DNA HOMOLOGY STUDIES IN *STREPTOMYCES* USING S1 NUCLEASE

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The optimal reaction conditions for the determination of DNA-homology in *Streptomyces* species were established in the presence of formamide using S1 nuclease. The melting temperature of *Streptomyces* DNA was 90°C in 0.42 M NaCl containing 20% formamide in which the denaturation was completed by boiling for 5 minutes. In the S1 reaction mixture consisting of 5 U of S1 nuclease, 0.168 M NaCl, 1 mM $ZnSO_4$ and 8% formamide at pH 4.8, single-stranded DNA was hydrolyzed by more than 98%, while the hydrolysis of double-stranded DNA was less than 3%. From the analysis of homoduplex formation, the C_0t 1/2 was found at 20 hours, when a mixture of unlabeled DNA and index DNA was used at a ratio of 500: 1.

In 1975, BARTH and GRINTER[1] investigated the assay conditions for DNA homology determination by S1 nuclease method and recommended their method as the most suitable and precise one applicable to most bacteria. However, there is some difficulty in applying this method to actinomycetes. For example, since melting temperature (Tm) of *Streptomyces* DNA ranges from 98 to 100°C due to its high GC content[2,3,4], the denaturation reaction should be carried out at a temperature as high as 110°C or even higher. COYKENDALL and MUNZENMAIER[5] used 115~120°C for denaturation and 70°C for reassociation of an *Actinomyces* DNA in 0.42 M NaCl. On the other hand, as MARMUR and DOTY pointed out[6], for the reassociation reaction, it is desirable to start at 25°C below the Tm to obtain the maximal rate. Conditions for assaying DNA homology in *Streptomyces* using S1 nuclease must thus be optimized because the determination of DNA homology is indispensable to identify or classify various species in actinomycetes.

We undertook a series of studies employing nuclease S1 and a denaturing agent formamide. In this paper we describe a simplified and convenient method for a DNA homology test in *Streptomyces*.

Materials and Methods

Microorganisms and Media

Streptomyces dimorphogenes NR-320-OM7HB[7], which produces trestatins[8], was grown for 48 hours at 28°C in 100 ml of Trypticase Soy Broth (BBL) containing 0.1% glycine. *Escherichia coli* K-12 was grown for 16 hours at 37°C in 100 ml of LB broth consisting of Bacto-tryptone 0.1%, Bacto-yeast extract 0.05% and NaCl 0.05%.

Isolation and Purification of DNA

DNA of *E. coli* was extracted and purified by the method described by MARMUR[9]. For isolation and purification of *Streptomyces* DNA, a modification of the method of CHATER *et al.*[10] was used as described below.

Cells, harvested by centrifugation for 15 minutes at 7,500×*g* at 4°C, were washed with P_3 medium[11], suspended in 20 ml of the same medium containing 20 mg of lysozyme (Sigma) and 1 mg of *N*-acetylmuramidase (Seikagaku Kogyo), and then incubated for 0.5~1 hour at 30°C. To protoplasts thus obtained was added 3 ml of 0.1 M Tris buffer (pH 8) containing 10% sodium dodecyl sulfate (SDS) and 1 ml of 0.25 M ethylenediaminetetraacetate (EDTA). The mixture was incubated for 20 minutes at 50°C and then chilled in ice for 10 minutes. The DNA was extracted twice with 15 ml of cold phenol - 0.1% 8-hydroxyquinoline saturated with TE (10 mM Tris, pH 7.5 plus 1 mM EDTA) containing 0.1 M NaCl and 5 ml of cold chloroform. After being centrifuged for 15 minutes at 3,700×*g*, the supernatant was recentrifuged for 20 minutes at 27,000×*g* at 4°C. The DNA was precipitated with two volumes of cold ethanol, and collected with a glass-rod. This crude DNA was dissolved in 20 ml of 0.1×SSC (15 mM NaCl plus 1.5 mM sodium citrate, pH 7.0), and incubated for 20 minutes at 37°C with 50 μg/ml of ribonuclease A (Sigma) followed by incubation for 20 minutes at 37°C with Pronase E (Kaken Seiyaku Co., Ltd.) at 100 μg/ml. After phenol extraction, the supernatant was extracted with 20 ml of chloroform - isoamyl alcohol (24: 1), and then reprecipitated with cold ethanol. The DNA was dissolved in 10 ml of 0.1×SSC and dialyzed against 0.1×SSC overnight.

The concentration of DNA was determined by absorption of ultraviolet light at 260 nm. The ratios of absorption of 230: 260: 280 nm were about 0.42: 1: 0.52.

Preparation of ^{3}H-labeled DNA

The unsheared DNA purified by the procedure described above was labeled by the nick translation[12,13]. A 50 μl of reaction mixture contained 2 μg of DNA, 5 μl of 10×NT buffer (0.5 M Tris, pH 7.5, 0.1 M $MgSO_4$, 10 mM dithiothreitol, 0.5 mg of bovine serum albumin per ml and 25% glycerol), 5 μl of 0.3 mM each of deoxyadenosine 5′-triphosphate, deoxyguanosine 5′-triphosphate and thymidine 5′-triphosphate, and 5 μCi of deoxy-[5-^{3}H]cytidine 5′-triphosphate (19 Ci/mM, Amersham), as well as 7 ng of DNase I (Miles Lab., Inc.). The reaction was allowed to proceed for 2 minutes at room temperature, for 5 minutes in ice, and then kept for 1 hour at 14°C after adding 1 μl of *E. coli* DNA polymerase I (Takara Shuzo Co., Ltd., final concentration: 14 U/ml). The reaction was terminated by adding 50 μl of TE buffer and 10 μl of 0.25 M EDTA at pH 8. The mixture was extracted with TE saturated phenol, and after ethyl ether extraction, loaded onto a Sephadex G-50 (medium) column (0.7×30 cm), which was eluted with TE. Fractions in the first peak showing the highest specific activity were pooled. The radioactivity was measured in a cocktail composed of 0.4% 2,5-diphenyloxazole and 0.02% 1,4-bis[2-(5-phenyloxazolyl)benzene] in toluene by a Kontron MR300 liquid scintillation counter. The specific activity and an average molecular size of thus prepared DNA were approximately 10^6 cpm/μg DNA and 300 base pairs (bp), respectively.

DNA-DNA Hybridization by the S1 Nuclease Method

The modification trials were based essentially on the procedures described by BARTH[1] and by COYKENDALL[5]. The sheared DNA was prepared by the sonic treatment for 3 minutes under dry ice-water cooling using Soniprep 150 (MSE Scientific Instruments). The reassociation reaction of *Streptomyces* DNA was performed at 65°C, which was 25°C lower than its Tm.

Results and Discussion

Denaturation of DNA in the Presence of Formamide

MCCONAUGHY[14] reported that 1% increase of the formamide (FA) concentration lowered the Tm of a DNA duplex by 0.7°C. We therefore first measured the Tm of *Streptomyces* and *E. coli* DNAs in 0.42 M NaCl *versus* various concentrations of FA (from 0 to 40% v/v, ultra pure grade, Bethesda Res. Lab., Inc.) in Beckman DU-8 spectrophotometer equipped with the Tm system. As Fig. 1 indicates, exactly the same slope was obtained for *Streptomyces* and *E. coli* DNAs, and the Tm of native DNA in this solution was reduced by 0.53°C per increase of 1% FA. Furthermore, it was found that *Streptomyces* DNA was completely denatured in the presence of more than 20% FA

Fig. 1. Tm of *S. dimorphogenes* DNA and *E. coli* DNA in the presence of formamide.

●: *S. dimorphogenes* DNA, ○: *E. coli* DNA.

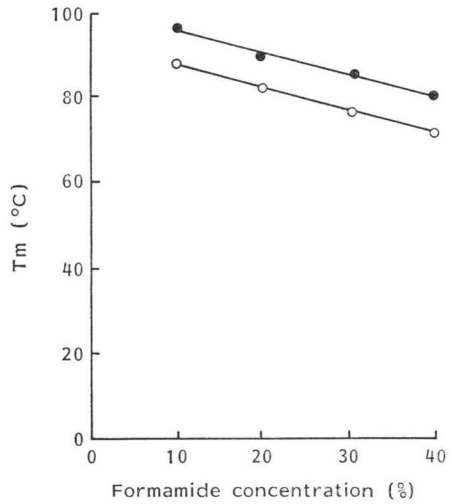


Table 1. Action of S1 nuclease on native and denatured *S. dimorphogenes* DNA in the presence of various formamide concentrations.

Formamide (%, v/v)	DNA recovered by TCA-precipitation (%)	
	Native DNA	Denatured DNA
0	100.0	NT
4	94.4	4.9
8	88.1	5.0
16	86.2	8.9

NT: Not tested.

Duplicate 1 ml of the reaction mixture consisted of 0.168 M NaCl, 1 mM $ZnSO_4$, 30 mM of sodium acetate (pH 4.8), 20 μg of sheared and denatured salmon sperm DNA, 5 U of S1 nuclease, the given concentration of formamide as well as 50 ng of labeled *S. dimorphogenes* DNA, either native or denatured. The mixture was incubated for 30 minutes at 65°C. TCA-precipitated DNA was counted as described. Recovered DNA was expressed as a percentage of the amount obtained in the absence of FA (a value taken as 100).

by boiling for 5 minutes (data not shown).

Next, we examined the effect of FA on the S1 nuclease activity using native and denatured *Streptomyces* DNA as a substrate. As seen in Table 1, the addition of 8% FA elicited lesser reduction of the S1 nuclease activity than that of

Fig. 2. Effect of S1 nuclease on native and denatured *S. dimorphogenes* DNA.

1 ml of the reaction mixture (in duplicate) consisting of 0.168 M NaCl, 1 mM $ZnSO_4$, 30 mM of sodium acetate (pH 4.8), 20 μg of sheared and denatured salmon sperm DNA, 8% formamide, the given unit of S1 nuclease as well as 50 ng of labeled *S. dimorphogenes* DNA, either native or denatured, was incubated at 65°C. TCA-precipitated DNA was counted as described.

○: 3 U, ●: 5 U.

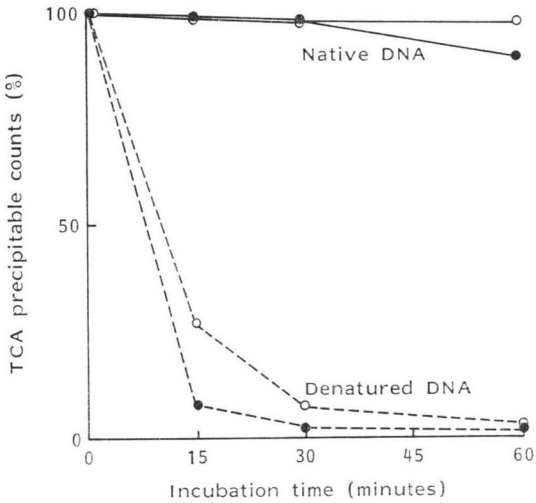
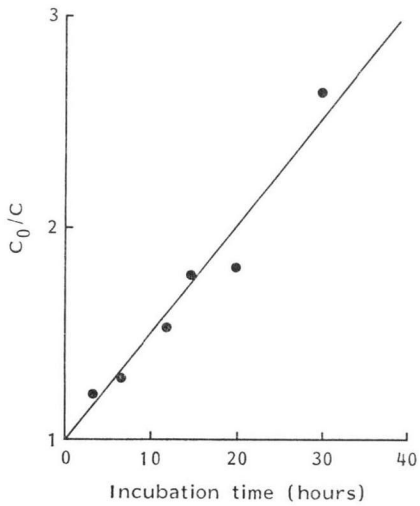


Fig. 3. Kinetics of *S. dimorphogenes* DNA reassociation.

Each hybridization mixture containing 20 ng of labeled DNA and 10 μg of unlabeled-sheared DNA. Other components and reaction conditions for the denaturation and reassociation are described in Results and Discussion.

C_0 is the initial DNA concentration, and C is the SS-DNA concentration.



16% FA, when judged by the recovery from denatured DNA. The reassociation reaction proceeded more favorably with 8% FA than with 16% FA, as reported by CASEY and DAVIDSON[15]. On the other hand, 4% FA that gave little effect on S1 nuclease activity was insufficient for the denaturation. Therefore, we chose 8% as the most suitable FA concentration.

Effect of S1 Nuclease Concentration

We tested the S1 nuclease concentration to optimize the activity of this enzyme so as to obtain not only the maximal hydrolysis of the single-stranded DNA (SS-DNA), but also the minimal hydrolysis of the double-stranded DNA (DS-DNA). The results in Fig. 2 show the time course of the S1 nuclease action measured at 65°C. In the presence of 5 U of this enzyme, the hydrolysis of SS-DNA was 98% by 30 minutes treatment whereas more than 97% of DS-DNA remained intact.

Kinetics of Homoduplex Formation

In order to find the reaction time for 50% reassociation (C_0t 1/2) of *S. dimorphogenes* DNA, the kinetics of homoduplex formation was measured during the incubation at 65°C. Fig. 3 shows that C_0t 1/2 was around 20 hours when the reaction mixture contained a 500: 1 ratio of unlabeled DNA (10 μg) and index DNA (20 ng). Thus, the incubation time for the reassociation reaction was set at 40 hours ($=2\times C_0t$ 1/2) taking into account the results of COYKENDALL and MUNZENMAIER[16]. It was also confirmed that this incubation time could be reduced to 16 hours when a 1,250: 1 ratio of unlabeled DNA (25 μg) and index DNA (20 ng) was used (data not shown), showing a good correspondence with the theoretical data obtained from the C_0t 1/2 analysis.

From the above findings, the reaction conditions for *Streptomyces* DNA hybridization using S1 nuclease were established as follows:

The reassociation mixture was made of 10 μg of unlabeled-sheared DNA and 20 ng of labeled DNA in 400 μl of 0.42 M NaCl plus 20% FA in a 5-ml capped tube (siliconized). The mixture was denatured by boiling for 5 minutes, and then incubated for 40 hours at 65°C ($2\times C_0t$ 1/2 time). After incubation, 600 μl of S1 buffer (50 mM sodium acetate, pH 4.8, 1.67 mM $ZnSO_4$, 33 μg of sheared and denatured salmon sperm DNA per ml), and 5 U of S1 nuclease were added. The mixture was further incubated for 30 minutes (at 65°C) at which time the reaction was terminated by an addition of 1 ml of cold 10% trichloroacetic acid (TCA). The mixture was kept on ice for 30 minutes. The precipitated DNA, which had been collected onto a Whatman GF/C filter, rinsed twice with 10 ml of cold 5% TCA containing 200 μM thymine and once with 2.5 ml of acetone, was dried for 30 minutes at 65°C before counting in a scintillation cocktail.

This method has been successfuly employed for DNA homology comparison of *S. dimorphogenes* with *Streptomyces olivaceus*, *Streptomyces nigrifaciens*, *Streptomyces plicatus* and other strains[7].

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