1.0	39	84.5 ± 1.5	9
E. coli/S. typhimurium* 7823	74.0[b]	38	83.0[b]	9
E. coli/S. typhi* 643	75.0 ± 1.0	43	83.0 ± 1.0	9
E. coli/A. aerogenes*	75.0 ± 1.0	37	85.5 ± 1.5	8
E. coli/P. mirabilis*-1	73.5 ± 1.5	6	—[c]	1
E. coli/P. mirabilis*-1 *F-lac*$^+$	80.0 ± 1.0	8	87.0 ± 0	3
E. coli/S. flexneri*	86.5 ± 0.5	86	86.5 ± 0.5	80
E. coli/S. typhi* 643 lac_3	76.0 ± 1.0	52	84.0 ± 1.0	16
E. coli/S. typhi* 643 X30T	80.0 ± 1.0	59	87.0 ± 1.0	32
E. coli/S. typhi* 643 X30W	83.0 ± 2.0	58	87.5 ± 0.5	38

[a] All reactions were carried out with hydroxyapatite. Asterisks designate the source of radioactive fragments.

[b] Only one experiment. All other reactions were carried out from 3 to 20 times.

[c] Too little reassociation to allow accurate assay.

incubation temperature, but only about 9% at the 75 C temperature. The duplexes formed between DNA from *E. coli* and *S. typhimurium* at 60 C had a $T_{m(e)}$ some 13.5 C below that of *E. coli* DNA duplexes. The product formed at 75 C, however, exhibited a $T_{m(e)}$ only about 3 C lower than that of *E. coli* DNA duplexes. Similar changes in relative binding and $T_{m(e)}$ were also noted in the interspecies DNA duplexes formed between *E. coli* and *S. typhi*, or *E. coli* and *A. aerogenes*. In the case of *E. coli-P. mirabilis* duplexes, at the 60 C incubation temperature there was approximately 6% relative reassociation with a $T_{m(e)}$ some 14 C below that of *E. coli* DNA. At 75 C, the relative reassociation was about 1%, which was too little to permit an accurate assay of thermal stability.

The thermal elution profiles show that interspecies duplexes were formed at the 60 C incubation temperature, as well as at the 75 C temperature (Fig. 1). The difference was that virtually all of the reaction product was quite stable in a 75 C incubation (Fig. 1A), whereas at 60 C duplexes with a markedly decreased thermal stability were also permitted. In fact, reassociated nucleotide sequences with low thermal stability make up the bulk of the reaction product formed at 60 C (Fig. 1B) in the case of duplexes formed between DNA from *E. coli* and DNA from *Salmonella*, *Aerobacter*, and *Proteus* species. In contrast, as shown in Table 3 and Fig. 2, at both the 60 and 75 C incubation temperatures, the *E. coli* and *S. flexneri* reaction product was highly stable and contained only a very small proportion of DNA that eluted at low temperatures.

Studies with synthetic polynucleotides have demonstrated that the thermal stability of double-stranded nucleic acid complexes is very sensitive to quite small proportions of unpaired bases (2, 9, 30). The presence of approximately 1% unpaired bases within a reassociated nucleotide sequence lowers its thermal stability by 1 C (2). On this basis, under our experimental conditions, the degree of unpaired bases tolerated in *E. coli-S. typhimurium* duplexes would be about 1 in 7 at an incubation temperature of 60 C, about 1 in 9 at 66 C, and less than 1 in 30 at 75 C. Therefore, the higher the temperature of incubation (i.e., the stricter the criterion of reassociation) presumably the greater is the discrimination and complementarity of reassociated nucleotide sequences. The alternative, but less likely, explanation that decreased thermal stability is predominantly due to a preferentially high percentage of A + T base pairs within interspecies duplexes is not considered in the argument. This alternative has been discussed elsewhere (6, 28).

Detection of E. coli-specific DNA in the genomes of heterologous organisms. It was assumed that the presence of increasing amounts of *E. coli*-specific nucleotide sequences in the genome of a heterologous organism would be detectable in reassociation and thermal stability experiments with *E. coli* DNA. Two kinds of genetic hybrids were employed to test this assumption. Three *S. typhi-E. coli* hybrids were used (1, 18): *S. typhi* 643 lac_3, which had substituted 7 to 13% of the *E. coli* chromosome for its own; *S. typhi* 643 X30T, which had substituted between 20 and 28% of the *E. coli* chromosome; and *S. typhi* 643 X30W, which contained between 39 and 44% of the *E. coli* chromosome.

^{32}P-labeled *E. coli* DNA fragments (0.1 μg) were incubated with approximately 400 μg of unlabeled DNA fragments from *E. coli*, *S. typhi*, *S. typhi* 643 lac$_3$, *S. typhi* 643 X30T, and *S. typhi* 643 X30W contained in 1 ml of 0.12 M PB. Labeled *E. coli* fragments incubated with *N. gonorrhoeae* DNA fragments and labeled *E. coli* fragments incubated in the absence of unlabeled DNA served as controls. Both the 60 and 75 C reactions were incubated for 24 hr. The reaction mixtures were passed through hydroxyapatite columns, and reassociated DNA

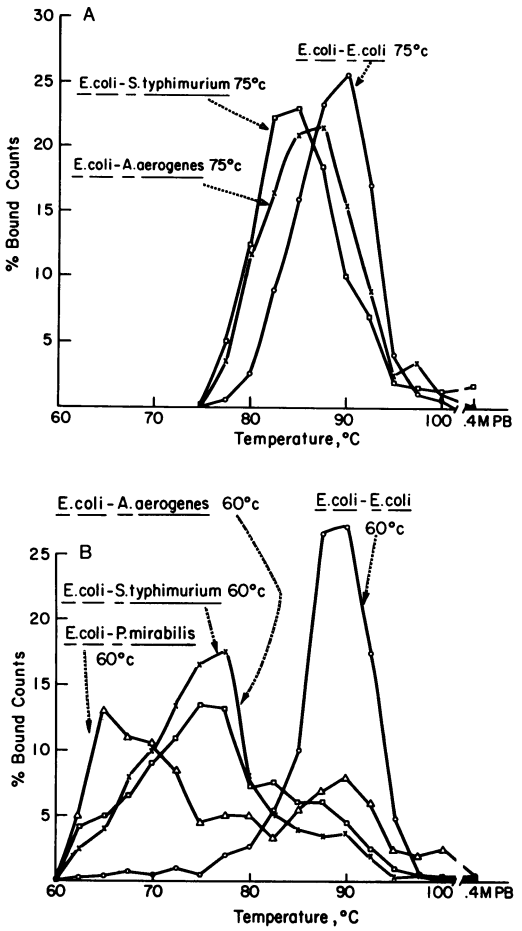

FIG. 1. *Thermal elution profiles of reassociated interspecies enterobacterial DNA duplexes. (A) Profiles obtained from 75 C reactions. (B) Profiles obtained from 60 C reactions. A 0.1-μg amount of ^{32}P-labeled E. coli DNA fragments was incubated with approximately 500 μg of unlabeled DNA fragments from the indicated organisms in 1 ml of 0.12 M PB for 18 hr at 60 or 75 C. The samples were loaded onto hydroxyapatite columns and eluted and assayed as described in Materials and Methods.*

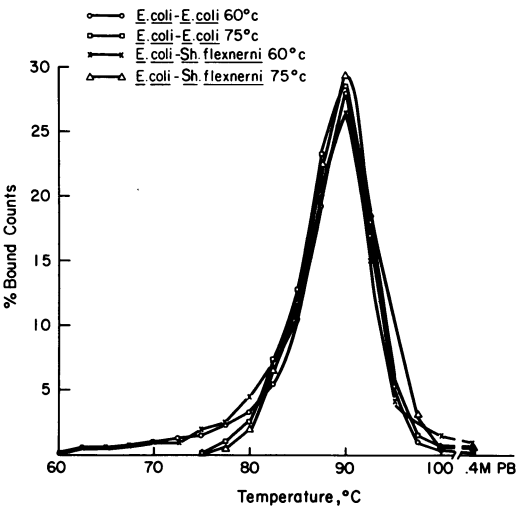

FIG. 2. *Thermal elution profiles of interspecies E. coli-S. flexneri DNA duplexes formed at 60 and 75 C. The reactions were carried out identically to those described in Fig. 1.*

was eluted as described in Materials and Methods.

The ability of the unlabeled DNA fragments to reassociate under our experimental conditions was confirmed by optically assaying the amount of input DNA sticking to the column after incubation. In reactions carried out at 60 C, unlabeled DNA fragments, regardless of origin, were 80 to 95% reassociated. An average 5 to 7% decrease in reassociation of unlabeled DNA fragments was observed in samples incubated at 75 C. Control reactions, in which 0.1 μg of labeled *E. coli* DNA fragments was incubated at 60 and 75 C, showed 2.0 and 1.8% binding to hydroxyapatite. Relative binding percentages and the thermal stability of duplexes formed between *E. coli* DNA and DNA from these *Salmonella* strains are listed in Table 3. Reaction specificity was assured by the inability of *E. coli* fragments to reassociate with *N. gonorrhoeae* DNA, in excess of control values, at either reaction temperature. The base composition of both the *E. coli* and *N. gonorrhoeae* DNA is approximately 50 mole per cent A + T (Kingsbury and Brenner, *unpublished data*).

The thermal elution profile obtained after reassociation of *E. coli* DNA fragments at 60 C (Fig. 1B) tends toward a Gaussian distribution with a $T_{m(e)}$ of about 88 C. In contrast, a profile obtained from an *E. coli-S. typhi* reaction at 66 C was very broad and exhibited a $T_{m(e)}$ some 13 C below that of the intraspecific 60 C *E. coli* reaction. The presence of increasing amounts of

E. coli-specific DNA in *S. typhi* strains lac_3, X30T, and X30W was evident from the increased relative percentage of reassociation and the increased $T_{m(e)}$ (Table 3). Thermal elution profiles from *E. coli-S. typhi* hybrid reactions at 60 C were biphasic in nature, with the appearance of a thermally stable *E. coli*-like peak (Fig. 3A).

An increase in thermal stability was apparent in elution profiles obtained from all interspecies reactions at 75 C (Table 3). These increases were due to the loss of reassociated sequences which were stable at 60 C, but could not withstand the more stringent incubation temperature. The loss of these sequences resulted in sharper, more stable elution profiles (Fig. 3B). Table 4 summarizes the results obtained with *S. typhi-E. coli* hybrid strains. The data indicate that the degree of reassociation at 75 C may be used to obtain a reasonable estimate as to the degree of specific *E. coli* genetic substitution in these strains.

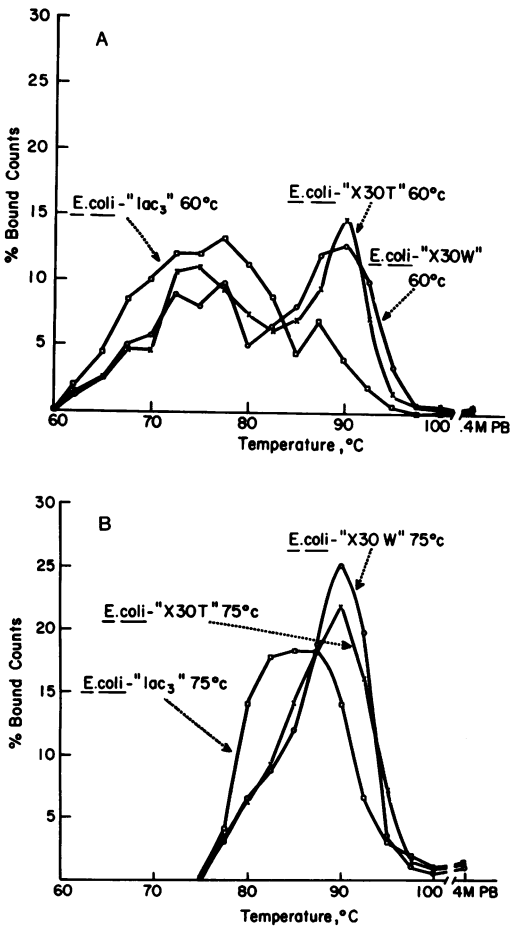

FIG. 3. *Detection of E. coli-specific DNA in the genome of S. typhi genetic hybrids. ^{32}P-labeled E. coli DNA fragments were incubated with unlabeled DNA from the indicated organisms as described in the text. The thermal elution and assay procedures are described in Materials and Methods. (A) Profiles obtained from 60 C reactions. (B) Profiles obtained from 75 C reactions.*

Approximately 9% relative reassociation occurred between DNA from *E. coli* and *S. typhi* at 75 C. This "background" problem was largely avoided by using a strain of *P. mirabilis* that carried the extrachromosomal element F-*lac*$^+$ (21). The F-*lac*$^+$ element is equivalent to about 2.5% of the *E. coli* genome and is present in *Proteus* as an addition to the *Proteus* genetic material (17). These experiments were carried out under conditions identical to those described for the *S. typhi* hybrid strains. Control binding values for *E. coli* labeled DNA fragments incubated in the absence of unlabeled DNA were

TABLE 4. *Detection of E. coli-specific DNA in the genomes of S. typhi hybrid strains*

Source of unlabeled DNA	Estimated *E. coli* genetic substitution[a]	Relative binding of *E. coli* DNA		Estimated *E. coli* DNA substitution[b]
		60 C	75 C	
	%	%	%	%
S. typhi 643	0	43 ± 4	9 ± 3	0
S. typhi 643 *lac*$_3$	7–13	52 ± 2	16 ± 1	7
S. typhi 643 X30T	20–28	59 ± 1	32 ± 3	25
S. typhi 643 X30W	39–44	58 ± 5	38 ± 2	33

[a] The estimate of the degree of *E. coli* substitution was calculated on the basis of *E. coli* characters stably present in *E. coli* × *S. typhi* hybrid assuming uniform substitution of *E. coli* material into the genome of *S. typhi*. It was assumed that the chromosomes of *E. coli* K-12 and *S. typhi* 643 are both 2.5 × 10^9 daltons. The genetic analysis of these hybrids was presented in detail by Falkow, Rownd, and Baron (18).

[b] The estimate of the degree of *E. coli*-specific nucleic acid substitution into the *S. typhi* chromosome was calculated by subtracting the "background" reaction between *E. coli* and *S. typhi* at 75 C from the percentage of *E. coli* DNA bound by the *S. typhi* hybrid strains at this temperature. It was also assumed that the "background" binding is distributed at random along the *S. typhi* chromosome and that the genetic substitution replaces a proportional amount of this "background" reaction. All reactions were carried out with hydroxyapatite. *E. coli**/*E. coli* reactions at 60 and 75 C were arbitrarily designated 100%; all binding percentages are relative to these intraspecies reactions.

2.1% at 60 C and 1.5% at 75 C. The presence of F-*lac*$^+$ in *P. mirabilis* did indeed increase the relative reassociation of *E. coli* DNA and *P. mirabilis* DNA by about 2% and, concurrently, significantly increased the thermal stability of this reaction product (Table 3). *P. mirabilis* F-*lac*$^+$ formed duplexes with about 3% of the *E. coli* DNA, and the $T_{m(e)}$ of this reaction product was similar to that of reassociated *E. coli* DNA duplexes (Table 3).

Martin and Hoyer (40) used the ratio of binding in agar at different incubation temperatures to determine relationships between DNA preparations from different animals. The ratio of relative reassociation at 75 and 60 C, which we have called the thermal binding index (TBI), has been useful in gauging the presence or absence of highly related genetic material in interspecies DNA reassociation reactions. The TBI values of *E. coli* DNA with DNA from strains of *Salmonella*, *Aerobacter*, and *Proteus* species were all about 0.2 (Table 5), whereas that with *S. flexneri* DNA was 0.93. The increased amounts of *E. coli* material in *P. mirabilis* F-*lac*$^+$ and in the *E. coli-S. typhi* genetic hybrids were readily apparent from the TBI.

Conservation of ribosomal RNA genes. Although the degree of nucleic acid binding is a measure of overall relatedness between organisms, it is clear that different parts of the chromosome may not have evolved at the same rate. In several groups of bacteria, it has already been demonstrated that the DNA sequences that code for ribosomal RNA (rRNA) appear to be conserved in base sequence as compared with the rest of the genome (14, 15, 29, 42). Using rRNA (42) and using isolated and purified DNA sequences that code for rRNA (29), other investigators have shown that the rRNA cistrons in enterobacteria are more highly conserved and form more stable interspecies hybrids than does bulk DNA from these organisms. DNA sequences that code for soluble RNA also appear to be more highly conserved than the bulk of enterobacterial DNA (24). Table 6 summarizes similar data obtained with labeled 23*S* rRNA from *E. coli* reacted with unlabeled DNA molecules immobilized on nitrocellulose filters. In every case, the relative binding of *E. coli* 23*S* rRNA to DNA from a heterologous organism was greater than the relative binding of *E. coli* DNA or mRNA to DNA from the heterologous organism. When 23*S* rRNA was tested on DNA from organisms previously studied (42), the two sets of results were in excellent agreement.

Divergence of the lactose operon among enteric bacteria. In the near future, it should be feasible to study the distribution of other well-defined parts of the chromosome, rather than depending upon bulk mRNA or the entire DNA complement. DNA fractions isolated from intergenetic hybrids (23) or from synchronous populations (12) should be useful in carrying out such experiments. As a first approximation, pulse-labeled RNA was isolated from *E. coli* under conditions where there was a preferential synthesis of β-galactosidase (43). A portion of the bulk pulse-labeled RNA was incubated with filter-immobilized DNA from several enterobacteria for measurement of the overall relative binding of the RNA preparation. Another portion of the RNA preparation was repeatedly reacted with the DNA of an *E. coli* strain with a genetic deletion which included the entire lactose (*lac*$^+$) operon. The RNA that remained after reaction with the deletion strain (about 3% of the original

TABLE 5. *Thermal binding index*[a]

Nucleic acid reaction	Thermal binding index
*E. coli**/*S. typhimurium* LT2	.23
*E. coli**/*S. typhimurium* 7823	.24
*E. coli**/*S. typhi* 643	.21
*E. coli**/*A. aerogenes*	.22
*E. coli**/*P. mirabilis*-1	.17
*E. coli**/*P. mirabilis*-1 F-*lac*$^+$	.38
*E. coli**/*S. flexneri*	.93
*E. coli**/*S. typhi* 643 lac_3	.31
*E. coli**/*S. typhi* 643 X30T	.54
*E. coli**/*S. typhi* 643 X30W	.66

[a] The data used in this table were taken from the hydroxyapatite experiments shown in Table 3. Thermal binding index = relative binding at 75 C/relative binding at 60 C. Asterisks designate source of labeled DNA.

TABLE 6. *Relative binding of E. coli 23S RNA with the DNA of other enterobacteria*[a]

Source of unlabeled DNA	Relative binding of *E. coli* 23*S* RNA
	%
E. coli	100
A. aerogenes	80
S. typhi	79
S. marcescens	74
P. mirabilis	76
P. morganii	52
S. flexneri	92

[a] Membrane filters containing 100 μg of the indicated DNA were incubated with 30 μg of 23S ribosomal RNA (4.3×10^4 counts per min per μg) overnight at 75 C in 2 × SSC. After incubation, the samples were treated with ribonuclease (60 min; in 2 × SSC; at 26 C).

radioactivity) was presumed to be relatively enriched for *lac*+ mRNA and was tested for its binding to heterologous DNA species. In the main, the per cent relative binding with the *lac*+ mRNA fraction was lower than the binding of the bulk mRNA (Table 7). In the case of *S. flexneri* DNA, the relative binding decreased by 13%. A decrease was expected, since it has been reported that the *y* region of the *lac*+ operon is deleted in strains of *Shigella* species (31). The *lac*+ fraction did not react measurably with DNA from *P. mirabilis*, *P. morganii*, or *S. marcescens*. An unexpected finding was that the reaction with the *lac*+ RNA fraction was about 70% higher with *Salmonella* sp. (ser.) *ballerup* and about 10% higher with *S. typhimurium* than was the bulk mRNA reaction with DNA from these organisms. Most genetic data (45) suggest that the *lac*+ region is deleted from *S. typhimurium*. Several years ago, however, it was reported that *lac*+ variants of *S. typhimurium* could be selected (46). Similar mutants have been isolated in this laboratory and are currently being characterized genetically. Some preliminary evidence suggests that there may be at least part of the structural gene for β-galactosidase present in *S. typhimurium*. There is no evidence, however (on the basis of complementation with *lac*+ episomes) for functional *i* or *y* loci. A study of the *lac*+ region of *Salmonella* sp. (ser.) *ballerup* has not been attempted. No data have been obtained to support the experimental inference that the *lac*+ operon of *Salmonella* sp. (ser.) *ballerup* is essentially identical to that of *E. coli*, although the strain employed here fermented lactose slowly.

It should also be emphasized that the *lac*+ operon was only part of the genetic deletion in the strain employed in these experiments. Portions of the genome adjacent to the *lac*+ operon may account, therefore, for part (or all) of the observed reaction. The deletion, however, was not more than 2%, as determined by interrupted mating experiments. At any rate, it does appear that, assuming a common ancestor, particular regions of the genome of enteric organisms have diverged at quite different rates.

TABLE 7. *Binding of pulse-labeled E. coli "lac+" RNA to enterobacterial DNA*[a]

Source of unlabeled DNA on filter	Bulk mRNA		Adsorbed mRNA	
	Ribonuclease-resistant counts/min bound	Relative binding	Ribonuclease-resistant counts/min bound	Relative binding
		%		%
E. coli K-12 *lac*+	39,267	100	1,320	100
E. coli K-12 *lac*Δ	39,156	99	27	2
P. mirabilis	1,149	3	28	2
A. aerogenes	7,945	20	190	14
S. typhi	8,599	22	220	17
S. typhimurium	8,864	22	460	34
S. flexneri	32,149	82	910	69
S. marcescens	3,215	8	29	2
Bethesda-10	8,864	22	340	26
Salmonella sp. (ser.) *ballerup*	8,334	21	1,230	93
Salmonella sp. (ser.) *arizonae*	9,868	25	270	20
P. morganii	2,464	6	26	2
None	130	1	28	2

[a] See preparation of lactose mRNA in Materials and Methods.

DISCUSSION

We presume that the interactions we measure at the level of DNA reasonably reflect both genetic and evolutionary relationships among enterobacteria. If DNA from two organisms is unable to reassociate under ideal conditions, it is clear that these organisms are no longer related. The ability to form specific DNA duplexes between two organisms is evidence for relatedness.

The degree of interspecies DNA reassociation and the stability of these duplexes varies greatly as the experimental conditions are altered. The case of *E. coli* and *S. typhimurium* can be used to illustrate this point. Initially, the degree of DNA reactions between these organisms was determined by allowing a mixture of the two types of DNA, one of which was uniformly substituted with a heavy isotope, to reassociate (47). The presence of reassociated duplexes composed of one "light" strand and one "heavy" strand in CsCl density gradients was the criterion employed to determine DNA similarity. Under these experimental conditions duplex formation was not detected between DNA from *E. coli* and *S. typhimurium*. A more quantitative estimate of nucleic acid similarity between these organisms was obtained by measuring the binding of DNA fragments (as well as mRNA) to DNA immobilized in agar (33). Under these conditions, *E. coli* and *S. typhimurium* DNA showed approximately 70% relative relatedness. A more recent study (6), and our results obtained by use of hydroxyapatite, filter, and DNA-agar techniques, show that the relative relatedness of *E. coli* to *S. typhimurium* varied between 40 and 9% as the temperature was varied.

One should question (indeed, one must consider) just what a DNA reassociation experiment

implies and which is the "correct" degree of binding between two organisms? In fact, each of the results (as long as the reaction conditions are stringent to rule out nonspecific reassociation) is the "correct" answer for the criteria employed. It is simply that the percentage of DNA fragments bound in any experiment does not by itself yield enough information concerning the amount of paired bases present in the reassociated DNA duplexes.

The optimal temperature for DNA reassociation is approximately 30 C below the T_m of that DNA (37, 39). If we apply this rule to the enteric bacteria and to our data, then the optimal temperature for these reactions would be about 60 C. Yet, at this temperature, the reassociated duplexes formed between the DNA of *E. coli* and the DNA of several other enteric species had $T_{m(e)}$ values 13 to 14 C below that of the homologous reaction. It was not until these reactions were carried out at 75 C that the DNA heteroduplexes showed thermal stabilities close to that of reassociated *E. coli* DNA duplexes. In DNA reactions between *E. coli* and *S. flexneri*, the situation seems reasonably straightforward. The majority of their DNA was quite similar and formed thermally stable duplexes according to any criteria employed. Less than 15% of the DNA sequences of these organisms have diverged to a point where they cannot reassociate. Approximately an additional 5% of their DNA has diverged sufficiently to prevent interspecies duplex formation at the stringent 75 C criterion. The data clearly indicate that the vast majority of the nucleotide sequence of strains of *E. coli* and *P. mirabilis* or *S. marcescens* were not related by any criteria employed. Furthermore, with few exceptions, most nucleotide sequences held in common among enteric organisms show significant divergence, as judged by the low degree of interspecies duplex formation and by the decreased stability of interspecies duplexes.

There are no formal guidelines available with which to correlate genetic and molecular information to taxonomic groupings. Therefore, any attempt to equate the extent and stability of interspecies duplexes with lines of speciation would be somewhat premature and certainly arbitrary. A reasonable rule of thumb is to use less stringent, but specific, reaction conditions to detect overall relatedness between species. Highly related or specific interspecies DNA duplexes should be studied under stringent, in this case 75 C, reaction conditions. The data obtained in this study correlate well with existing taxonomic groupings. The exceptions which occur will, it is hoped, present the taxonomist with a new view.

The use of recombinant strains of *S. typhi* and *P. mirabilis* merodiploids allows an assessment of similarities or differences within specific portions of the genome. *Proteus* strains carrying specific regions of the *E. coli* chromosome (23) or specific episomes as in *P. mirabilis* F-*lac*$^+$ appear to be ideal for these experiments because of the very slight relatedness between parental *P. mirabilis* and *E. coli* in 75 C reactions.

The experiments carried out with isolated RNA from the lactose operon of *E. coli* provide a preliminary assessment of its distribution among several enterobacteria. It is not surprising that the distribution of this operon was found to be quite variable. The stability of these interspecies lactose operon duplexes remains to be tested. We feel that, even with the relatively crude techniques now at our disposal, nucleic acid reassociation experiments may be used to great advantage in determining relationships between specific genetic regions.

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