Journal of Experimental Microbiology and Immunology (JEMI) Vol. 8:47-53

INVESTIGATION OF THE LIGATION EFFICIENCY OF DIGESTED Λ FRAGMENTS OF DIFFERENT SIZES AND COMPATIBILITY OF ENDS

ALAN TSANG
Department of Microbiology and Immunology, UBC

T4 Ligase is an enzyme derived from the T4 bacteriophage. Its remarkable ability to catalyze a ligation reaction by covalently joining two DNA molecules has allowed it to become a very useful tool in recombinant DNA technology. A previous study has indicated that not all λ DNA fragments generated from a restriction digest ligate at the same efficiency. This finding led to an investigation to determine the effects of fragment size and fragment end compatibility on the ligation efficiency of digested λ DNA fragments. In this study, *Hind III*, *Nco I/ApaL I*, and *Acc I* digests of λ DNA were prepared to generate different DNA fragments for a time-course ligation experiment. Regardless of the enzymes used, it was found that larger fragments preferentially ligate over smaller fragments. Also, fragments with incompatible ends were used up more rapidly to form ligation products than those with compatible restriction ends. These results suggest that while size and end compatibility of fragments have some control over preferences for ligation, the structural characteristics and sequence variations of fragment ends may have a more profound effect on ligation efficiency.

Ligase is an important enzyme that can be found in many organisms (3). One of the primary and important roles of ligase can be seen during DNA replication. In both eukaryotic and prokaryotic cells, ligases function by covalently joining short strands of newly synthesized DNA by forming a phosphodiester bond between the 5' phosphate of one strand of DNA and the 3' hydroxyl of another (3). DNA ligases are particularly useful tools in recombinant DNA technology because of their ability to specifically recognize and initiate ligation of two different DNA fragments generated from restriction digests (2). Fragments generated by the same restriction enzyme produce compatible ends that allow them to bind at those ends and re-anneal upon reaction with DNA ligase. Hence, Ligases and restriction enzymes together form a group of important enzymes that are widely used in DNA cloning applications (2).

T4 DNA ligase derived from the T4 bacteriophage is commonly used to clone specific DNA fragments into plasmid vectors for transformation into bacterial cells (2). It has been observed in another study that an 8371 bp fragment identified from an *Nde1* digest of λ persisted after 48 hours of reaction with T4 ligase (1). Generated from a single cut in λ by the *Nde1* restriction enzyme, this 8371 bp fragment has two incompatible ends (one *Ndel* restriction end and one λ end). Compared to λ DNA cut twice by *Nde1* to produce two compatible restriction ends, fragments with incompatible ends are expected to ligate with less efficiency due to the lower number of possibilities for their ends to bind and interact. In addition to the end compatibility of fragments, it is believed that ligation efficiency may also be affected by the size of fragments.

In this study, a time-course ligation of DNA fragments generated from three restriction digests of λ DNA was carried out to identify specific fragments that ligate preferentially over others, as well as those that demonstrate little or no ligation. The sizes and end compatibility of each digested DNA fragment were determined to investigate any potential effects these factors may have on the ligation of λ DNA.

MATERIALS AND METHODS

Restriction Digest. Three separate digest reactions were carried out using the following restriction enzymes: *Hind III* (#15207-012, Invitrogen), *Nco I/ApaL I* (#R0193S/ #R0507S, New England Biolabs), and *Acc I* (#15415-029, Invitrogen). Different enzyme buffers were used in each digest: Buffer R (#LK27, Fermentas) for Hind III digest, Buffer Tango (#JZ28, Fermentas) for *Nco I/ApaL I* digest, and Buffer B (#LK26, Fermentas) for *Acc I* digest. Six units of enzyme(s) and 6 μL of 10X buffer for each digest were reacted with 6 μg of λ DNA with final volumes of 60 μL made up in nuclease-free water. The digests were incubated at 37˚C for 2 hours, followed by heat inactivation of enzyme at 65˚C for 20 minutes.

Ligation. λ DNA fragments prepared from the restriction digests were reacted at 22˚C with T4 DNA ligase (#EL0015, Fermentas) supplied with 10X ligase buffer. Three separate ligations of the fragments from

each restriction digest were carried out for different durations (Table 1). For each reaction, 0.5 units of T4 ligase and 1 µL of 10X ligase buffer were reacted with 0.5 µg of digested λ DNA in a final volume of 10 µL made up in nuclease-free water. Following ligation, each sample was heat inactivated at 65˚C for 20 minutes.

TABLE 1 Ligation times (L_1, L_2, and L_3) of λ DNA fragments generated from three restriction digests.

Restriction Digest	Ligation Time (min)		
	L_1	L_2	L_3
Hind III	20	40	60
Nco I/ApaL I	10	20	30
Acc I	30	60	90

Agarose Gel Electrophoresis. 1X TBE buffer diluted from a 10X stock (108 g Tris-base, 55 g Boric acid, and 9.3 g of EDTA in 1 L deionized water) was used to prepare 1% UltraPure agarose gel (#15510-019, Invitrogen). Two molecular weight standards were prepared by separately diluting 1 µg of 1 Kb Plus DNA Ladder (#10787-018, Invitrogen) and 1 µg of λ DNA/*Hin*d III Fragments (#15612-013, Invitrogen) in 1X TBE to volumes of 10 µL each. Four controls were prepared by separately diluting 1 µg of λ DNA (#25250-010, Invitrogen) and 0.5 µg of each three digest in 1X TBE to volumes of 10 µL each. Two micrograms of 6X gel loading buffer (0.5 g bromophenol blue, 0.5 g xylene cyanol, 30 mL glycerol, and 170 mL deionized water) were added to the 2 molecular weight standards, 4 controls, and 6 ligation samples. Prior to loading on the gel, all 15 samples were heated at 65˚C for 10 minutes. The gel was run in 1X TBE buffer at 105 V for 2.5 hours (or until the xylene cyanol dye front has migrated 5-6 cm from the wells) to completely separate the ligation products and substrates in the ligation samples.

Gel Staining and Imaging. The gels were stained in ethidium bromide solution (0.2 µg/mL) for 20 minutes, followed by visualization in the gel documentation system. An image of the gel was digitally captured using the gel documentation system, AlphaImager (AlphaInnotech, CA, USA).

Band Identification and Quantitation. Resolved bands on the gel were identified and quantitated using the 1D-Multi program from the AlphaImager software (AlphaInnotech, CA, USA). The sizes of the bands from the 1 Kb Plus DNA Ladder were used to construct a standard curve (point-to-point fit) for determining the sizes of the ligation substrates and products from each sample. The 1D-Multi program was specifically used to measure the intensity of each band relative to other bands in the same lane. Based on these band intensities, score values representing the relative amount of each DNA molecule (substrate or product) present in each sample were generated.

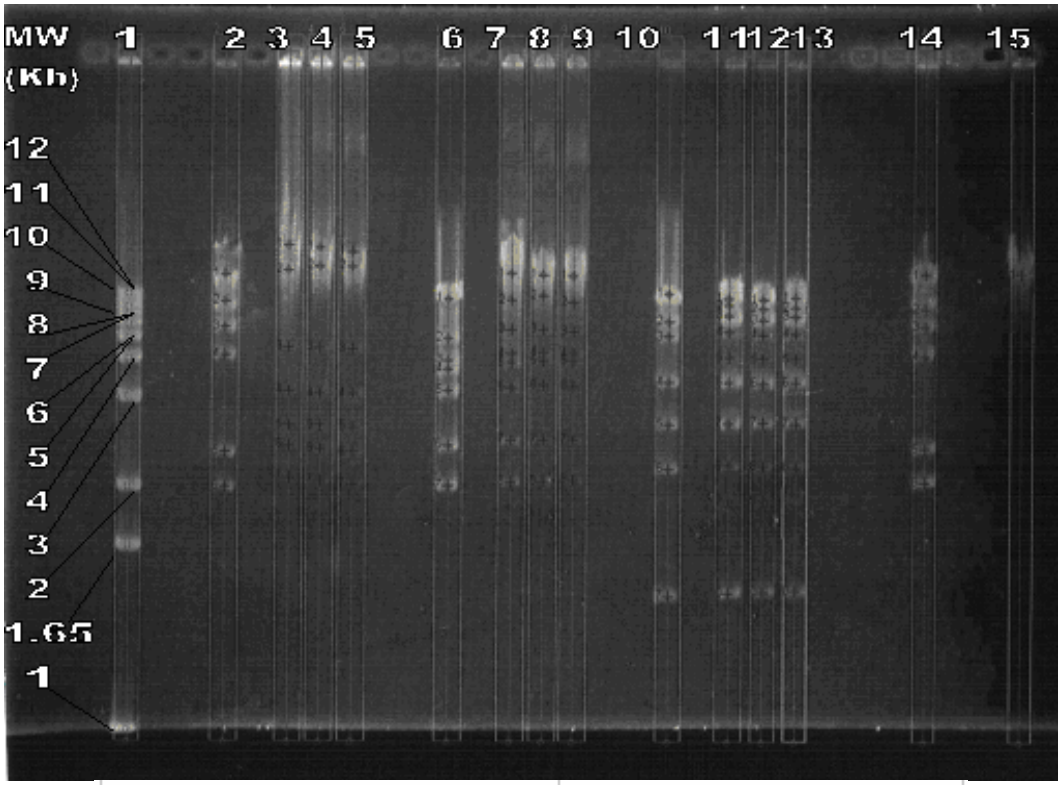
RESULTS

λ DNA Fragments generated from the restriction digests were reacted with T4 ligase for different durations to identify the λ substrates that readily ligate to form products (Fig. 1). Quantitation results show that λ substrates generated from the *Acc I* digest appeared to form the most products upon ligation, whereas those generated from the *Nco I/ApaL I* digest formed only one large product at 10.5 kb (Table 2). For each digest, it was found that larger substrates ligate preferentially over smaller substrates (Table 3). For the *Acc I* digests, ligation of incompatible end fragments with one λ end and one *Acc I* restriction end (5.6 kb and 2190 kb) were found to be preferred over ligation of compatible *Acc I* end fragments (Table 3). A 3.3 kb fragment generated from the *Nco I/ApaL I* digest was found to ligate exceptionally fast relative to other fragments with similar size and end compatibility (Table 3). For all three digests, at least one pair of substrates were identified that could possibly react to form each product observed (Table 4).

Considering the fact that products could be formed from multiple ligation reactions involving more than two substrates, there could be many different combinations of substrates that would form products with similar sizes. Therefore, the sizes and numbers of substrates that were directly involved in the formation of each ligation product could not be accurately identified (Table 4).

DISCUSSION

Results from the ligation of *Nco I/ApaL I* and *Acc I* digested λ fragments show the tendency of certain substrates to undergo multiple ligation reactions. Based on the quantitation data generated with the AlphaImager software, a single 10.5 kb product from the ligation of *Nco I/ApaL I* digested fragments was identified (Table 2). This indicates that multiple reactions involving multiple substrates were occurring simultaneously to form the 10.5 kb product. The inability to identify intermediate products suggests that those substrates rapidly and readily ligate with each other. The finding that a 3272 bp fragment ligated exceptionally fast relative to other fragments with



Lane 1: 1 Kb Plus DNA Ladder (MW Standard)	Lane 9: 20 min Ligation of *Nco I/ApaL I* Digested λ DNA
Lane 2: *Hind III* Digest (Control)	Lane 10: *Acc I* Digest (Control)
Lane 3: 20 min Ligation of *Hind III* Digested λ DNA	Lane 11: 30 min Ligation of *Acc I* Digested λ DNA
Lane 4: 40 min Ligation of *Hind III* Digested λ DNA	Lane 12: 60 min Ligation of *Acc I* Digested λ DNA
Lane 5: 60 min Ligation of *Hind III* Digested λ DNA	Lane 13: 90 min Ligation of *Acc I* Digested λ DNA
Lane 6: *Nco I/ApaL I* Digest (Control)	Lane 14: λ DNA/*Hind III* Fragments
Lane 7: 10min Ligation of *Nco I/ApaL I* Digested λ DNA	Lane 15: Non-digested λ DNA (Control)
Lane 8: 15 min Ligation of *Nco I/ApaL I* Digested λ DNA	

FIG. 1 Ligation of *Hind III*, *Nco I/ApaL I*, and *Acc I* digested λ DNA for different time periods. Bands identified by AlphaImager are indicated by a plus (+) sign.

similar size and end compatibility implies the presence of specific sequences within the fragment that may affect the ligation efficiency of digested λ DNA.

The observation that larger fragments ligate preferentially over smaller fragments was expected (Table 3). Since larger fragments experience more frequent collisions than smaller fragments, there is a higher probability of two large fragments binding at the ends and reacting with ligase than two small fragments (4). Of the three restriction digests, *Hind III* digested fragments clearly illustrate the high ligation preference of larger substrates (Table 3). Substrates with sizes 4361 bp and higher appeared to have reacted completely to form very large products that could not be accurately identified and quantitated by AlphaImager (Table 3). We could see from Figure 1 that the substrate bands were no longer visible after ligation, confirming the finding from previous studies that *Hind III* λ fragments ligate quite readily (1). These findings show that larger substrates from the *Hind III* digest ligate at a higher efficiency than smaller substrates.

TABLE 2 Relative scores (S) of products formed from the ligation of λ fragments generated from the three restriction digests. For each digest, three ligation reactions (L_1, L_2, L_3) were carried out for different durations. The bands representing the products were identified and quantitated using AlphaImager.

Digest	Product (kb)			Relative Score (S)		
	L_1	L_2	L_3	L_1	L_2	L_3
Hind III	4.6	4.4	4.5	12	17	12
	3.1	3.1	3.1	9	12	8
	2.7	2.7	2.7	8	9	6
Nco I/ApaL I	10.5	11.5	10	44	31	11
Acc I	11	10.8	11.3	262	96	67
	9.5	8.9	9	119	49	25
	8	7.5	7.9	113	40	0.2
	4.1	4	4	16	0.1	2
	1.9	1.9	1.9	6	2	5

* L_1 = 20 min ligation (Hind III Digest), 10 min ligation (Nco I/ApaL I Digest), or 30 min ligation (Acc I Digest)
L_2 = 40 min ligation (Hind III Digest), 15 min ligation (Nco I/ApaL I Digest), or 60 min ligation (Acc I Digest)
L_3 = 60 min ligation (Hind III Digest), 20 min ligation (Nco I/ApaL I Digest), or 90 min ligation (Acc I Digest)

It should be noted that for each digest, there were smaller fragments that could not be accurately detected by AlphaImager due to poor resolution on the gel. Therefore, it is possible that some of the ligation products listed in Table 2 may have been produced from substrates not listed in Table 3. The consequence of this is that there is the possibility of ligation amongst these smaller fragments to form products that are approximately similar in size to the larger substrates. For instance, consider the score values for the 1444 bp and 3574 bp substrates generated from the *Acc I* digest (Table 3). The scores and score ratios at L_1, L_2, and L_3 were exceptionally high compared to other substrates, suggesting the possibility that smaller substrates (499 bp and 639 bp) generated from the digest but not resolved on the gel may be involved in reactions that yielded products with similar sizes to the 1444 bp and 3574 bp substrates.

The investigation of fragment end compatibility on ligation efficiency showed that incompatible end fragments containing a λ end and a restriction end ligated at similar or higher efficiency than compatible restriction end fragments (Table 3). Based on this unexpected finding, it is possible that there may be specific sequences within λ that could promote strong ligation interactions at the ends. While no significant preference for ligation was observed for incompatible end fragments from *Hind III* and *Nco I/ApaL I* digests, the low scores and score ratios of incompatible end fragments from the *Acc I* digest (5581 bp and 2190 bp) suggest that those fragments were used up rather quickly (Table 3). Given the low amounts of these two fragments remaining at L_3, it is expected that these substrates are actively involved in ligation. In fact, results suggest they may ligate with each other to form an approximately 7900 bp product (Table 4). As ligation time was increased, this product appeared to have reacted further with other substrates to form larger products that could not be identified (Table 2). It should be noted that the 7900 bp product generated from ligation of the two incompatible end fragments is identical to a hypothetical 7900 bp substrate with two compatible *Acc I* ends. The observation that this 7900 bp fragment was used up rapidly from suspected further ligations with other substrates is consistent with the observation that large fragments with compatible ends ligate more readily than small fragments with similar endcompatibility.

TABLE. 3 Relative scores (*S*) of substrates remaining from the ligation of λ fragments generated from the three restriction digests. For each digest, three ligation reactions (L_1, L_2, L_3) were carried out for different durations, and no ligase was added to the control (C). Also shown is the ligation to control score ratios (S_{L1}/S_C) for the substrates remaining in each ligation sample. The bands representing these substrates were identified and quantitated using AlphaImager.

Digest	Substrate (bp)	Relative Score (S)				S_{L1}/S_C (%)		
		C	L_1	L_2	L_3	L_1	L_2	L_3
Hind III	23130	98	-	-	-	-	-	-
	9416	71	-	-	-	-	-	-
	6557	40	-	-	-	-	-	-
	4361	64	-	-	-	-	-	-
	2322	53	4	10	2	7	18	4
	2027	44	6	4	6	14	10	13
	564	-	-	-	-	-	-	-
	125	-	-	-	-	-	-	-
Nco II/*Apa L I*	12348	133	44	31	11	33	23	8
	5619	71	20	3	5	27	5	7
	4254	71	27	8	4	38	11	6
	4032	75	30	10	8	39	13	11
	3272	83	15	1	0.5	18	1	1
	2469	89	26	10	4	29	11	5
	2103	80	36	16	13	45	20	16
	695	-	-	-	-	-	-	-
Acc I	11828	163	262	96	67	161	59	41
	6957	18	113	40	0.2	634	222	1
	5581	20	40	0.2	0.1	206	1	1
	3574	62	125	77	45	202	123	72
	2720	60	50	36	23	82	60	37
	2190	53	16	15	7	31	28	14
	1444	46	75	52	48	163	113	105
	639	-	-	-	-	-	-	-
	499	-	-	-	-	-	-	-

* C = Control (No Ligase)
L1 = 20 min ligation (HindIII Digest), 10 min ligation (NcoI/ApaLI Digest), or 30 min ligation (AccI Digest)
L2 = 40 min ligation (HindIII Digest), 15 min ligation (NcoI/ApaLI Digest), or 60 min ligation (AccI Digest)
L3 = 60 min ligation (HindIII Digest), 20 min ligation (NcoI/ApaLI Digest), or 90 min ligation (AccI Digest)

TABLE 4 Substrate pairs potentially involved in ligation to form products. Substrates were paired based on their size similarity to the products observed from ligation of digested λ fragments.

Digest	Products (kb)	Substrate Pairs (bp)
Hind III	4.5	(4361 + 125); (2027 + 2322)
	3.1	(2322 + 564)
	2.7	(2027 + 564)
Nco I/ApaL I	10.5	(5619 + 4254); (5619 + 4032)
Acc I	11	(6957 + 3574); (5581 + 5581)
	9	(6957 + 2190); (5581 + 3574)
	7.8	(6957 + 639); (5581 + 2190)
	4	(3574 + 499); (2720 + 1444)
	1.9	(1444 + 499); (1444 + 639)

* Each product listed is the mean of the products identified at L_1, L_2, and L_3 in Table 2

FUTURE EXPERIMENTS

Fragments that demonstrated exceptional ligation efficiency should be further investigated to determine if their restriction sequences have any function in promoting ligation. These include the 3272 bp fragment generated from the *Nco I/ApaL I* digest, as well as the 3574 bp and 1444 bp fragments generated from the *Acc I* digest. Looking up the sequences surrounding the cut sites of those fragments might provide information that could be of structural significance, such as a modified ligase recognition site that would change the binding affinity of the enzyme. Although results indicate that ligation efficiency is affected by fragment size, it is not clear if fragments with different end compatibilities affect ligation. To confirm these findings, it might be worthwhile to carry out an experiment to determine the ligation efficiency of compatible versus incompatible end fragments that are structurally identical (except for sequence variations at the ends). This could be achieved by using PCR to synthesize compatible end fragments that extend from a primer located at the end of λ. Incompatible end fragments structurally identical to the PCR fragments could also be generated from λ restriction digests. The results could be used to compare the rate of product formation between ligation of incompatible end fragments and ligation of PCR-generated compatible end fragments.

ACKNOWLEDGEMENTS

This study was supported by the University of British Columbia's Department of Microbiology and Immunology. I would like to thank Dr W. Ramey and Jennifer Sibley for their ongoing support and useful advice leading to the successful completion of this project.

REFERENCES

1. **Chang, E., B., Ge., M. Lee, M., So., and W., Wang.** 2005. Investigation of the Ligation Efficiency of *Nde I* Digested Fragments. J. Exp. Microbiol. Immunol. **7:**68-72.
2. **Guilfoyle, R.A., C.L., Leeck, K.D., Kroening, L.M., Smith, and G., Zhen.** 1997. Ligation-mediated PCR amplication of specific fragments from a class-II restriction

endonuclease total digest. Nucleic Acids Res. **25:**1854-1858.

3. **Liu, P., A., Burdzy, L.C., Sowers.** 2004. DNA ligases ensure fidelity by interrogating minor groove contacts. Nucleic Acids Res. **32:**4503-4511.
4. **Miki, T., T., Matsui, M.A. Heidaran, and S.A. Aaronson.** 1989. Gene. An efficient directional cloning system to construct cDNA libraries containing full-length inserts at high frequency. **83:**137-146.
5. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.