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Conservation of Transfer Ribonucleic Acid and 5S Ribonucleic Acid Cistrons in *Enterobacteriaceae*

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The genes for transfer ribonucleic acid (tDNA) and 5S ribonucleic acid (5S DNA) were isolated from the total deoxyribonucleic acid (DNA) of *Escherichia coli*. The relatedness of tDNA and 5S DNA from *E. coli* and other species of *Enterobacteriaceae* was determined by reassociation of the isolated genes labeled with $^{32}PO_4$ to unlabeled, unfractionated DNA. Double-stranded DNA was separated from unreacted DNA by hydroxyapatite chromatography. Thermal elution profiles were done to determine the amount of unpaired bases present in related DNA sequences. Relative to total DNA, both 5S DNA and tDNA were highly conserved throughout the *Enterobacteriaceae*, including the genera *Yersinia* and *Proteus*.

Present techniques enable us to isolate deoxyribonucleic acid (DNA) that specifies any gene or group of genes that can be selected for (7, 10, 18). Genes that specify structural and transfer ribonucleic acid (tRNA) were among the first to be isolated and characterized (8, 13, 16, 18). The genes for ribosomal ribonucleic acid (rDNA), 5S ribonucleic acid (5S DNA), and tRNA (tDNA) were of great interest because of their importance in protein synthesis and regulation. They were successfully isolated because their RNA transcription products were relatively simple to isolate in large quantities.

Now that almost any bacterial gene or gene region can theoretically be isolated, one can determine nucleotide sequence divergence of specific genes in a group of organisms. This has already been done with the lactose genes of *Escherichia coli* (2) and with the α-chain structural genes of the tryptophan synthetase of *E. coli* (14). The divergence of these genes is similar to the divergence of bulk DNA in species of *Enterobacteriaceae* (2; D. J. Brenner, unpublished observations). rDNA, however, is highly conserved among *Enterobacteriaceae* (2, 13, 17). In fact, rDNA from *E. coli* is more than 90% related to DNA from other enteric bacteria, including *Proteus mirabilis*, whereas bulk DNA from *E. coli* is less than 10% related to DNA from *P. mirabilis* (2, 13).

Since rDNA is highly conserved, several laboratories used rDNA interspecies hybridization to assess relatedness in groups of organisms where bulk DNA has extensively diverged (1, 9, 19-22). Only one attempt has been made to assess relatedness of tDNA genes between species (12). In our study, using isolated genes, we assessed interspecies relatedness of tDNA and 5S DNA among *Enterobacteriaceae*. Both 5S DNA and tDNA were highly conserved as compared to total DNA, although they were conserved to a lesser degree than rDNA genes.

MATERIALS AND METHODS

The isolation and purification of tRNA and 5S RNA were carried out as described by Doctor and Brenner (8). The isolation and purification of tDNA (6, 8) and 5S DNA (6) from *E. coli* by using multiple cycles of RNA-DNA hybridization on hydroxyapatite (HA) has been described, as has the isolation of total labeled or unlabeled DNA (3).

For hybridization, $^{32}PO_4$-labeled, sheared, denatured, unfractionated DNA (0.1 μg/ml), tDNA (0.001 μg/ml), or 5S DNA (0.001 μg/ml) was incubated with approximately 1 mg of unlabeled DNA per ml from *E. coli* B and other species. The incubations were carried out in 0.14 M phosphate buffer (PB; an equimolar mixture of Na_2HPO_4 and NaH_2PO_4; pH 6.8) for 16 h at either 60 or 75°C. After incubation, the reaction mixtures were passed through HA held at either 60 or 75°C and were equilibrated with 0.14 M PB plus 0.4% sodium dodecyl sulfate (4, 5). This procedure removed single-stranded DNA. Double-stranded DNA binds to HA in 0.14 M PB. It was eluted as double-stranded DNA with 0.4 M PB or as single-stranded DNA in a series of 0.14 M PB washes at increasing 2.5°C-temperature increments. This latter procedure is termed a thermal elution profile and was used to determine the thermal stability [$T_{m(e)}$] of the double-stranded DNA. $T_{m(e)}$, the temperature at

which one-half of the double-stranded DNA elutes from HA, is calculated for the homologous control reaction and for heterologous reactions. The difference in thermal stability, $\Delta T_{m(e)}$, is obtained by subtracting the $T_{m(e)}$ of the heterologous DNA duplex from the $T_{m(e)}$ of the homologous DNA duplex. The percentage of unpaired bases is obtained from the $\Delta T_{m(e)}$ by assuming that each 1°C decrease in $T_{m(e)}$ is caused by approximately 1% of the unpaired bases (15).

RESULTS

Isolated, labeled *E. coli* 5S DNA and tDNA were reacted with homologous and heterologous RNA preparations to determine the purity and specificity of these gene preparations (Table 1). Both tDNA and 5S DNA gave 90% or higher reassociation with homologous RNA and 2% or less binding to HA in controls without RNA. tDNA gave a slight reaction with *E. coli* 5S RNA (6%) and no reaction with rat liver tRNA, yeast tRNA, or *E. coli* rRNA. 5S DNA slightly reacted with tRNA from *E. coli* (10%) but not with rat liver tRNA. As expected, there was considerable reaction (50%) between 5S DNA and unfractionated *E. coli* rRNA that contained 5S RNA.

Labeled, isolated tDNA and 5S DNA were reacted with total DNA from representative species to determine the extent of conservation of these genes in *Enterobacteriaceae*. Labeled DNA from which tDNA and 5S DNA had been removed during the gene isolation procedure (6, 8) was used to assess the relatedness of bulk DNA in these species. In 60°C reactions (Table 2), tDNA and 5S DNA from *E. coli* were 60% or more related to all species tested, whereas bulk *E. coli* DNA gave relatedness values as low as 7%. The percentage of unpaired bases within related sequences (15) also was significantly less in heterologous tDNA and 5S DNA duplexes than in heterologous bulk DNA duplexes. A more emphatic demonstration of the conservation of tDNA and 5S DNA was obtained in 75°C reactions, where only highly complementary DNA sequences could form stable duplexes (Table 2). The relatedness between bulk *E. coli* DNA and DNA from all other species except *Shigella flexneri* was between 1 and 15%, but tDNA and 5S DNA were 40 to 65% related among these species. Thermal elution profiles of representative tDNA duplexes obtained in 60 and 75°C reactions are shown in Fig. 1. In 60°C reactions, all heterologous reactions contain duplexes that are mainly stable above 80°C. This is in marked contrast to thermal elution profiles obtained from bulk DNA reactions between *E. coli* and *Salmonella typhimurium* or *P. mirabilis*, where most DNA elutes below 80°C and very few nucleotide sequences can form duplexes in 75°C reactions (3). This relationship is graphically shown in Fig. 2 where relative binding of the bulk DNA and tDNA of *E. coli* in heterologous 60 and 75°C reactions is compared. Bulk DNA reactions fall into three categories. (i) Organisms to which *E. coli* is highly related exhibit a small decrease in binding at 75°C as compared to that at 60°C (*S. flexneri*). (ii) Moderately related organisms (*S. typhimurium, Enterobacter aerogenes*) exhibit a 2- to 4-fold drop in binding at 75°C as compared to that at 60°C, and (iii) distantly related organisms (*Enterobacter hafniae, Yersinia pestis, P. mirabilis*) usually exhibit 5- to 10-fold decreases in binding at 75°C. tDNA binding is much more stable at 75°C, exhibiting less than a 2-fold decrease, even among the most distantly related species within *Enterobacteriaceae*.

TABLE 1. *5S DNA and tDNA hybridization reactions*[a]

Source of RNA	Hybridization reactions (%)	
	5S DNA	tDNA
None	2.0	1.5
E. coli 5S RNA (50 μg/ml)	91	6
E. coli tRNA (50 μg/ml)	10	94
Rat liver tRNA (200 μg/ml)	1.5	1.0
E. coli rRNA (200 μg/ml)	50	2.0
Yeast tRNA (200 μg/ml)		0

[a] A 0.001-μg portion of DNA in 0.12 M PB, either alone or with RNA at the indicated concentration, was denatured by heating at 100°C for 4 min, cooling quickly to 60°C, and incubating for 15 min (*E. coli* tRNA and 5S RNA) or 2 h (rat liver tRNA, rRNA, no RNA). The samples were then assayed at 60°C on HA equilibrated with 0.12 M PB plus 0.4% sodium dodecyl sulfate.

DISCUSSION

Both tDNA and 5S DNA cistrons are highly conserved as compared to total DNA among enteric bacteria. This fact can be quite useful in assessing evolutionary divergence in distantly related bacteria and also in establishing a system of classification based on gene similarity. There are now four levels at which DNA relatedness can be measured: rDNA, 5S or tDNA, total DNA, and DNA from genes that specify a specific enzyme, such as tryptophan synthetase. rDNA is the DNA that is conserved to the highest degree. It can, therefore, be used to group organisms at the family or suprafamily level. tDNA or 5S DNA relatedness can be used to separate these organisms at the family or tribe level. Species separation is best obtained

TABLE 2. *Conservation of tRNA cistrons and 5S RNA cistrons*

Source of unlabeled DNA	Source of labeled DNA					
	Unfractionated *E. coli* B DNA		*E. coli* B tRNA cistrons		*E. coli* B 5S RNA cistrons	
	% Relating binding	% Unpaired[a] bases	% Relative binding	% Unpaired bases	% Relative[b] binding	% Unpaired bases
60°C reactions						
E. coli B	100[a]		100[b]		100[b]	
E. coli O128	97		100			
E. coli O7	91		100			
E. coli AD-O6	88		96			
S. flexneri	84	2.4	86	2.0	82	3.7
S. typhimurium	46	12.3	75	4.0	79	4.4
E. aerogenes	46		78			
E. hafniae	19		67			
P. mirabilis	7	13.8	67	8.0	71	5.0
Y. pestis	13		63			
75°C reactions						
E. coli B	100[c]		100		100	
E. coli O128						
E. coli O7						
E. coli AD-O6						
S. flexneri	81	1.3	82	1.4	76	1.3
S. typhimurium	15	3.3	65	2.6	66	3.2
E. aerogenes	15		66			
E. hafniae	3		56			
P. mirabilis	1		42	4.8	42	4.1
Y. pestis	1		43			

[a] The percentage of unpaired bases is calculated on the assumption that a 1°C decrease in the $T_{m(e)}$ of a heterologous DNA duplex compared to that of a homologous DNA duplex is caused by each 1% of unpaired bases within the heterologous duplex (15). The $T_{m(e)}$ of homologous *E. coli* B duplexes was approximately 90°C in both 60 and 75°C reactions.

[b] The average actual reassociation of homologous *E. coli* B DNA at 60°C was 90%.

[c] The average actual reassociation of homologous *E. coli* B DNA at 75°C was 83%.

by determining total DNA relatedness. Subspeciation may best be determined by testing relatedness within specific genes common to a given species. Strict quantitative rules are not yet available; however, bulk DNA relatedness is already in extensive use for speciation, and rDNA is frequently used to determine relatedness among distantly related bacteria.

It must be remembered that the observed degree of relatedness is dependent upon many parameters, some of which are incubation temperature, salt concentration, DNA concentration, time of incubation, and DNA fragment size. These are especially important in studying organisms that are not 80% or more related (1–3). The reaction conditions must be carefully considered before comparing results from different laboratories. Our comparisons to the results of DNA studies by Kohne (13) are valid, because the methods used were identical to those of this study, except for a small difference in salt concentration. The other studies (2, 14, 17) to which the present data are compared used filter hybridization systems at a somewhat lower salt concentration (0.36 M sodium compared to 0.42 M sodium) and a somewhat higher optimal incubation temperature (65°C compared to 60°C). These two differences tend to offset one another, since high salt stabilizes the duplexes and high temperature precludes the formation of some of the thermally unstable DNA duplexes. Thus, these comparisions are meaningful, if not exact.

We know of no proposed theory to explain the fact that the tDNA and 5S of enteric bacteria, though conserved, show more divergence than rDNA. As an integral part of the protein-synthesizing system, it is not surprising that rDNA is highly conserved. 5S DNA is also intimately associated with the ribosomes, and tDNA is certainly essential in protein synthesis. Why, then, do these molecules exhibit greater divergence than rDNA?

We propose that tDNA and 5S DNA may be conserved to almost the same degree as rDNA and that the higher extent of divergence seen is due in a large part to spacer regions between the tDNA and 5S DNA cistrons. It is well known from both genetic and hybridization studies that tDNA cistrons are clustered in *E.*

coli and presumably in all enteric bacteria (see reference 11). Fournier et al. (11) performed the following experiment to determine whether tDNA cistrons are contiguous or whether they are separated by spacer DNA. ^{32}P-labeled tDNA (an average single-strand fragment size with a molecular weight of 125,000), which is approximately 4.5 times as large as a tRNA molecule, was hybridized to ^{35}S-labeled tRNA (6). The hybrids were treated with a single-strand-specific endodeoxyribonuclease. If the hybridized segments were contiguous, without spacer sequences, the resulting hybrids would contain three or four tRNA molecules. If the hybridized segments were separated by single-stranded gaps, the endonuclease treatment would generate hybrids that contain only one tRNA molecule and its corresponding cistron. After endonuclease treatment, 30% of the DNA was digested, and the ratio of RNA to DNA in the remaining hybrid was approximately 1.0. The molecular weight of these hybrids was approximately 60,000, the value expected for a single tRNA and its corresponding cistron. These results are consistent with the presence of approximately 20% of the DNA between clustered tDNA cistrons.

Although comparable experiments were not done for 5S DNA, it is assumed that there is

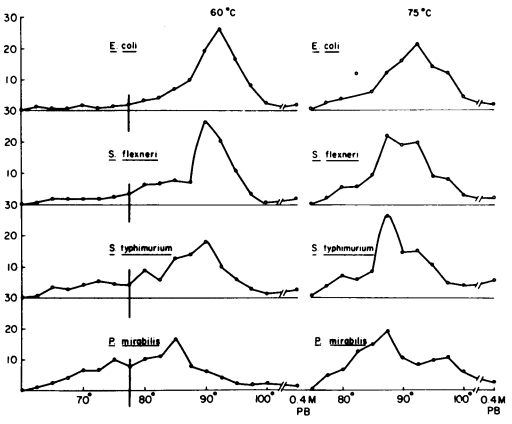

FIG. 1. *Thermal elution profiles from E. coli tDNA duplexes. $^{32}PO_4$-labeled tDNA from E. coli (0.001 μg/ml) and 1,000 μg of unlabeled DNA per ml in 0.14 M PB were incubated at either 60 or 75°C for 16 h. The reaction mixtures were passed through HA and washed with four 15-ml portions of 0.14 M PB to remove single-stranded DNA. The reassociated DNA was denatured and eluted in a series of 0.14 M PB washes at increasing 2.5°C-temperature increments to 100°C, followed by a wash with 0.4 M PB. The ordinate represents the percentage of counts bound to the eluted HA. The abscissa represents the temperature (°C). Relatedness data are shown in Table 2.*

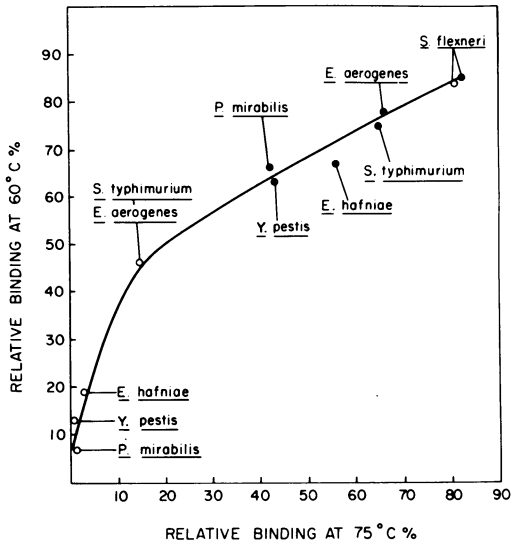

FIG. 2. *Comparison of binding in 60 and 75°C reactions. Open circles are bulk DNA reactions, and closed circles are tDNA reactions. These data were taken from Table 2 (60°C reaction). In all cases, the homologous reaction was E. coli B DNA.*

spacer DNA present between the 5S DNA cistrons. Since 5S RNA is approximately a 25% larger molecule than tRNA, one would expect somewhat less spacer DNA per DNA fragment with a molecular weight of 125,000 than is present on the tDNA fragment. We hypothesize that tDNA and 5S DNA may be conserved to almost the same extent as rDNA, but that the spacer DNA is species or group specific and diverges at a rate close to that of total DNA. Spacer DNA is probably located next to rDNA cistrons. Its effect is not seen, because rDNA cistrons with molecular weights of 500,000 to 1,000,000 are among the largest cistrons in bacteria, and, therefore, most DNA fragments with a molecular weight of 125,000 contain no spacer DNA.

It is also possible that the divergence of tDNA and 5S DNA over that of rDNA is also due entirely, or in part, to divergence within the cistrons themselves or to diverged DNA other than spacer DNA. One way to test this is to use single-stranded DNA fragments with molecular weights of about 25,000. Unfortunately, it is extremely difficult to shear DNA to this size using pressure or sonic oscillation.

One can test whether spacer DNA or intracistron divergence is responsible for any or all of the divergence of tDNA and 5S DNA among species of *Enterobacteriaceae*. Spacer DNA should not be transcribed; therefore, hybridiza-

tion of labeled tRNA and 5S RNA to DNA from various species would test this hypothesis. Spacer DNA is not the cause of divergence if the amount of divergence for tRNA and 5S RNA is similar to that for tDNA and 5S DNA. If the interspecies RNA-DNA reactions exhibit significantly less divergence than the comparable interspecies DNA-DNA hybridizations, then it is likely that spacer DNA is responsible for much of the divergence.

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