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Investigation of the Relationship between Terminal Sequence Variation and the Ligation Efficiency of T4 DNA Ligase on Blunt-ended DNA

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It is known that the rate of ligation for DNA digested with different restriction enzymes is variable. In order to further investigate this observation, pUC19 DNA was digested with one of seven different restriction enzymes to produce different DNA termini. All termini used in this study were blunt-ended either by restriction digest, by end filling, or excision of digested DNA with the Klenow fragment or T4 DNA polymerase. The resulting DNA was ligated with T4 DNA ligase and the effect of incubation time on the ligation efficiency was examined. Highest ligation efficiencies were observed at 0.5 hr whereas further incubation times lead to decreases in ligation efficiency. Additionally, *Sma*I, *Sca*I, and *Xmn*I digested blunt-ended DNA fragments displayed the highest ligation efficiencies in comparison to those fragments that underwent 5' end filling or 3' end excision to become blunt-ended. At 0.5 hr, the efficiency of ligation was greatest for *Sma*I digested sample and lowest for the *Sal*I and *Sap*I digested samples.

Restriction enzymes are important components of the restriction modification system in bacteria, archaea, and in viruses of certain algae (1, 5). Among other functions, the main role of restriction enzymes is to protect the host cell against foreign DNA. Restriction enzymes cleave DNA molecules at specific sequences known as restriction sites, thereby generating new ends that are either blunt or cohesive (3). The ends can be joined *in vitro* by DNA ligases such as the T4 DNA ligase, an enzyme encoded by the *Eschericia coli* bacteriophage T4 (11). DNA ligases are separated into two classes based on the cofactor they use (6). The first class of ligases uses NAD^+ as a cofactor and is only found in bacteria while the second class uses ATP as a cofactor and is found in eukaryotes, viruses and bacteriophages. Both ATP and Mg^{++} are required for the catalytic activity of T4 DNA ligase (2, 6).

Generally, ligation between blunt ends is more difficult to achieve than is ligation between cohesive ends of DNA (8, 9). The ability of T4 DNA ligase to catalyze the efficient joining of blunt-ended double-stranded DNA has lead to its widespread use. The catalytic reaction involves the formation of a phosphodiester bond through the joining of DNA ends containing a 3'- hydroxyl and those containing a 5'-phosphate group. More specifically, this reaction is characterized by three successive nucleotidyl transfer reactions: (i) enzymatic activation through the formation of a covalent enzyme-AMP intermediate followed by the release of PP_i; (ii) formation of an inverted (5')-(5') pyrophosphate bridge structure by the transfer of the AMP nucleotide to a phosphorylated 5'-end of the nick; (iii) attack on the AMP-DNA bond by the 3'- hydroxyl (transesterification) which results in joining of the nick and release of free AMP (6).

Biologically, DNA ligases are essential for the joining of Okazaki fragments during DNA replication, and for finishing the short-patch DNA synthesis which takes place in the DNA repair process (4). The ability to digest and ligate ends of DNA is also important in the construction of recombinant DNA molecules. Currently, very little is known about the effects of the terminal DNA sequence on the ligation efficiency of T4 DNA ligase. This study illustrates that the ligation efficiency of T4 DNA ligase is sensitive to sequence variation at the DNA terminals when joining blunt-ended DNA. The efficiency of ligation may be affected by various factors such as: the position of the restriction sites, the type of restriction site and its sequence, and the enzymes that cleaved at the restriction site. This study examined the ligation efficiency of blunt-ended pUC19 DNA generated by various single digests containing different sequences at their cut sites.

MATERIALS AND METHODS

Enzymes. *Sma*I (15228-L18), *Sca*I (15436-017), *Sal*I (15217-011), and *Xba*I (15226-012) were products of Invitrogen. *Ahd*I (R0584S), *Sap*I (R0569S), and *Xmn*I (R0194S) were products of New England Biolabs. The restriction enzymes were used with the supplied buffers recommended for optimal activity. T4 DNA ligase (15224-017), T4 DNA Polymerase (18005-025) and Large Fragment DNA Polymerase I (18012-021) were from Invitrogen.

Isolation of pUC19. pUC19 was isolated from an overnight culture of DH5α cells (UBC Teaching Lab, frozen stock), in 100 ml of LB media containing 100 μg/ml or 50 μg/ml ampicillin. Both concentrations

TABLE 1. Restriction digestion conditions for pUC19.

Restriction enzyme	Recognition site[a]	Terminal sequences[b] ligated	Location of cut site on pUC19	Units enzyme used per µg of pUC19	Reaction buffer[c]	Reaction temp. (°C)
*Sma*I	CCC/GGG	C-G	414	1	React 4	30
	GGG/CCC	G-C				
*Sca*I	AGT/ACT	T-A	2179	3.7	React 6	37
	TCA/TGA	A-T				
*Xmn*I	GAACG/TTTTC	G-T	2298	5.2	Buffer H	37
	CTTGC/AAAAG	C-A				
*Sal*I	G/TCGAC	A-T	429/433	8.6	React 10	37
	CAGCT/G	T-A				
*Ahd*I	GACGGG/GAGTC	G-G	1671/1672	4	NEBuffer 4	37
	CTGCC/CCTCAG	C-C				
*Sap*I	GGA/AGCG	C-A	2677/2680	3.6	NEBuffer 4	37
	CCTTCG/C	G-T				
*Xba*I	T/CTAGA	G-C	423/427	5.3	React2	37
	AGATC/T	C-G				

[a]the recognition site is described from the 5' to 3' end of DNA and the / indicates the cut site
[b]the terminal sequences are provided from the 5' to 3' end of DNA
[c]the React buffers were from Invitrogen, Buffer H and NEBuffer 4 were from New England Biolabs

were equally effective, and 50 µg/ml were subsequently used for convenience. Plasmid isolation was carried out using the Qiagen Maxi Plasmid Purification Kit (12262, Qiagen). The resulting DNA was resuspended in approximately 100 µl of deionized H_2O and quantified using the Ultrospec 3000 (Pharmacia Biotech).

Single site restriction digestion of pUC19. One to three micrograms of pUC19 were used in each digestion reaction. The reactions were made up to final volumes of either 30 µl or 50 µl in distilled H_2O depending on the amount of pUC19 used in the digestion reaction. The reaction mixture was also composed of 100 µg/ml BSA (B7004S, New England BioLabs), 1x concentration of the appropriate reaction buffer, and the appropriate units of restriction enzyme (Table 1). All reactions were incubated at the specified temperature for 2 hours (Table 1). After incubation, the restriction enzymes were heat inactivated at 65°C for 20 min. *Xba*I and *Sal*I were known to be resistant to heat inactivation while *Sma*I was partly resistant. Therefore, all restriction enzymes were removed from the digested products using the PCR Cleanup Kit (28104, Qiagen) or a standard phenol extraction and ethanol precipitation procedure prior to ligation (7). In all cases the efficiency of digestion was monitored using agarose gel electrophoresis.

Blunt-ending of restriction digests. Blunt-end formation through filling or excision was based on the methods previously described by Wartell and Reznikoff (10). Protruding 5' ends created by *Sal*I, *Sap*I, and *Xba*I digestions were filled in using the Large Fragment of DNA Polymerase I (Klenow Fragment). The reaction mixture consisted of digested DNA, 1x React 2 (Y92500, Invitrogen), and 80 µM oligo deoxynucleoside triphosphates (oligo dNTPs, 10297-018, Invitrogen) in a 50 µl total reaction volume containing distilled H_2O. Two units of the Klenow Fragment were added and the reaction mixture was incubated at 22°C for 30 min. Following incubation, the Klenow Fragment was heat inactivated at 70 °C for 5 min. Protruding 3' ends created by *Ahd*I digestions were excised using T4 DNA Polymerase. The reaction mixture consisted of digested DNA, 1x T4 DNA Polymerase buffer (Y02284, Invitrogen), and 80 µM oligo dNTPs (10297-018, Invitrogen) in a 50 µl total reaction volume containing distilled H_2O. Approximately 7.5 units of T4 DNA Polymerase were added and the reaction mixture was incubated at 37 °C for 5 min. Two microlitres of 0.25 M EDTA were used to stop the reaction, and the DNA was purified using phenol extraction followed by ethanol precipitation.

Ligations. Ligation reactions were carried out in 50 µl total reaction volumes containing 1x T4 ligation buffer (Y90001, Invitrogen), digested DNA diluted in distilled H_2O, and 1.2 units of T4 DNA ligase were used per microgram of digested pUC19 DNA. The reactions were performed at 24°C in a thermal cycler (Techne DHC-3) for varying periods of time ranging from 0.5 hours to 23 hours.

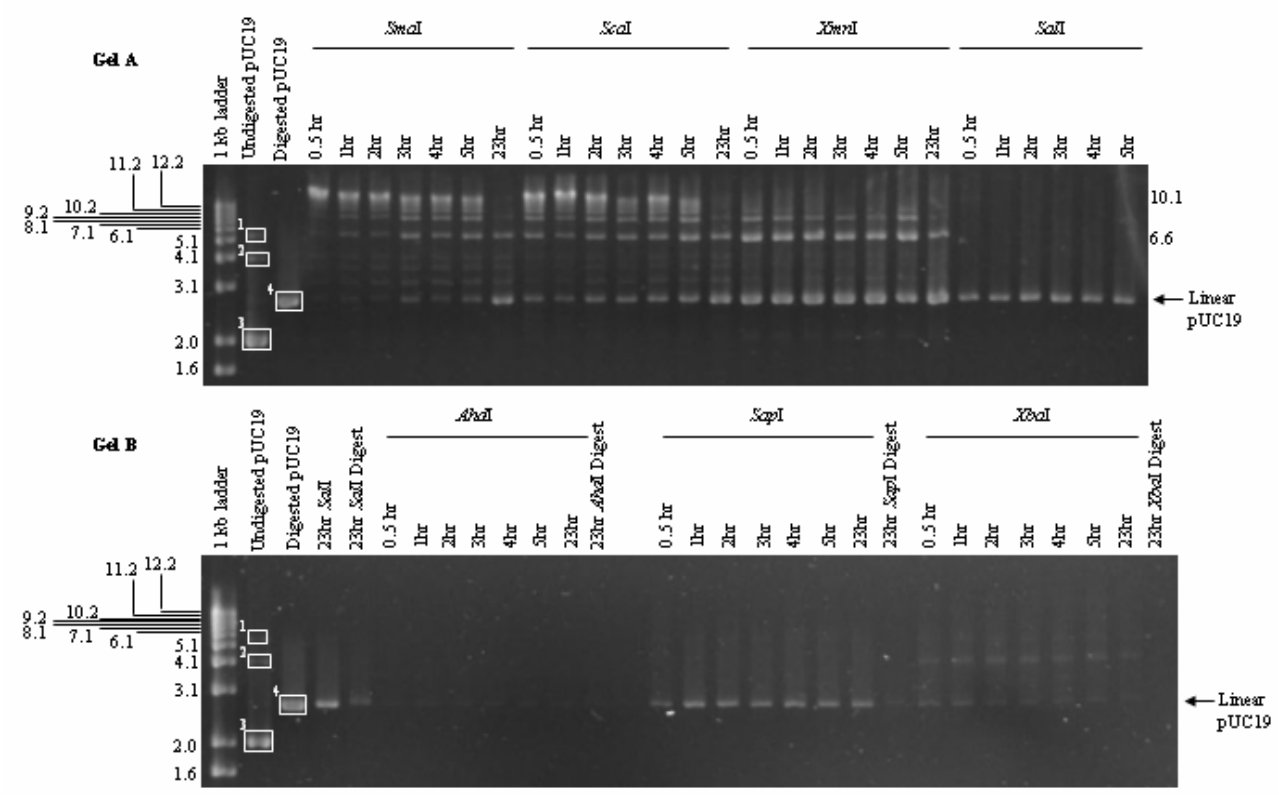

FIG. 1 Time course evaluation of ligation efficiency of T4 DNA ligase. 0.7 µg of pUC19 was digested with each of the restriction endonucleases and subsequently purified. After 5' end filling and 3' protrusion excision, DNA was ligated at 24°C. 0.1 µg samples were taken at 0.5hr, 1hr, 2hr, 3hr, 4hr, 5hr, and 23hr post ligation. Samples were electrophoresed on 1% agarose gels at 100V and stained in 0.2µg/ml ethidium bromide for 30 min. Digestion was repeated on the 23hr ligation samples that had undergone blunt end formation prior to ligation. Gel A bands 1, 2, 3 and 4 of pUC19 were 6.6, 5.2, 2.2 and 3.3 respectively. Gel B bands 1, 2, 3 and 4 of pUC19 were 7.0, 5.5, 2.5 and 3.7 respectively. All mol wts have been reported in kilo base pairs (kBp).

Agarose gel electrophoresis. Six times gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol) was added at a final concentration of 1x to the DNA samples. Electrophoresis was performed in a 1% agarose gel in 1x TBE (5x TBE was prepared using 54 g Tris base, 27.5 g boric acid, and 20 ml EDTA to a final volume of 1L in distilled H_2O). Electrophoresis was performed at 100 to 120 volts for 1.5 hr to 3.0 hr depending on the size of the gel. The gels were stained in 0.2µg/ml ethidium bromide (EtBr) for 30 min and visualized with a UV transilluminator. The AlphaEase^R FC StandAlone Software (AlphaInnotech User's Manual, AlphaInnotech, San Leandro, CA) was used to quantitate the bands of DNA based on integrated data values (IDVs). IDV values were based on the equation IDV = ∑ (each pixel value – avg background). In order to facilitate the comparisons between different data sets, times above background values were calculated by dividing the IDV value by the avg background.

RESULTS

Time course evaluation of ligation efficiency at different DNA terminal sequences. pUC19 that was completely digested by one of the seven restriction enzymes was ligated from 0.5 hr to 23 hr after all ends had been converted into blunt ends (Figure 1). The ligation efficiency of the various ends was markedly different. At 0.5hr the ligation efficiency of *Sma*I > *Sca*I > *Xmn*I > *Xba*I > *Sal*I, *Sap*I, where the *Sal*I and *Sap*I DNA ran parallel to digested pUC19 and displayed no ligation (Figure 1). However, DNA quantitation of the band corresponding to linear DNA by the AlphaEase^R FC StandAlone Software indicated that the ligation efficiency of *Sma*I > *Sca*I, *Xba*I > *Sap*I > *Sal*I > *Xmn*I at 0.5 hr with corresponding IDV times above background values of 0, 0.5, 0.5, 0.7, 1.3, and 2.2 times respectively. Gel A had an avg background of 1545940 ± 42761 IDV, whereas gel B had an avg background of 8069 ± 647 IDV (Figure 1). The ligation efficiency of the *Ahd*I sample was not determined as no

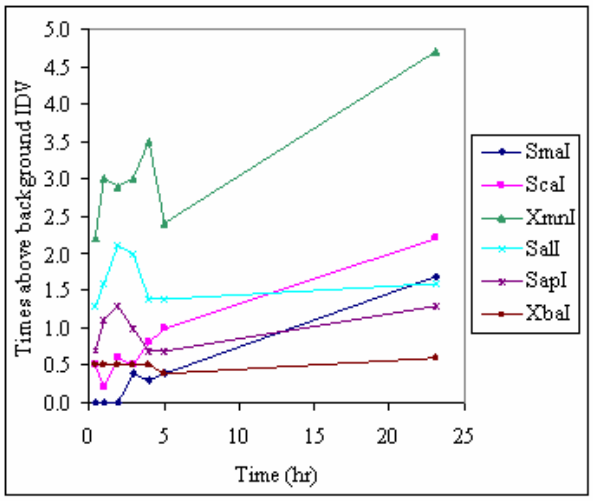

FIG. 2 Time course of ligation for *Sma*I, *Sca*I, *Xmn*I, *Sal*I, *Sap*I, and *Xba*I digested, ligated pUC19. Ligation was performed at 24°C for 0.5 hr to 23 hr, and measured as the relative intensity above background.

DNA was seen on the gel. A repeated experiment at the 0.5 hr and 23 hr time points showed similar results; however, in this trial the ligation efficiency of *Ahd*I was similar to *Sal*I and *Sap*I (results not shown).

Restriction enzyme removal assay. Over time, a decrease in the amount of ligated DNA was observed in all samples characterized by the appearance of increased amounts of linear pUC19 (Figure 1). The appearance of linear DNA at 3.3 kBp on gel A and at 3.7 kBp on gel B was indicated by an increase in times above background IDV (Figure 2).

To test whether the decrease in ligation was due to the presence of residual amounts of restriction enzyme in the ligation reaction, purified digests were combined, incubated, and visualized by agarose gel electrophoresis (Figure 3). Cleavage of pUC19 at a second site was not observed in this assay. The *Xmn*I + *Sal*I sample contained 2 bands, but this effect was due to incomplete digestion by *Xmn*I. For this sample, bands corresponding to approximately 0.8 kBp and 1.9 kBp would have been observed if residual amounts of restriction enzyme were present. However, bands of this size were not detected (Figure 3 and Table 1).

DNA topology analysis. Isolated, undigested pUC19 contained 3 bands (Figures 1 and 4). From Figure 4, the low molecular weight band at approximately 1.4 kBp was thought to represent supercoiled pUC19 as this form of DNA was expected to migrate the furthest. The two higher mol wt bands were thought to signify multimerized linear, relaxed circular, or closed circular dimer and trimer concatenated forms of superhelical DNA. The mol wt of these bands is 5.9 kBp and 8.4 kBp, which corresponds to approximately 2 and 3 times the mol wt of 2.9 kBp linear pUC19 respectively (data not shown). Although supercoiled and dimeric forms of undigested pUC19 were also seen in Figure 1, no trimeric form of undigested pUC19 was present. Instead a form that corresponds to about 1.5 times the molecular weight of linear pUC19 was observed at about 5.2 kBp to 5.5 kBp.

Multiple high mol wt bands were seen in the time course ligation study (Figure 1). Bands at 10.1 kBp and 6.6 kBp of trimeric and dimeric pUC19 respectively were observed along with several intermediate bands. Intermediate bands were thought to represent different degrees of relaxation of pUC19. In a separate run, 10 µg/ml EtBr was used to supercoil the pUC19 DNA (Figure 4). Although a slight upwards shift in the banding pattern of pUC19 appeared to occur in the presence of EtBr, this effect was thought not to be real. Supercoiling of DNA was not observed in any of the samples. All samples contained a band at approximately 2.9 kBp that corresponded to linear DNA. A band that represented a tetrameric form of DNA was detected at roughly 12.0 kBp in the *Sma*I, *Sca*I, and *Ahd*I digested, 0.5 hr ligated samples. Another band corresponding to trimeric DNA was detected at approximately 8.4 kBp in the *Sma*I, *Sca*I, and *Xmn*I digested, 0.5 hr ligated samples. These bands signified open circular or closed circular, concatenated DNA, or linear, multimerized DNA. As seen previously, the high mol wt bands disappeared at 23 hr post ligation. Additionally, a bright band at approximately 17.1 kBp was observed in the *Sca*I digested, 0.5 hr ligated sample containing EtBr. It is unknown as to what this band represents as it appears to originate from the linear form of pUC19.

DISCUSSION

DNA quantitation by the AlphaEase^{R} FC StandAlone Software was based solely on the quantitation of remaining linear DNA in the ligated samples where large values indicated low levels of ligation and small values indicated high levels of ligation. Because all bands were not taken into account for the quantitation, the results were not used to predict the efficiency of ligation. Instead they were used to outline trends in the data (Figure 2). The background IDV values were significant indicating that high levels of non-specific spot density could have skewed the results. The results provided by the software were highly sensitive to the size and location of the area that had been manually selected. The relative error for measurements was predicted to range from 3% to 8% based on the error measured in the background IDVs. The *Ahd*I sample did not show any DNA bands. Since the DNA was not monitored during the treatment and isolation, the fragments may have been lost during the

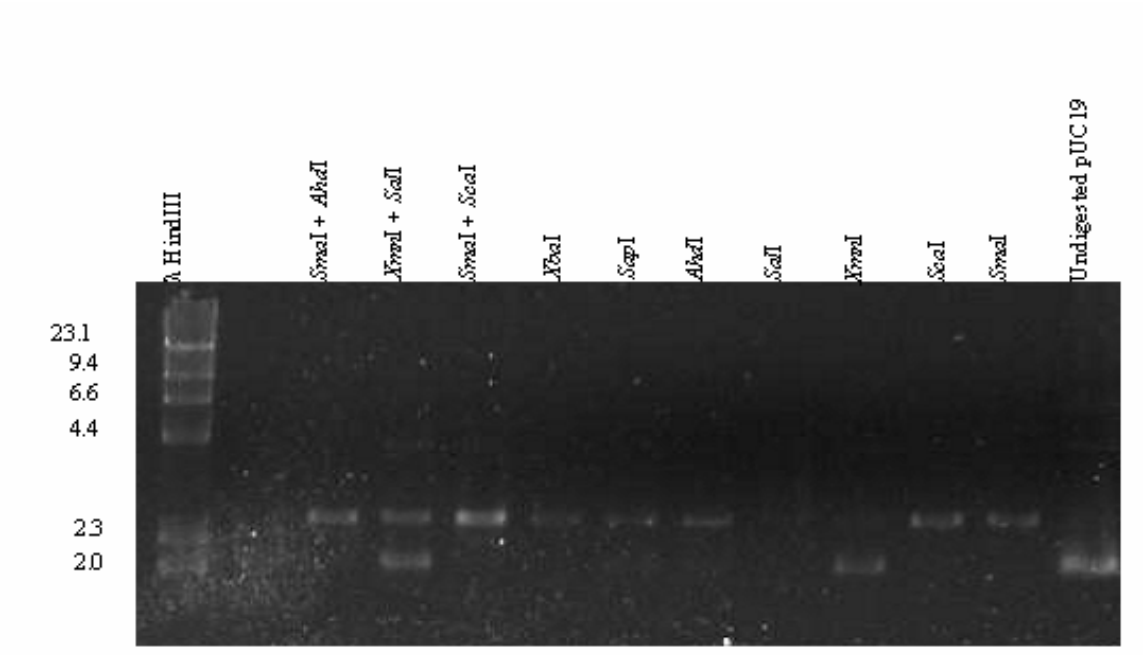

FIG. 3 Representative combined and single digests of pUC19. 2 µg of pUC19 were digested with each of the seven restriction enzymes and purified by phenol extraction and ethanol precipitation. 0.05 µg of each of the digests was combined with another digest and incubated at 37°C for 1hr. 0.1 µg of combined DNA and singly digested DNA was electrophoresed on a 1% agarose gel at 100V and stained in 0.2 µg/ml ethidium bromide for 30 min. The mol wts of the λ HindIII ladder have been reported in base pairs.

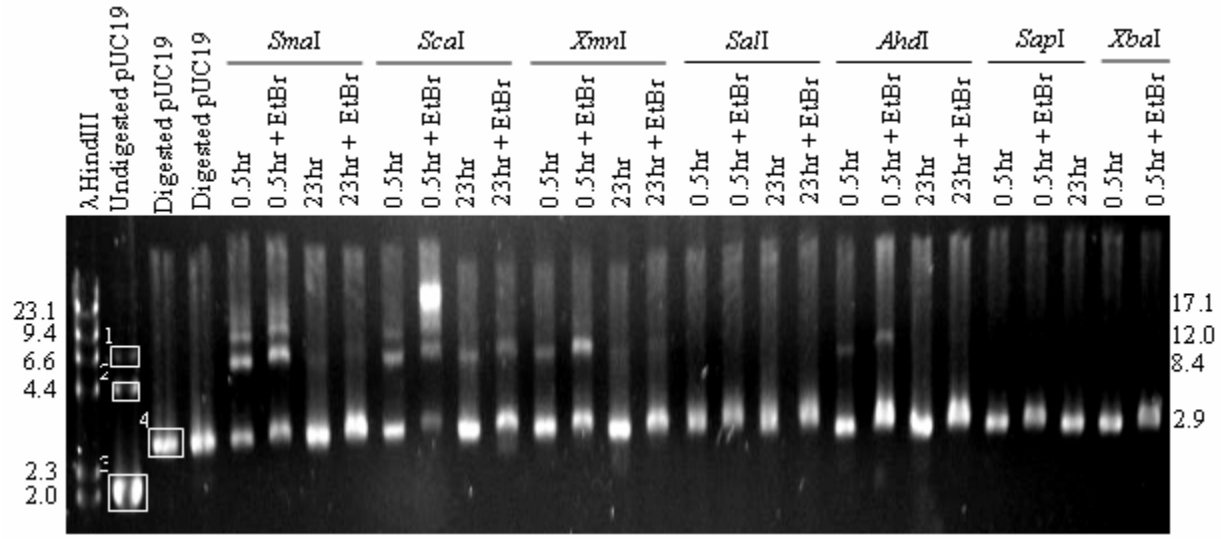

FIG. 4 Two µg of pUC19 was digested with each of the restriction endonucleases and subsequently purified by phenol extraction and ethanol precipitation. After blunt end filling of *Sal*I, *Sap*I, and *Xba*I digests and 3' protrusion excision of *Ahd*I digests, DNA was ligated at 24°C. 0.4µg samples were taken at 0.5hr and 23hr. One half of each sample was separately combined with ethidium bromide (EtBr) at a final concentration of 10 µg/ml and heated at 65°C for 10 minutes prior loading. Resulting DNA was separated on a 1% agarose gel at 120V and stained in 0.2µg/ml ethidium bromide for 30 min. Digestion was repeated on the 23hr ligation samples that had undergone blunt end formation prior to ligation. Bands 1, 2, 3 and 4 of pUC19 and digested pUC19 were 9.5, 8.4, 1.4, and 2.9 respectively. All mol wts have been reported in kilo base pairs.

phenol extraction and ethanol precipitation procedure that followed 3'protrusion excision (Figure 1).

In order to check for the fidelity of blunt-end formation, the ligated samples from *Ahd*I, *Sal*I, *Sap*I, and *Xba*I digests were re-digested with their respective enzymes. Upon successful blunt end formation, these restriction enzymes should have been incapable of cleaving the ligated DNA due to alteration of the recognition sites. Because a significant amount of ligation was not observed in these samples, the efficiency of blunt end formation could not be assessed in this manner. Although, if blunt end formation had not taken place, greater amounts of ligation would be expected in these samples as cohesive ends are more rapidly and efficiently ligated than are blunt ends due stabilizing interactions between unpaired bases (8,9).

In all ligated samples, the majority of high molecular weight bands found early in ligation disappeared by 23 hr at which point mainly linear DNA remained (Figure 1 and 3). An assay was conducted to test for the presence of residual amounts of restriction enzyme. This assay tested for restriction enzymes that were either left behind free in solution or those that bound DNA and were capable of cleaving DNA in *trans* (W. Ramey, personal communication). The assay did not account for those restriction enzymes that bound DNA and cleaved it in the *cis* configuration. Little work has been done to characterize this property of restriction enzymes.

In terms of undigested pUC19 topology, others have reported the presence of 4 bands on 0.9% agarose gels (G. Bola, unpublished data). Their analysis of topology suggested that the highest molecular weight band at 11.1 kBp (not present in the current study) shifted upwards on an agarose gel upon the addition of 7.5 µg/ml EtBr and corresponded to either the open circular or closed circular form of the plasmid. They also reported bands at approximately 5.4 kBp and 6.7 kBp that shifted downwards upon the addition of 7.5 µg/ml EtBr and corresponded to closed circular pUC19. Similarly, we expect that bands labelled 1 and 2 in Figure 1 and Figure 4 represent closed circular forms of undigested pUC19. Moreover, bands corresponding to the similar molecular weights in the ligated samples should also represent closed circular pUC19.

The time-dependent decrease in ligation efficiency of T4 DNA ligase may have been related to the presence of residual amounts of oligo dNTPs. Deoxyadenosine triphosphate is known to competitively inhibit T4 DNA ligase by decreasing its activity by 60% at concentrations of 66 µM (T4 DNA Ligase Technical Bulletin, Invitrogen). End filling of the 5' protrusions required the presence of 80 µM oligo dNTPs and the protocol designed by Wartell and Reznikoff did not require removal of non-incorporated nucleotides prior to ligation (10). It is thought that the presence of the oligo dNTPs led to the absence of significant amounts of ligation in the *Sal*I, *Sap*I, and *Xba*I digested, ligated samples (Figures 1 and 4) .

In a study on mouse testicular extracts, Sathees and Raman also report the multimerization of blunt-ended and sticky-ended DNA (8). They show that although the efficiency of end joining and multimerization was termini-dependent, the orientation of joining was random with a lack of preference for homologous ends. The joining of linear blunt or sticky-ended DNA could be either head-to-head, tail to tail, head-to-tail, or a random 1:2:1 combination of the above. In their study, head-to-tail joining was found to be more common than the other combinations. They found that the relative efficiency of end joining with different DNA termini was as follows: *Sal*I > *Eco*RI > *Hind*III > *Pst*I > *Xmn*I> *Sma*I > *Hinc*II > *Nde*I. However, their results did not correspond with those presented in this study as a greater amount of multimerization and ligation was observed in the *Xmn*I and *Sma*I digested, ligated samples than in the *Sal*I digested, ligated sample. However, based on DNA intensity, it is evident from Figure 1 that the total DNA in each of the seven different samples was variable. If equal amounts of DNA had been loaded into each of the wells the results would be more reliable and thus comparable.

In summary, the ligation efficiency of T4 DNA ligase at the different DNA terminals was variable and showed a decreasing trend in ligation over time. Maximal ligation was observed in the *Sma*I digested sample containing $\overset{C}{G}$ and $\overset{G}{C}$ at its termini followed by the *Sca*I digested sample containing $\overset{T}{A}$ and $\overset{A}{T}$ at its termini, and the *Xmn*I digested sample containing $\overset{G}{C}$ and $\overset{T}{A}$ at its termini. The lowest ligation efficiencies were seen in samples that were blunt-ended by means other than simple restriction digestion. Although the ligation efficiency of T4 DNA ligase is thought to be termini dependent, in this study the technical processing of the DNA after digestions contributed to the differences seen in DNA ligation efficiency.

FUTURE EXPERIMENTS

An alternative approach for checking for blunt end formation from *Ahd*I, *Sal*I, *Sap*I, and *Xba*I digests would be to sequence the DNA after blunt-end formation. Also, radiolabeled oligo dNTPs may be used for blunt-end filling of 5' protruding ends (8). The incorporation of radiolabeled oligo dNTPs could be assayed for by performing a Southern blot followed

by autoradiography. In the future oligo dNTPs should be removed from all reaction mixtures after blunt-end creation and prior to ligations. The topology of ligated DNA should be characterized by performing a titration with concentrations of EtBr greater than 10μg/ml. This should supercoil any closed circular DNA and lead to an increase in its rate of migration. Supercoiling would allow for a more thorough characterization of ligation efficiency of the different ends of DNA. Additionally, other terminal, blunt-ended DNA sequences may be included to broaden this study.

The fidelity of the end joining process should be assayed by redigesting the recovered multimeric forms of pUC19 with the respective linearising enzymes and gel electrophoresis of the digested products. This would indicate those products that are completely recleavable and it may also indicate the orientation of multimeric forms of DNA or the presence of an alteration/deletion that may have occurred.

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