Use of a Single-Strand Specific Nuclease for Analysis of Bacterial and Plasmid Deoxyribonucleic Acid Homo- and Heteroduplexes

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Bacterial and plasmid homo- and heteroduplexes have been analyzed with a single-strand specific endonuclease, S1, of *Aspergillus oryzae*. Under appropriate assay conditions, there was a high degree of correlation between the degree of deoxyribonucleic acid (DNA)-DNA homoduplex formation assessed by the S1 endonuclease and by hydroxyapatite (HA). Heteroduplexes which contain extensive regions of polynucleotide sequences in common are similarly recognized by the S1 endonuclease and HA. In instances where there is little or imperfect complementarity between heterologous DNA strands, the S1 endonuclease and the HA method give slightly different estimates. From DNA duplex thermal stability experiments assayed with the S1 endonuclease, there is preliminary evidence that well-matched sequences identified by the enzyme are not similarly recognized by HA. The assay of homo- and heteroduplexes with the S1 endonuclease permits an accurate, reproducible and rapid determination of polynucleotide sequence relationships and may be seriously considered as a method of choice for survey work and for investigations which require a large number of DNA-DNA hybridization assays.

The dissociation of deoxyribonucleic acid (DNA) strands and their specific reannealing with single-stranded nucleic acid from a homologous or heterologous source is the basis for all nucleic acid relationship studies. Over the past few years most investigators have utilized immobilized membrane filter-bound DNA or columns of hydroxyapatite (HA) for the separation of reassociated nucleic acid hybrid duplexes from unreacted DNA (3, 7, 8).

A number of enzymes have been described which specifically degrade single-stranded DNA at a much higher rate than double-stranded DNA. Obviously, an enzyme of this type would be most useful in performing DNA-DNA duplex studies. Initially, Ando (1) reported an endonuclease, called S1, which is easy to prepare from a commercially available crude extract of *Aspergillus oryzae*. Endonuclease S1 has been further studied by Sutton (14), who applied its selective activity on single-stranded DNA to the examination of the kinetics of reassociation of

DNA from eukaryotes. Other investigators have employed preparations of this enzyme to determine the length distribution of repetitive DNA sequences (D. E. Graham, M. Henerey, and R. J. Britten, unpublished data), to estimate the double-helical content in single-stranded nucleic acids (12), and to detect oncornavirus ribonucleic acid (RNA) in tumor virus infected cells (15).

In the present communication, we report the use of this enzyme for the determination of nucleotide sequence relatedness between homologous and heterologous bacterial and plasmid DNA. Our results show that DNA-DNA hybridization can be accurately assayed with endonuclease S1 and that the results are highly correlated with results obtained with the widely employed HA method.

MATERIALS AND METHODS

Preparation of the S1-endonuclease. Crude α-amylase powder from *A. oryzae* (Sigma Chemical Co.,

St. Louis; Mo.) was used as a source of endonuclease S1 and was stored at -20 C until used. The enzyme was partially purified by a one-step fractionation procedure on a diethylaminoethyl-cellulose column (Whatman DE-52, preswollen), essentially as described by Sutton (14). Fractions eluted at 0.1 M NaCl which exhibited (in 5-μliter samples) more than 90% digestion of single-stranded ^{14}C-thymine λ phage DNA, but less than 5% activity on double-stranded DNA, were pooled and kept at -20 C in 25% (wt/vol) glycerol.

Nuclease assay. The standard reaction mixture in a total volume of 1 ml consisted of a radioactive DNA substrate in: 0.1 mM $ZnSO_4$; 0.15 M NaCl; 30 mM sodium acetate buffer, pH 4.5 $\pm$ 0.1. Where indicated, the reaction mixture was supplemented with 20 μg of calf thymus DNA (Sigma Chemical Co., type V) which was sonically treated and heat denatured. After the addition of S1 nuclease (1-50 μliters/ml), reaction mixtures were incubated at 50 C for 20 min. The reaction was terminated by chilling in an ice bath, and any remaining DNA duplexes were precipitated by the addition of 0.25 ml of ice-cold 20% trichloroacetic acid. The trichloroacetic acid precipitate was collected on membrane filters (type HA, Millipore Corp., Watertown, Mass.) which were dried at 70 C and counted in a Packard liquid scintillation spectrometer, model 3200. One unit of enzyme activity was defined as the amount of enzyme that could solubilize 1% of the initial trichloroacetic acid-precipitable counts (0.1 μg of denatured lambda phage ^{14}C-DNA per ml) in the standard conditions plus 20 μg of sonically treated, heat-denatured calf thymus DNA per ml.

Preparation of DNA. Lambda phage DNA labeled with ^{14}C-thymine (specific activity 25 mCi/mmol) was prepared as described by Korn and Weissbach (10). ^{32}P-labeled and unlabeled bacterial DNA was prepared as described previously (3). The DNA was sheared in a pressure pump at 50,000 psi to an average molecular weight of 2.5 $\times$ 10^6 and filtered through a cellulose acetate filter (Metricel, 0.45 μm, Gelman Instrument Co., Ann Arbor, Mich.). Labeled DNA fragments were denatured by heating and were passed through an HA column equilibrated at 60 C in 0.14 M phosphate buffer (PB, an equimolar mixture of NaH_2PO_4 plus Na_2HPO_4) plus 0.4% sodium dodecyl sulfate (SDS). Labeled material bound to the column under these conditions was discarded. This procedure decreased the zero-time binding (label bound to HA immediately after the DNA is denatured) to 2% or less (3). The specific activity of the ^{32}P-DNA was 0.2 $\times$ 10^5 to 1.5 $\times$ 10^5 counts per min per μg.

^{3}H-thymine-labeled DNA of the I-like R factor, R-144, was prepared by isolating covalently closed circular molecules from a culture of *Escherichia coli* J5-R144. The circular molecules were separated from chromosomal DNA by the Brij 58 lysis technique of Clewell and Helinski (6) and further purified by banding in a CsCl-ethidium bromide gradient as described previously (13). Purified, covalently closed circular molecules (as judged by sedimentation in neutral and alkaline sucrose gradients) were extracted with isopropanol to remove the ethidium bromide, and then dialyzed against 0.14 M PB.

The plasmid DNA was sonically treated to an approximate molecular weight of 2.5 $\times$ 10^5, denatured, and passed through an HA column as described above. The specific activity of the ^{3}H-thymine R144 DNA was 5 $\times$ 10^5 to 1 $\times$ 10^6 counts per min per μg.

Preparation of homologous and heterologous DNA-DNA duplexes for assay. All DNA was exhaustively dialyzed against distilled water and then 0.42 M NaCl before use. Homologous and heterologous bacterial DNA duplexes were prepared by incubating 0.1 μg of sheared, denatured ^{32}P-labeled DNA from *E. coli* B with 150 μg of sheared, denatured, unlabeled DNA from *E. coli* B, *E. coli* K-12, *Shigella flexneri* 24570, *Salmonella typhimurium* LT2, *Enterobacter liquifaciens*, *Proteus mirabilis* 1, and *P. mirabilis* PM-1 Flac$^+$. The source of these cultures has been described (2, 3).

Plasmid DNA duplexes were prepared by incubating 0.002 μg of sheared, denatured, ^{3}H-labeled DNA from R144 with 150 to 300 μg of sheared, denatured DNA per ml from the *E. coli* J5-F$^-$ strain or strains of *E. coli* J5 harboring R144; ColI; the F-like R factor, R1; the I-like transfer factor, JR66A; the N-like R factor, N3; the W-like R factor, Sa; the Ent plasmid P307 (9; Guerry et al., manuscript in preparation).

All DNA reassociation reactions were carried out at both 60 and 75 C in 0.42 M NaCl to a C_0t (5) of about 100 for the unlabeled bacterial DNA component.

Parallel reassociation reactions were assayed on HA using a batch method (4), and by the nuclease assay to determine the extent of DNA duplex formation.

RESULTS

Figure 1 shows the activity of partially purified endonuclease S1 on native and denatured λ ^{14}C-DNA. Under the assay conditions, the enzyme specifically degrades about 95% of the single-stranded material, while only about 5% of the double-stranded DNA is solubilized. As reported by others (1, 15), we found that the activity of the S1 endonuclease was optimally stimulated by Zn^{2+} ions and was most active at a pH of 4.5; at pH 6.4 activity decreased 50-fold. The enzyme was relatively insensitive to the ionic strength of the assay mixture. Optimal enzyme activity was measured at about 0.1 M NaCl. However, in our hands, the partially purified enzyme preparations at 0.1 M NaCl slowly degraded double-stranded DNA. Subsequently, we found that the effect on double-stranded DNA could be maintained at the 5% level or less by increasing the NaCl concentration in the assay mixture to about 0.15 to 0.2 M without significantly affecting the activity on single-stranded DNA.

Initial experiments employing the enzyme to assess the degree of DNA duplex formation between heterologous DNA samples often gave highly variable results. For example, the degree of DNA duplex formation between ^{3}H-labeled R-144 DNA and the DNA of *E. coli* J5-F$^-$ was

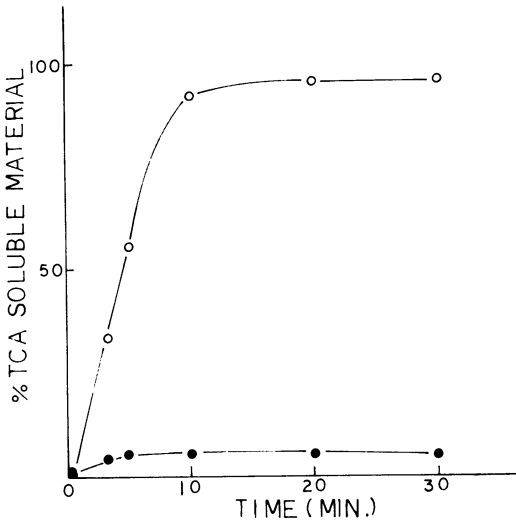FIG. 1. *Action of endonuclease S1 upon denatured and native* λ *phage* [14]*C-DNA. Five-microliter amounts (95 U) of S1 endonuclease, prepared as described in Materials and Methods, were added to tubes containing 0.1 mM ZnSO₄, 0.2 M NaCl, 30 mM sodium acetate buffer (pH 4.5), plus 20 µg of sonically treated, denatured calf thymus DNA per ml, and 0.1 µg of either native or denatured (5 min at 100 C) λ phage* [14]*C-DNA (10,000 counts/min). At the end of the indicated times, reactions were stopped by icing and then precipitated by adding 0.25 ml 20% trichloroacetic acid and counted. Symbols:* ●, *native* λ *phage* [14]*C-DNA;* O, *denatured* λ*-phage* [14]*C-DNA.*

consistently measured at $9 \pm 1.0\%$ by the HA procedure. In contrast, various preparations of the *E. coli* J5-F⁻ DNA exhibited widely differing levels of apparent reassociation with R144 DNA when measured by the enzyme procedure. These erratic results were eventually traced to the presence of relatively small amounts of SDS, PB, or chelating agents present in some DNA preparations as a consequence of purification procedures. Thus, endonuclease S1 activity is very sensitive to contaminating agents which may influence the Zn^{2+} concentration and/or the pH of the reaction. As reported by M. Vogt (15), we found that sodium phosphate buffer (pH 4.6) significantly inhibits S1 nuclease even at low concentrations. The influence of SDS was assessed by reconstruction experiments which showed, for example, that the reaction between R144 and *E. coli* J5-F⁻ DNA was raised from a "true" value of 8.6% to an apparent value of 18% at concentrations of 0.05% SDS, and was over 70% at concentrations of 0.1% SDS. It was, therefore, quite important to exhaustively dialyze all DNA preparations prior to their assay with endonuclease S1.

The action of S1 nuclease upon denatured DNA is strongly dependent upon DNA concentration. At very low concentrations of ^{32}P-labeled, sheared, denatured DNA from *E. coli* B (0.01-0.02 µg/ml), the amount of DNA hydrolyzed by the enzyme was only about 75%. However, after sheared, denatured calf thymus DNA (carrier) was added, the enzyme exhibited increasing activity on the labeled DNA up to a carrier concentration of 20 µg/ml, so that 90 to 92% of the denatured DNA was rendered trichloroacetic acid soluble (Fig. 2). The "core" of single-stranded DNA resistant to endonuclease S1 (8-10%) could not be reduced further under our experimental conditions.

The small amount of double-stranded hydrolyzing activity in the partially purified S1 preparation was also affected by the addition of added carrier DNA (9% hydrolysis in the absence of carrier DNA to 5% hydrolysis in the presence of 20 µg of carrier DNA per ml). The precise role of the added carrier DNA has not
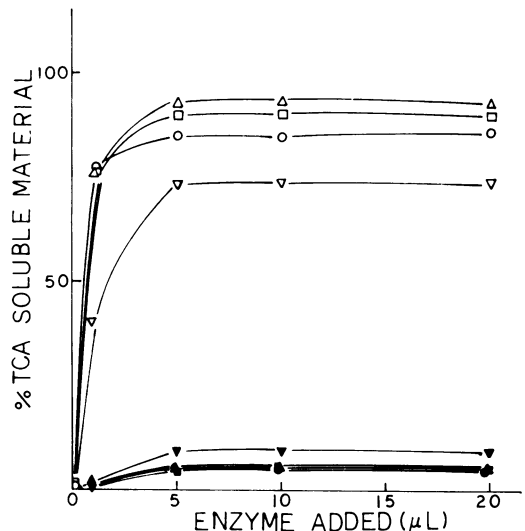

FIG. 2. *Action of endonuclease S1 upon denatured and native* ^{32}*P-labeled E. coli B DNA as a function of carrier DNA concentration. A 0.1-µg amount (20,000 counts per min per µg) of* ^{32}*P-labeled E. coli B DNA per ml was denatured by heating. Samples (0.3 ml) were assayed with increasing amounts of endonuclease S1 (1 µliter corresponds to about 19 U of nuclease) in the presence of different quantities of sonically treated, denatured calf thymus DNA. Control reactions using native* ^{32}*P-labeled DNA were performed similarly. Open symbols, denatured* ^{32}*P-labeled E. coli B DNA (0.03 µg/ml); closed symbols, native* ^{32}*P-labeled E. coli B DNA (0.03 µg/ml). Symbols:* ▽▼, *no carrier DNA;* O ●, *1 µg of carrier DNA per ml,* □ ■, *10 µg of carrier DNA per ml;* △ ▲, *20 µg of carrier DNA per ml.*

been established. It was determined, however, that neither native DNA nor unsheared, denatured DNA could substitute efficiently for the sheared, denatured product. The same effect has been reported by Sutton (14).

Having established reproducible conditions to distinguish between single-stranded and double-stranded DNA with endonuclease S1, we turned our attention towards the action and specificity of this enzyme for reassociated DNA of homo- and heteroduplexes. Figure 3 shows the action of increasing amounts of endonuclease S1 on *E. coli* B homoduplexes and *E. coli* B-*P. mirabilis* heteroduplexes as a function of carrier concentration. The reassociation reactions were carried out at 60 C as described in Materials and Methods, and samples of the reassociation mixture were added to assay mixtures containing varying amounts of enzyme and carrier DNA. The control reaction consisted of labeled DNA incubated in the absence of unlabeled DNA. The total amount of radioac-

FIG. 3. *Action of endonuclease S1 upon reassociated homologous and heterologous enterobacterial DNA hybrids. Samples (0.3-0.5 ml) of homologous (E. coli B-E. coli B) and heterologous (E. coli B-P. mirabilis 1) DNA-DNA duplex formed after 16 h of incubation at 60 C in 0.42 M NaCl were assayed in the presence of increasing amounts of S1 endonuclease (1 μliter corresponds to about 19 U of nuclease) and sonically treated, denatured calf thymus DNA. Open symbols, heterologous DNA hybrid; closed symbols, homologous DNA hybrid. Symbols: ▽ ▼, no carrier DNA; ○ ●, 1 μg of carrier DNA per ml; □ ■, 10 μg of carrier DNA per ml; △ ▲, 20 μg of carrier DNA per ml. The values shown are neither normalized nor corrected for the background resistant to S1 endonuclease.*

tivity in each sample was determined by diluting a sample into an assay tube devoid of enzyme. After incubation, only 15 to 23% of the label was hydrolyzed by S1 endonuclease in the *E. coli* B-*E. coli* B reaction, i.e., there was 77 to 85% homoduplex formation (Fig. 3, plateau of the curves). The degree of homoduplex formation as measured by the S1 nuclease assay was rather minimally affected by added carried DNA, since an identical sample of the reassociation mixture assayed by the HA method showed 88% homoduplex formation. In reassociated *E. coli* B-*P. mirabilis*, from 73 to 85% of the DNA was hydrolyzed by the S1 nuclease, depending upon the amount of carrier DNA, i.e., there was 15 to 27% heteroduplex formation. In this case the added carrier strongly affected the sensitivity of the heteroduplex to S1 endonuclease activity. Corrected for the background S1 resistant core and normalized to the *E. coli*-*E. coli* homoduplex value, the degree of polynucleotide sequence relationship between *E. coli* and *P. mirabilis* was 3 to 5% in the presence of 20 μg of carrier DNA per ml and 15% in the absence of the carrier DNA. An identical sample of the *E. coli*-*P. mirabilis* reassociation mixture assayed by the HA method showed 6 to 8% heteroduplex formation (normalized to the *E. coli*-*E. coli* reaction). Thus, even in the presence of a large amount of unlabeled reassociated DNA in enzyme assay mixtures, it was necessary to include added carrier DNA to ensure efficient S1 endonuclease activity. In all subsequent experiments, therefore, 20 μg of sonically treated and denatured calf thymus DNA per ml was added to the enzyme reaction, with at least 95 U of nuclease (5 μliters of our preparation) per ml of reaction mixture.

Table 1 shows the extent of DNA-DNA duplex formation at 60 or 75 C as estimated by the endonuclease S1 assay method and the HA batch procedure. There was an obvious high degree of correlation between results obtained at 60 C with both procedures. Moreover, the S1 endonuclease was sufficiently specific to consistently detect the small amount of additional homologous DNA present in *P. mirabilis* carrying an *E. coli* plasmid. The results obtained at 75 C with the two procedures were more divergent, particularly with regard to DNA pairs which share relatively few nucleotide sequences in common. It has been generally assumed that the reduction in heteroduplex formation at 75 C compared to 60 C measured with HA reflected an increased discrimination of imperfectly matched complementary strand pairs (2, 5, 11). The values of heteroduplex formation measured by the S1 endonuclease assay
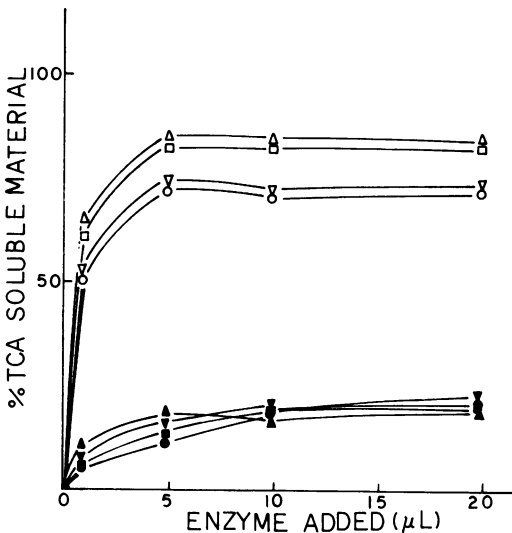
TABLE 1. *Action of nuclease S1 on reassociated bacterial DNA*[a]

Reaction[b]	Relative reassociation at 60 C (%)		Relative reassociation at 75 C (%)	
	S1	HA	S1	HA
E. coli B*/*E. coli* B	100	100	100	100
E. coli B*/*E. coli* K-12	89	94	98	88
E. coli B*/*S. flexneri* 24570	65	76	68	70
E. coli B*/*S. typhimurium* LT2	37	44	20	11
E. coli B*/*E. liquifaciens*	20	19	13	4
E. coli B*/*S. marcescens* SM6W2	21	24	13	4
E. coli B*/*P. mirabilis* FLAC	6	8	16	3
E. coli B*/*P. mirabilis* I	3	6	5	2

[a] A 0.1-μg amount (20,000 counts per min per μg) of sheared, denatured ^{32}P-labeled *E. coli* B DNA was incubated with 150 μg of unlabeled, sheared, denatured DNA per ml from each of the indicated organisms. DNA reassociation was performed in 0.42 M NaCl at either 60 or 75 C for 16 h. At the end of the incubation period, samples were assayed on HA or by the S1 endonuclease assay as described in Materials and Methods. The homologous *E. coli* B reaction as determined by the HA method was about 90% at 60 C and 82% at 75 C. These values were corrected for the zero time label bound to the HA column immediately after the DNA has been denatured (0.5–2.5%), and all other reactions were normalized to these values taken as 100%. The homologous reaction was estimated by the S1 nuclease by determining the percentage of trichloroacetic acid-insoluble counts remaining after incubation of a 0.3-ml sample of the reassociation mixture in the standard conditions, plus 20 μg of carrier DNA per ml and 95 U (5 μliters) of enzyme per ml of reaction mixture. This homologous reaction contained approximately 85% trichloroacetic acid-insoluble counts/min for the 60 C homoduplex and 89% for the 75 C homoduplex. These values were corrected for an 8 to 10% background of single-stranded DNA resistant to endonuclease S1 after reassociation in the same conditions. These corrected values were taken as 100%, and all other reassociation values were similarly corrected and normalized to this figure.

[b] Asterisk indicates source of labeled DNA.

method were reduced at 75 C as compared to 60 C in only a few instances. Moreover, this reduction, when present, was never so marked as that observed with the HA method. The significance of these findings is uncertain at present except to show that the HA method and enzyme treatment apparently discriminate against imperfectly base-paired structures in different ways.

The extent of base pair mismatching of DNA-DNA duplexes has been assessed by determin-

ing their thermal stability T_m (the temperature at which 50% of the reassociated DNA no longer binds to HA or becomes hydrolyzable by the S1 endonuclease). In our laboratory, a decrease in T_m of 1 C in a heteroduplex relative to the T_m of the homologous DNA-DNA duplex has been taken to indicate 1% mismatched base pairs (11). Thermal stability profiles of 60 C reassociated *E. coli* B-*E. coli* B homoduplexes and 60 C *E. coli* B-*S. typhimurium* DNA heteroduplexes, as assayed by the S1 endonuclease, are shown in Fig. 4. It can be seen that the T_m (80 C) of the heteroduplex was some 10 C lower than that of the *E. coli* B homoduplex (T_m, 90 C). This result is in good agreement with the 13
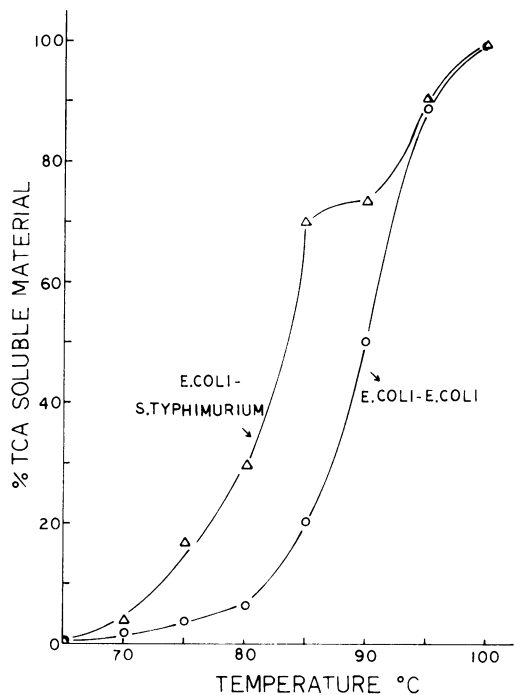

FIG. 4. *Thermal stability profiles for 60 C E. coli-E. coli and E. coli-S. typhimurium DNA duplexes. After reassociation, samples were assayed for the amount of trichloroacetic acid-precipitable counts after incubation with endonuclease S1 (95 U per ml of reaction mixture). The thermal stability of the hybrids was assayed by heating and determining at each temperature (in 0.3-ml samples) the amount of DNA hydrolyzable by the S1 endonuclease in the standard conditions plus 20 μg of calf thymus DNA per ml using 95 U (5 μliters) of enzyme per ml of reaction mixture. Values in the graphs were obtained by subtracting the amount of trichloroacetic acid precipitable counts present immediately after reassociation and incubation with the S1 endonuclease. Symbols:* $\triangle$, *E. coli-S. typhimurium LT2;* $\bigcirc$, *E. coli-E. coli B.*

C difference in T_m obtained previously on HA for the same DNA pairs (3). The data with both techniques indicated, therefore, that roughly one base pair in ten of the heteroduplex was mismatched following reassociation at 60 C. This average divergence in base pairing was not random, however, as can be seen by examination of the melting profile of the *E. coli* B-*S. typhimurium* DNA hybrid in Fig. 4 which clearly shows that a significant proportion of the heteroduplex sequences has the same thermal stability as that of the *E. coli* B homoduplexes. The results are similar when the thermal stability of the homologous and heterologous duplexes are measured on HA (3).

When similar thermal stability profiles were performed with the same DNA pairs reassociated at 75 C (Fig. 5), the T_m of both the homo- and heteroduplexes were essentially the same (90 C). Similar results were obtained with HA (3). These data suggest, therefore, that the endonuclease and HA recognized similar structures with a high degree of base-pairing specificity even though the absolute amount of DNA recognized as double-stranded was higher by

the S1 assay than by the HA method when applied to 75 C reassociated DNA (see Table 1). Thus, despite the observed differences in the extent of duplex formation, it is, nonetheless, clear that the determination of heteroduplex formation by the S1 endonuclease yields the same relative order of divergence (percentage of unpaired bases in the sequences held in common) between DNAs of heterologous origin as does the HA method. Table 2 reinforces this contention by showing the degree of nucleotide sequence similarity between the DNA of the R-factor R144 and other plasmids. These reactions, carried out at a reassociation temperature of 75 C, showed a very high degree of correlation between the two methods. By both methods the data showed that R144, a member of plasmid compatibility group I, has a high level of nucleotide sequence similarity with other group I plasmids such as ColI and JR66a but is almost totally unrelated to plasmids of other compatibility groups. This appears to be a rather general phenomenon (Guerry et al., manuscript in preparation). It also should be noted that DNA-DNA duplexes formed between plasmids display a high degree of precise pairing, and the degree of reassociation is essentially unaffected by the temperature of reassociation (9); thus, the very high degree of correlation between the HA and enzyme methods at 75 C in identifying plasmid-plasmid heteroduplexes, in contrast to that seen between some bacterial DNAs, reinforces the view that the two methods are essentially identical in detecting well-matched extensive regions of reassociated DNA, but differ
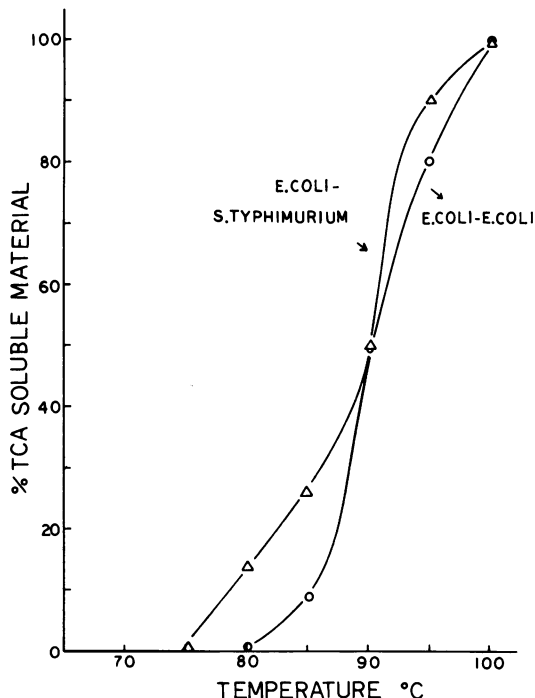

FIG. 5. *Thermal stability profiles for 75 C E. coli-E. coli and E. coli-S. typhimurium. The same experiment as described in the legend to Fig. 4 except that reassociations were carried out at 75 C. Symbols:* Δ, *E. coli B-S. typhimurium LT2;* O, *E. coli B-E. coli B.*

TABLE 2. *Action of nuclease S1 on plasmid DNA-DNA duplexes*[a]

Reaction	Relative homology at 75 C (%)	
	S1	HA
*R144/R144	100	100
*R144/chromosomal DNA	10	10
*R144/R1	9	15
*R144/colI	85	78
*R144/JR66A	79	
*R144/N3	11	11
*R144/Sa	10	14
*R144/307 Ent	7	

[a] Reassociation reactions were carried out at 75 C for 16 h in 0.42 M NaCl and assayed as described in footnote a, Table 1. Homologous reaction on HA = 90% reassociation; homologous reaction by S1 assay = 89% reassociation. Control reactions and calculations were as described in Table 1.

[b] Asterisk indicates source of labeled DNA.

in their discrimination of imperfectly matched regions.

DISCUSSION

The major intent of this investigation was to assess the potential utility of an easily prepared single-strand specific nuclease for the determination of bacterial and plasmid DNA-DNA duplexes. The *Aspergillus* nuclease can be prepared inexpensively and in large quantities. It has been prepared as an essentially pure protein (15), but most of the work with it has been done with partially purified preparations (14, 15; Britten et al., *Methods in enzymology*, in press). Even after a partial purification, the enzyme is sufficiently free of nucleases that digest double-stranded DNA (our preparations of S1 endonuclease resulted in more than 95% hydrolysis of single-stranded lambda phage ^{14}C-DNA, while the action on double-stranded substrate was 5% or less). The enzyme method enjoys many of the advantages of the HA method in the sense that it is reproducible and one can obtain virtually complete detection of the reassociated DNA product. Moreover, the reassociated fraction can be characterized by thermal denaturation and assaying the loss in trichloroacetic acid precipitability in controlled steps. Varying amounts of DNA can be handled routinely, and it is easy to maintain experimental conditions. Thermal-jacketed columns or a heated centrifuge are not required and the enzyme assay requires a somewhat shorter time to carry out. The disadvantage of the method is that non-associated DNA is irreversibly lost and that many of the same agents that are normally part of the procedures for isolation of DNA (SDS, chelating agents, high pH, etc.) can interfere with the assay unless carefully removed by dialysis. The nitrocellulose filter method for assessing DNA-DNA duplex formation (7) also gives reproducible results and, like the enzyme method, can be applied to large numbers of samples. However, the enzyme method does not require the (often tedious) preparation of immobilized DNA filters nor does one need worry about the possibility of DNA leaching from the filters either during reassociation or during the thermal dissociation of duplexes.

Although DNA-DNA duplexes assayed on HA and by the S1 endonuclease method show a high degree of correlation, our data suggest that, in instances where there is imperfect complementarity, the HA method and the S1 assay give slightly different estimates. Of course, there have been no definitive studies on the nature of

the imperfect complementarity between heterologous enterobacterial DNA. Thermal stability studies on HA suggest that at a reassociation temperature of 60 C up to 22% of the base pairs in a strand may be mismatched but still be recognized as double-stranded by HA (Brenner, unpublished observation). At a reassociation temperature of 75 C, 8% or fewer unpaired bases have been detected (Brenner, unpublished observation). Of course, sequences recognized as noncomplementary could result from randomly scattered imperfect base sequences or a short complementary region within an otherwise unpaired sequence. Presumably, the distinction made between single- and double-stranded DNA by the enzyme and by the HA column differ under these circumstances. From the thermal stability experiments with the S1 endonuclease, there is some indication that sequences in a heterologous hybrid that are seen as well-matched regions by the enzyme are not similarly recognized by the HA column.

A number of uncertainties remain concerning the differences between the enzyme and the HA methods. Nonetheless, it is our judgement that the S1 endonuclease assay permits an accurate, reproducible, rapid determination of polynucleotide sequence relationships and should be seriously considered as a method of choice for survey work and for investigations which require a large number of DNA hybridization assays.

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