# Chapter 4 - Length Dependency Studies

## 4.1 Introduction

In Chapter 3 it was determined that DNA ligase appeared to adopted a double-displacement (ping-pong) mechanism for binding and reacting with substrates $NAD^+$ and nicked DNA. Order-of-addition experiments using Ligase on 100 bp and 601 bp DNAs (that is, varying [$NAD^+$] and [DNA]) were also carried out (data not shown). On longer lengths of DNA, Ligase had a slightly increased affinity for $NAD^+$ and a lower affinity for nicked DNA, compared to short lengths of DNA. What can not be determined from order-of-addition studies is whether Ligase uses facilitated diffusion to locate a nick. Length dependency studies would be useful to determine this, and this the focus of this chapter.

Previous experiments on DNA binding proteins (restriction enzymes EcoRI and EcoRV) have shown that increasing the length of DNA flanking the target increased the rate at which the protein associated and dissociated with its target site (Shimamoto, 1999). Previous work by the Modrich group (Jack et al., 1982) carried out length dependency experiments on EcoRI using 9 DNA lengths from 34 to 6,200 bp. Their results determined that increasing DNA length increased the rate at which EcoRI locates and leaves its target site.

Using similar length dependency studies, the rate at which Ligase fixes a nick in the DNA backbone could be determined. Shorter DNA lengths were used. However, these should give more accurate results as each pair of DNA lengths were in direct competition to each other. For these experiments, different length, radioactively labelled PCR substrates were made from plasmid PL1 containing a single central BbvCI site. Using different primer pairs as described in Materials and Methods (figure 2.4B) double-stranded PCR lengths were made ranging from 100's to 1000's of base pairs.

## 4.2 Extended method for DNA preparation.

For length dependency experiments it was decided to use 40 bp oligo duplex and 5 different PCR DNA lengths; 100 bp, 301 bp, 601 bp, 902 bp and 2707 bp. Each DNA substrate contained a single central nick in the bottom strand, induced using the mutated restriction enzyme BbvCI R1+. 601 bp was used as this has a single off-centre nick. It would be interesting to see if this would affect the Ligase reaction rate.

Initial experiments on denaturing polyacrylamide gels, revealed the appearance of 'intermediate bands'. For example, on 301 bp there was an intermediate band at about 284 b. Only two bands were expected to be seen, 301 b and 147 b, as only the bottom strand was radiolabelled. On a polyacrylamide gel, the intermediate band increased with intensity as Ligase reaction progressed. This meant it was a product of the Ligase reaction. The intermediate had an initial rate of 0.2 $nMmin^{-1}$ and was completed to 2.5 nM in 10 min. The 301 b band had an initial rate of 0.5 $nMmin^{-1}$ and Ligase reaction was completed to 5 nM within 20 min (data not shown). It could not be determined if the intermediate DNA substrate affected the rate at which Ligase reacted with the 301 bp DNA. It was decided to determine where these extra bands came from.

To determine where these extra DNA fragments came from, an aliquot of nicked PCR products were run on 1% agarose gels. Figure 4.1 Panels A and B show no intermediate DNA bands can be seen on agarose gels. However, when nicked PCR samples were run on denaturing polyacrylamide gels, the extra DNA bands are clearly visible. This suggested that the problem occurs in either the top or bottom DNA stand and not the dsDNA. Figure 4.1 Panel C represents a typical timecourse of Ligase reaction on 301 bp DNA. The bottom strands is nicked and on a denaturing polyacrylamide gel this is depicted as two individual DNA strands, 154 b ad 147 b. AT time zero, Ligase was added to a final concentration of 0.2 nM. As both top and bottom primers were radiolabelled at time zero a band is seen at 301 b. This

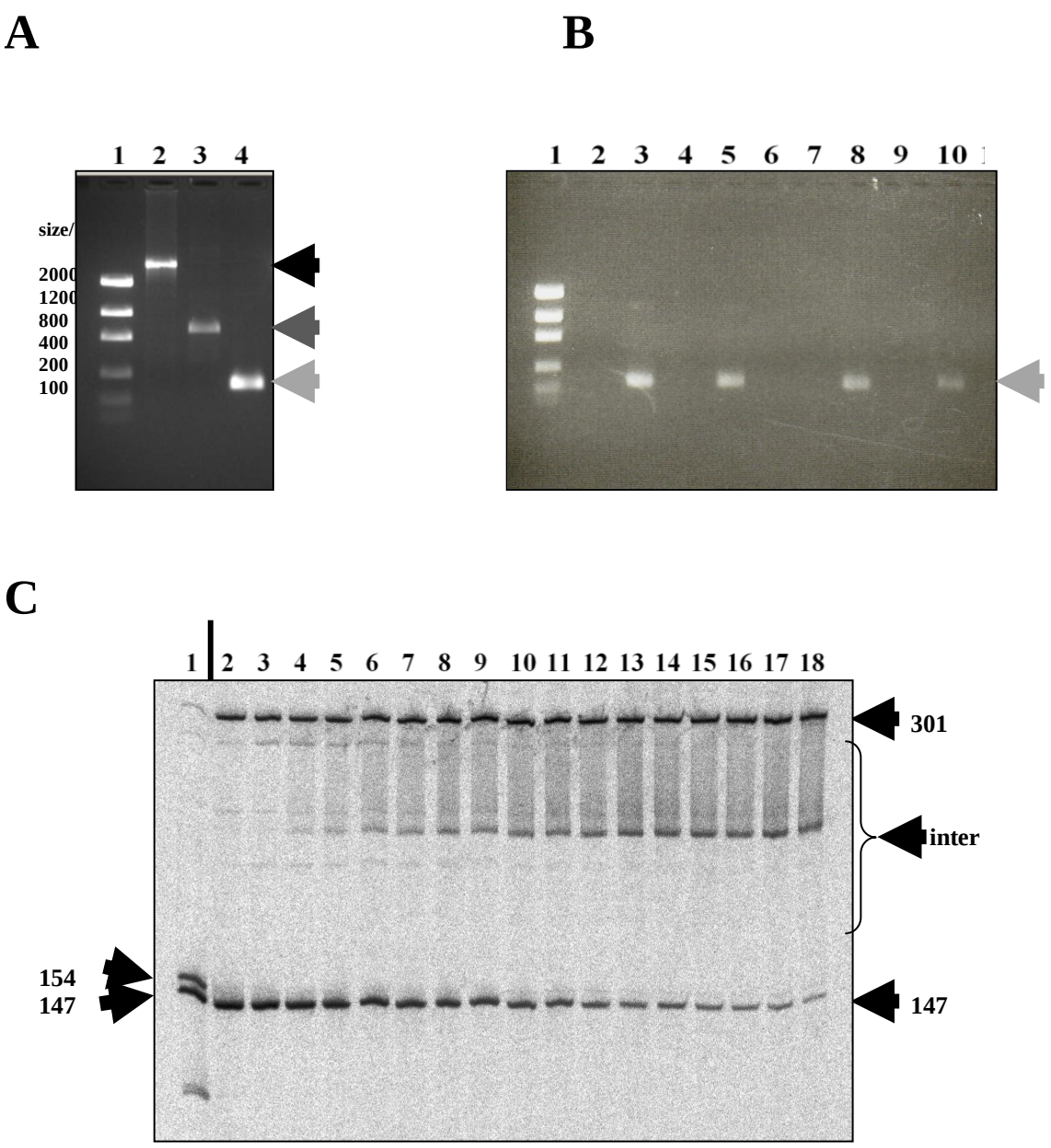


**Figure 4.1. Initial production of nicked PCR fragments.**
**Panel A**. 1% (w/v) agarose gel showing three PCR lengths. Lane 1 (low-mass DNA marker; sizes in bp shown on left), Lane 2 (2707 bp PCR from pL1, black arrow), Lane 3 (902 bp PCR from pL1, grey arrow), Lane 4 (301 bp PCR from pL1, light-grey arrow). Each lane contained ~ 200 ng PCR product; PCR was as per Materials and Methods.
**Panel B**. 1% (w/v) agarose gel showing two PCR lengths before and after nicking with BbvCI R1+. Lane 1 (low-mass DNA ladder), Lane 2 (277 bp intact PCR from pL1), Lane 3 (301 bp intact PCR from pL1), Lane 4 (277 bp singly-nicked PCR), Lane 5 (301 bp singly-nicked PCR).
**Panel C**. Ligation timecourse on 301 bp singly-nicked PCR, run on a 5% (w/v) denaturing gel. Reaction at 37°C, with 20 nM nicked DNA incubated with 0.5 nM ligase, in Ligase buffer. Lanes 1 (marker of 154 and 147 bases, from cutting with BbvCI wildtype), Lane 2 (marker of 147 bases, from R1+ nicking), Lanes 3-18 (timepoints 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 60 min). Arrows indicate DNA fragment sizes (in bases), with intermediate bands produced (labelled 'inter') during ligation.

band is the intact top strand of 301 bp. As time progressed, Ligase seals the nick. Band 147 b disappear as the nick is sealed to produce a single 301 b, therefore band 301 b increases in intensity. However, as time progresses, so does the appearance of other bands suggesting that these intermediates were a product of Ligase reaction. It was decided that the intermediate bands needed to be investigated further as they appeared to be a product of Ligase reaction.

First it was thought the pL1 plasmid was contaminated, so a sample was sent for sequencing to confirm its purity, the plasmid was clean (sequencing trace in Chapter 2.2). To determine if intermediate bands were a product of the Ligase reaction, timecourses were completed in the presence of ligase and without ligase. Also to check that external factors or contaminants were not affecting Ligase reaction, timecourses were completed by replacing ligase with either ligation diffusion buffer, ligase reaction buffer or buffer T.E (added at time = zero, ligase final concentration was 0.5 nM). As a negative control ligase timecourse was completed on intact 301 bp DNA. Time intervals were taken at time zero, 30 sec and 60 min (by which point ligase reaction should be completed). At each interval, aliquots of reaction were terminated in STOP buffer. Samples were denatured for 3 min at 99°C and then run on 5% denaturing polyacrylamide gels. Gels were dried, exposed, scanned and visualised (described in full in Chapter 2.16 and 2.17). Figure 4.2 panel A shows the results of these experiments. Only the bottom primer was radiolabelled, therefore only the bottom DNA strand can be seen. At time zero a faint band can be seen at 301 b. This was a result of incomplete digestion on the bottom strand by BbvCI R1+. When no ligase was added (lanes 1-3), intermediate bands are visible. When Ligase was added at time zero (lanes 4-6) the intermediate bands appear to form a second nick-sealed product other than the expected 301 b. When buffer replaced Ligase at time zero (lanes 7-15), as expected there was no

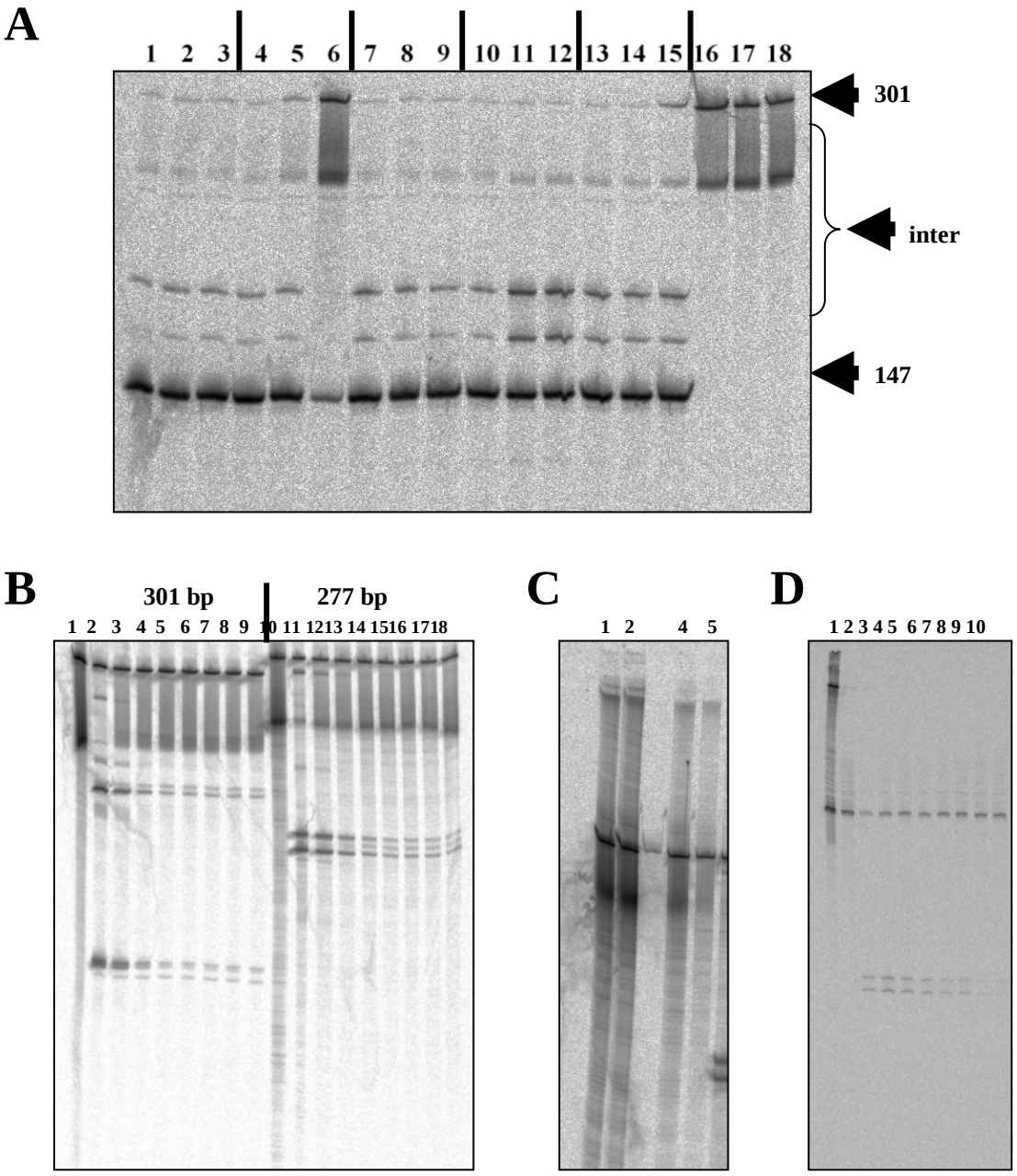


**Figure 4.2** Investigating source of intermediate band contaminants.
**Panel A**. Six ligation timecourses on 301 bp singly-nicked PCR, run on a 5% (w/v) denaturing gel. Reactions at 37°C, with 20 nM nicked DNA incubated in Ligase buffer for 0, 0.5 or 60 min, had one of six components to investigate if these caused the extra bands to appear. Lanes 1-3 (no ligase added), Lanes 4-6 (0.5 nM Ligase, positive control), Lanes 7-9 (Ligase dilution buffer), Lanes 10-12 (Ligase reaction buffer), Lanes 13-15 (TE buffer), Lanes 16-18 (0.5 nM Ligase on intact 301 bp PCR, negative control). Arrows to right indicate DNA fragment sizes (in bases), with intermediate bands produced (labelled 'inter') during ligation.
**Panel B**. Using alpha $^{33}$P-dATP in PCR reactions rather than end-labelled primers does not remove the intermediate bands during ligation**.** (Nor does moving the nick position slightly off-centre). Lanes 1- 9**,** 277 bp nicked with R1+ (277 un-nicked marker, 0,0.5,1,2,5,10,30,60 min). lanes 10-18, 301 bp nicked with R1+ (301 un-nicked marker, 0,0.5,1,2,5,10,30,60 min)
**Panel C**. 301 bp Raw PCR of alpha dATP-labelled 301 (lane 1 and 2). Lane 3 is blank. Lane 4 and 5 cleaned PCR product and result of reducing number of PCR cycles.
**Panel D**. Lane 1 before agarose gel purification. Lane 2 after agarose gel purification. Lanes 3-10 Ligase timecourse; 3 nM nicked DNA, 0.5 nM ligA (20 sec, 40 sec,1min,1.5,2,5,20 min).

ligase reaction. These results also show that the buffers used throughout the experiments do no effect or alter the formation of intermediate DNA bands. Lanes 16-18, Ligase reaction on intact 301 bp DNA shows that there is an intermediate band. This however is not a product of ligase reaction as it is present at time zero before Ligase is added, and the band intensity does not alter as the timecourse progresses. This suggests that the intermediate DNA is a PCR product.

To test whether the method by which DNA was radiolabelled could have an effect, PCR oligos were radiolabelled using $\gamma$-$^{33}$P before adding to the PCR mix. The next step was to label DNA during PCR using alpha $^{33}$P dATP. This method has the potential to label every adenosine in the double helix. An example of this is shown in figure 4.2 Panel B. Radiolabelling the DNA via an alternative method did not eliminate the intermediate bands. 277 bp DNA was made using different pair of primers (Chapter 2, Table 2.3, oligos 7 and 8) to determine if an off-centred nick site would eliminate intermediate bands. A timecourse was carried out using 0.5 nM Ligase, aliquots were run on a 5% denaturing polyacrylamide gel (Figure 4.2 Panel B). The gel shows that using alternative primers to make dsDNA does not eliminate the intermediate bands. Lanes 1 and 11 are intact 277 bp and 301 bp respectively. An intermediate band is present again suggesting that it is a PCR product.

A series of experiments were then conducted to determine if there was a problem during the production of DNA causing intermediate bands to be formed. The number of PCR cycles was reduced to ensure that the concentration of dNTPs wasn't depleted producing stunted PCR products. Instead of using Taq polymerase, Vent polymerase was used to ensure Taq doesn't have an exonuclease activity cutting the PCR product. Different PCR programs were used to confirm that the correct denaturing and annealing temperatures were used. These experiments had no effect on the production of intermediate bands (results not shown).

Since changing the PCR program used to make DNA had no effect on the formation of intermediate bands, it was decided to try and remove them after dsDNA was made. Figure 4.2 Panel C shows 1 µl aliquots of 301 bp PCR product before (lanes 1 and 2) and after (lanes 5 and 6) clean up by either Qiagen PCR cleanup kits or phenol chloroform ethanol precipitation. Aliquots were run on 5% denaturing polyacrylamide gels and visualised as previously described. Results show that the intermediate bands can not be removed in this way.

To remove intermediate bands, dsDNA was run on a 1% agarose gel. DNA bands were visualised on a phosphorimager and then cut out using a razor. DNA was extracted from the gel and purified using Qiagen gel extraction kit. DNA was nicked using BbVCI R1+ as previously described. Figure 4.2 panel D shows a ligase timecourse on gel purified 301 bp. Lane 1 is 301 bp before gel purification. Lane 2 is intact gel purified 301 bp. Lanes 3 -10 is Ligase reaction timecourse. The intensity of the intermediate bands is minimal, suggesting that the intermediate DNA has been removed. To improve the method of DNA purification, cleaned PCR DNA was run on polyacrylamide gels instead of agarose gels. Polyacrylamide gels separate similar DNA lengths more efficietly than agarose. Figure 4.3 Panels A, B and C show visualised polyacrylamide gels before DNA bands are excised. DNA bands were excised and placed in T.E and the DNA eluted out for 8 hr. DNA was concentrated by ethanol precipitation and resuspended in 50 µl T.E buffer. The purity and concentration were determined using Nanodrop spectrophotometry. A DNA marker was required to determine the sizes of each band visible on a denaturing polyacrylamide gel. Figure 4.3C shows a low molecular weight DNA marker. Either 0.5, 1, 2 or 4 ml of marker was loaded to determine the intensity of the bands when the dried gel was exposed for 8 hr (as per usual experimental practice). It was decided to use 0.5 ml as this gave sufficient intensity.

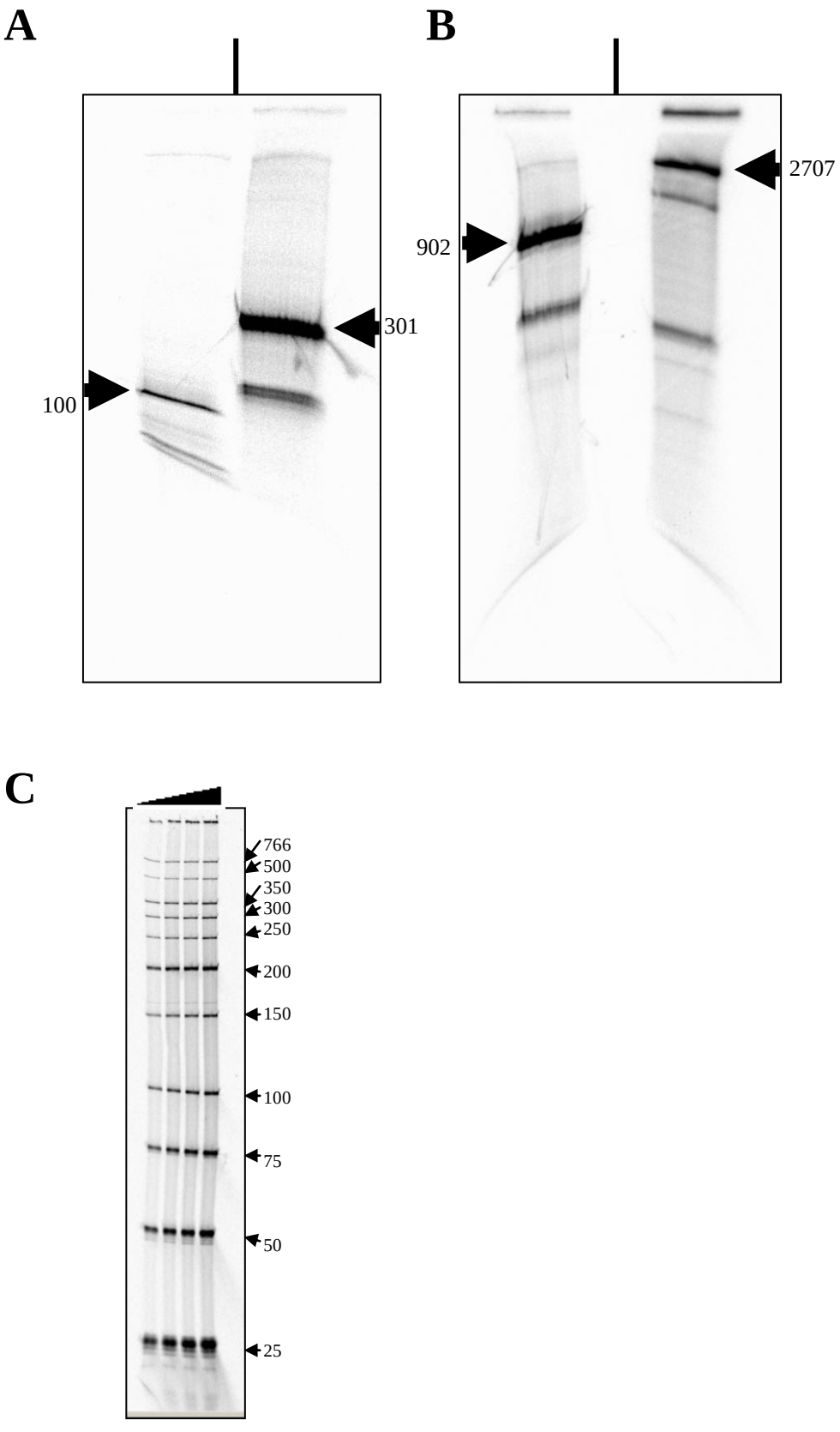


**Figure 4.3** Polyacrylamide gel purification
**Panel A**. Gel purified nicked 100 bp and 301 bp. Purified on 5% polyacrylamide gels, 15 mA 40 min. Black arrows indicate which bands were excised from the gel. DNA was extracted from these bands.
**Panel B**. Gel purified nicked 902 bp and 2707 bp.
**Panel C**. A maker lane was prepared by end-labelling the 11 fragments of a low molecular weight DNA marker (NEB) in the same way as for labelling oligonucleotides. When denatured for loading, these produced single-stranded DNAs of 766, 500, 350, 300, 250, 200, 150, 100, 75, 50 and 25 bases. Lane 1-4 0.5, 1, 2 or 4 ul of marker was added.

### 4.3 Length dependency competition experiments.

For length dependency studies a series of competition experiments were completed. For this, 6 different length PCR products were prepared as previously described. PCR's were gel purified on native polyacrylamide gels. This method proved to be effective in eliminating unwanted DNA bands that were only visible on denaturing polyacrylamide gels. This method, however, was expensive in terms of cost of radiolabelling DNA, and also because so much DNA was lost during the purification. Because of this, it was decided to use only a low concentration of DNA throughout length dependency studies. The concentration was set at 5 nM DNA. In competition studies, this meant 2.5 nM of each substrate was used per reaction.

Ligase reactions (50 µl) were achieved at 37°C with 2.5 nM of each DNA. Reaction buffer was 30 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM DTT, 50 µg/ml BSA and 25 µM $NAD^+$. Ligase reaction was initiated by adding Ligase to final concentration of 0.2 nM. Competition reactions were achieved by adding two different DNA lengths (Table 4.1).

| | 40 bp | 100 bp | 301 bp | 601 bp | 902 bp | 2707 bp |
|---|---|---|---|---|---|---|
| 40 bp | 40 v 40 | - | - | - | - | - |
| 100 bp | 40 v 100 | 100 v 100 | - | - | - | - |
| 301 bp | 40 v 301 | 100 v 301 | 301 v 301 | - | - | - |
| 601 bp | - | - | - | 601 v 601 | - | - |
| 902 bp | 40 v 902 | 100 v 902 | 301 v 902 | - | 902 v 902 | - |
| 2707 bp | - | 100 v 2707 | 301 v 2707 | - | 902 v 2707 | 2707 v 2707 |

**Table 4.1 Competition experiments. Shorter DNA lengths in columns vs longer DNA lengths in rows.**

At set time intervals the Ligase reaction was terminated by adding aliquots to STOP buffer. DNA samples were denatured by heating to 99°C for 3 min. Samples were then run on 5% or 8% denaturing polyacrylamide gels (described in detail in Chapter 2.16).

Gels were dried, exposed and visualised as described earlier. Figures 4.4 to 4.6 show the visualised gels of competition experiments.

Figures 4.4 to 4.6 show the results of each competition experiment. The gels and graphs are colour coordinated according to each DNA substrate; 100 bp (purple), 301 bp, (blue), 601 bp (green), 902 bp (orange) and 2707 bp (red). The Ligase reactions were completed as per Materials and Methods (section 2.11), and at set time intervals the reaction was terminated by adding to STOP buffer. DNA was denatured and run on 5% denaturing polyacrylamide gels.

Figure 4.4 shows the results for competition studies on 301 bp vs 2707 bp and 902 bp vs 2707 bp (Panel A). All PCR substrates were radiolabelled by incorporating 33P dATP. This shows as dark bands on the gel. It was assumed that every adenosine (A) in the DNA substrates was radiolabelled.The number of A's was also determined for each band seen on a denaturing polyacrylamide gel. The fraction of 'hot' A's was determined. The intensity of each band on the gel was determined, and multiplied by the fraction of hot A's, then multiplied again by the known DNA concentration (2.5 nM). This gave the total concentration within each DNA band on a denaturing polyacrylamide gel. However, at time zero, a dark DNA band can be seen for nick sealed product. This was the intact top DNA strand. This band can not be eliminated from the calculation. Instead it was incorporated into timecourse results. At time zero, the band is 100% intact top DNA strand and is equal to 2.5 nM. As time progresses the intensity of the band increases due to the nicked DNA being sealed to produce intact DNA up to a total of 5 nM DNA. The graphs in panel B show that the reaction started at 2.5 nM for intact product. This is the intact top strand, and remains at this concentration throughout the timecourse. Only the concentration of nick sealed DNA causes a rise. The intensity of DNA bands was plotted in Prism, and a single exponential rise was fitted to nick sealed product, and a single exponential decay was fitted to the two nicked

DNA fragments. Panel B shows the denaturing polyacrylamide gel for competition between 100 bp against 2707 bp. DNA fragment sizes are indictaed by black arrows and are in bases The nicked substrate for 2707 bp are 1357 and 1350; bands are not distinguishable on the gel as they do not migrate far on a 5% gel. The band at 1357* accounts for both of these DNA fragments. DNA was prepared by radiolabelling the 5' terminus of the bottom strand of DNA, therefore the number of A's was not required to be known. However, this labelling method means there is only one 'hit' of intensity in the DNA bands. Visualising the gels was problematic as the band intensity was so weak compared to other DNA substrates. To overcome this, gels were exposed for 2 days instead of the normal 8 hrs. This over exposed the bands for other DNA lengths. All gels shown in this section represent exposure for 8 hr for ease of reading gels. Gels were analysed at both exposure times to confirm that they produce the same results (data not shown). The over exposure of larger DNA lengths did not affect the outcome of results (results not shown). The gel shows the clear trend, as Ligase reaction progresses, the intensity of product bands 100 b and 2707 b increases. The intensity of each band was determined, and DNA concentration calculated in Excel. The initial rates were calculated to be 0.07 $nMmin^{-1}$ for 100 bp and 0.17 $nMmin^{-1}$ for 2707 bp. Again the reaction was faster on the longer DNA length

The three graphs in panel B show the reaction of 2707 bp against 301 bp; 2707 bp against 902 bp; 2707 bp against 100 bp. Ligase reaction was completed within 20 min for all reactions, visualised as a plateau in the fitted curves. The same time intervals were taken for every competition experiment, 0, 5, 10, 15, 20, 30, 45, 60 and 120 min. As the reaction was fast, for clarity of results, the graph for 902 vs 2707 bp only shows data points for up to 60 min. This enabled visualisation of the initial reaction more clearly. In all of the results, Ligase has a preference for the longer DNA length. The initial reaction

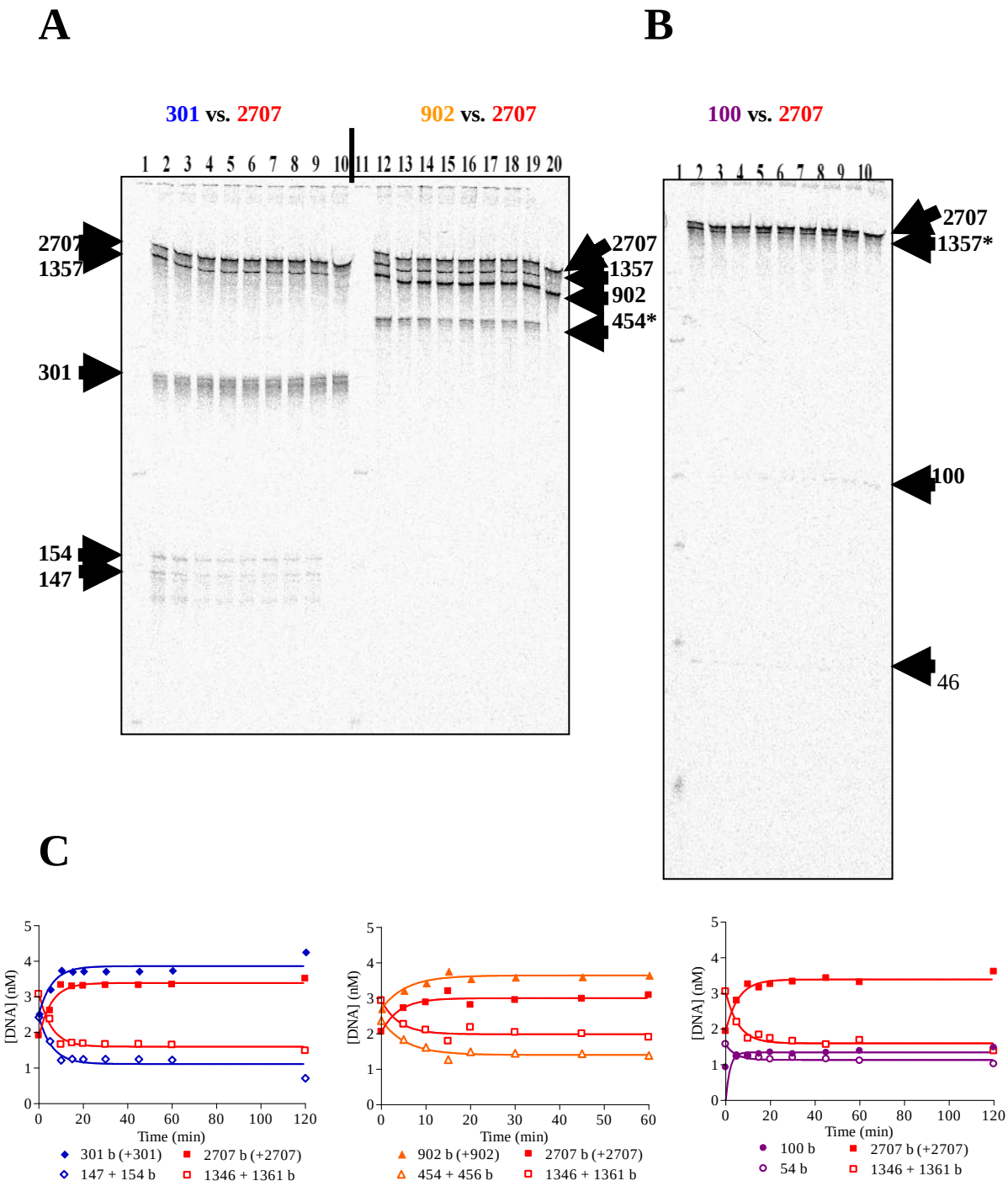


**Figure 4.4** 2707 competition results.
**Panel A**. 5% polyacrylamide gel showing two timecourses. Lanes 1 and 11 are the marker. Lanes 2-10 timecourse for 301 vs 2707 bp, and lanes 11-20 timecourse for 902 vs 2707 bp. Time points 0, 5, 10, 15, 20, 30, 45, 60 and 120 min. Black arrows indicate the position and size of DNA fragments.
**Panel B.** Polyacylamide gel showing timecourse 100 vs 2707 bp. Lane 1 marker, lanes 2-10 timepoints 0, 5, 10, 15, 20, 30, 45, 60 and 120 min.
**Panel C**. Three graphs showing quantitative results of above gels. The increase in ligation product is shown for 2707 b (filled red squares), 902 b (filled orange triangles), 301 b (filled blue diamonds) and 100 b (filled purple circles).
* indicates two nicked DNA fragments that could not be separated.

rate was determined by fitting a linear regression line to the first 10-15% of reaction in Excel. The initial rates for the competition between 902 bp and 2707 bp was 0.13 $nMmin^{-1}$ for 902 bp and 0.14 $nMmin^{-1}$ for 2707 bp. These results are very similar. The initial rates for 301 vs 2707 bp were 0.13 $nMmin^{-1}$ and 0.16 $nMmin^{-1}$ respectively. Again the reaction was faster for the longer DNA length.

Figure 4.5 shows the results for DNA substrates against the same DNA substrates. Panel A shows the 5% polyacrylamide gel for competition experiments for 902 vs 902 bp (left, lanes 2-10) and 2707 vs 2707 bp (right, lanes 12-20). The time intervals are 0, 5, 10, 15, 20, 30, 45, 60 and 120 min for both the Ligase reactions. DNA fragments are seen as bands and the sizes are indicated by black arrows. Fragments 454* and 1357* indicate there is another DNA fragment within that band intensity (457 b and 1350 b respectively). On a 5% denaturing polyacrylamide gel the bands do not separate. The intensity of each band was determined using Image Gauge and the data was transferred to Excel. The data was plotted and the DNA concentration in each band was calculated. The initial rates were determined by fitting a linear regression through the first 10% of the reaction (where the timecourse is seen as a straight line). For 902 vs 902 bp the initial rate was 0.54 nM/min. For 2707 vs 2707 bp the initial rate was 0.42 nM/min. According to these results the Ligase reaction is slower on longer lengths of DNA when there is no competition with shorter DNA lengths. The data was also fitted into Prism (to fit a single exponential rise and decay to data points), as shown in Panel B. Two graphs, the top is 902 vs 902 bp (orange triangles), the bottom graph shows the results for 2707 vs 2707 bp (red squares). Both the Ligase reactions were completed within 20 min. The 2707 vs graph indicates that the Ligase reaction was completed within 20 min but at the DNA concentration 7.5 nM. Figure 4.5C shows the 5% polyacrylamide gel for 100 vs 100 bp (left, lanes 2-10) and 301 vs 301 bp (right, lanes

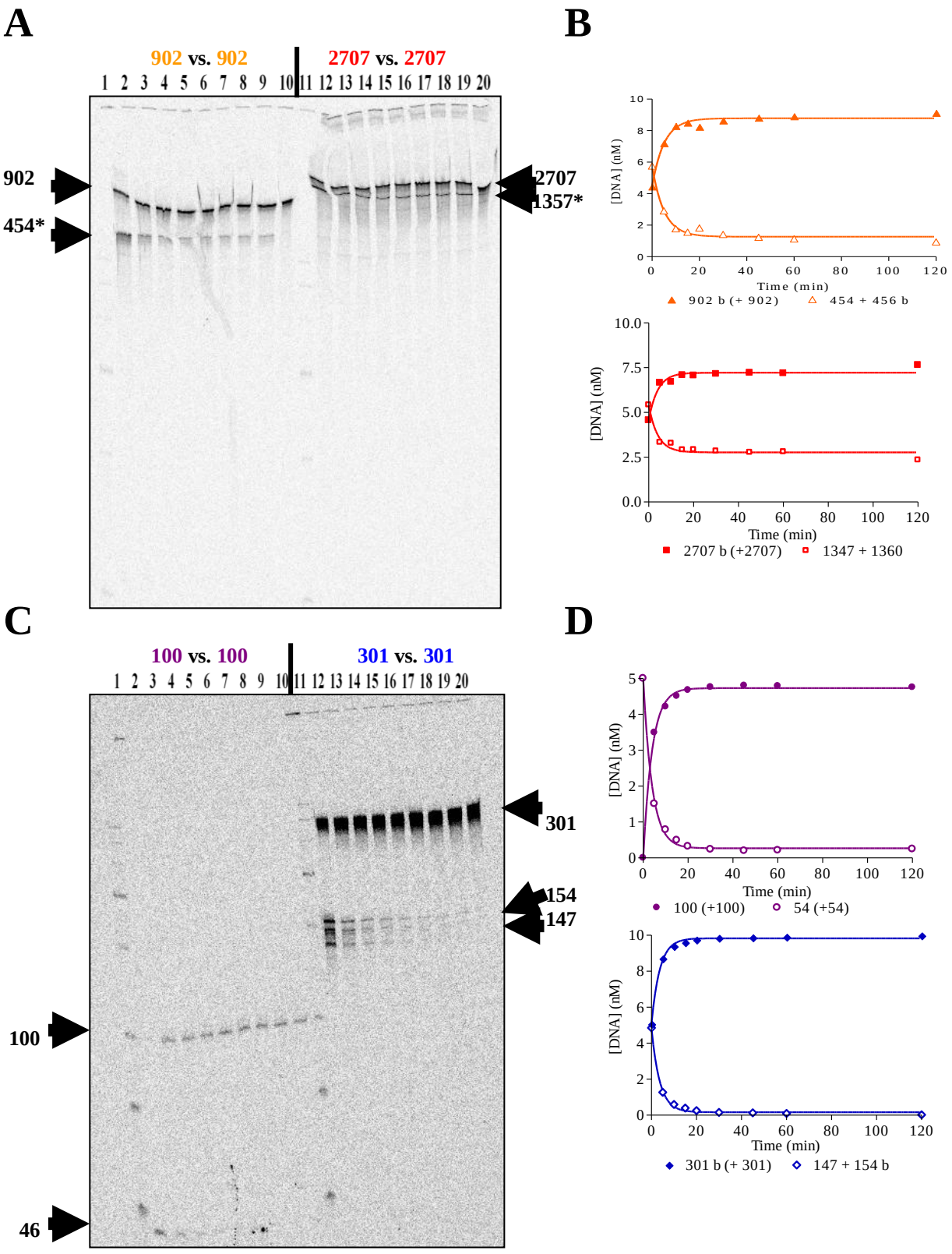


**Figure 4.5 Competition against same DNA**

**Panel A**. 5% polyacrylamide gel showing timecourse for 902 vs 902 bp; lanes 2-10 time points 0, 5, 10, 15, 20, 30, 45, 60 and 120 min. Lanes 11-20 timecourse for 2707 vs 2707 bp; time points 0, 5, 10, 15, 20, 30, 45, 60 and 120 min. Lanes 1 and 11 are the marker.. Black arrows indicate the position and size of DNA fragments. **Panel B**. Two graphs showing quantitative results of gels A. The increase in ligation product is shown for 902 b (filled orange triangles) and 2707 b (filled red squares). **Panel C** gel showing timecourse for 100 vs 100 bp; lanes 2-10, and timecourse for 301 vs 301 bp; lanes 11-20. Lanes 1 and 11 are the marker. Timepoints as above. **Panel D**. Two graphs showing results of gel C. The increase in ligation product is shown for 100 b (filled purple circles) and 301 b (filled blue diamonds).

12-20). Time intervals were 0, 5, 10, 15, 20, 30, 45, 60 and 120 min for both the Ligase reactions. Each DNA fragment can be seen clearly on the gel, and are indicated by the black arrows. The gel is slightly over exposed so we could see the 100 b and 46 b DNA fragments. These fragments are low in intensity because they were end labelled using gamma 33P. Therefore only one 'hit' of intensity is being

recorded compared to the 142 'hits' recorded by fragment 301 b. The intensity of each band was determined using ImageGuage and the data was transferred into Excel. The DNA concentration was calculated for every band and plotted into a graph (not shown). Initial rates were determined by fitting a linear regression to the first 10% of the Ligase reaction; for 100 vs 100 initial rate was 0.69 nM/min, for 302 vs 301 bp, 0.53 nM/min. The timecourse was then plotted into graphs using Prism (Panel D). The top graph shows 100 vs 100 bp (purple circles), the bottom graph shows 301 vs 301 bp (blue diamonds). Both the Ligase reactions were completed within 20 min.

Figure 4.6 Panel A shows the 5% polyacrylamide gel results for competition experiments for 100 vs 301 bp (left, lanes 2-10) and 100 vs 902 bp (right, lanes 12-20). The time intervals are 0, 5, 10, 15, 20, 30, 45, 60 and 120 min for both the Ligase reactions. DNA fragments are seen as bands and the sizes are indicated by black arrows. Fragment 454* indicate there is another DNA fragment within that band intensity (457 b). The intensity of each band was determined using ImageGuage and the data was transferred to Excel. The data was plotted and the DNA concentration in each band was calculated. The initial rates were determined by fitting a linear regression through the first 10% of the reaction (where the timecourse is seen as a straight line). For 100 vs 301 bp the initial rate was 0.10 nM/minfor 100 b, and 0.28 nM/min for 301 b. For 100 vs 902 bp the initial rate was 0.10 nM/min for 100 b, and 0.33 nM/min for 902 b. The ligase reaction was faster on the longer lengths of DNA in both competitions. The data

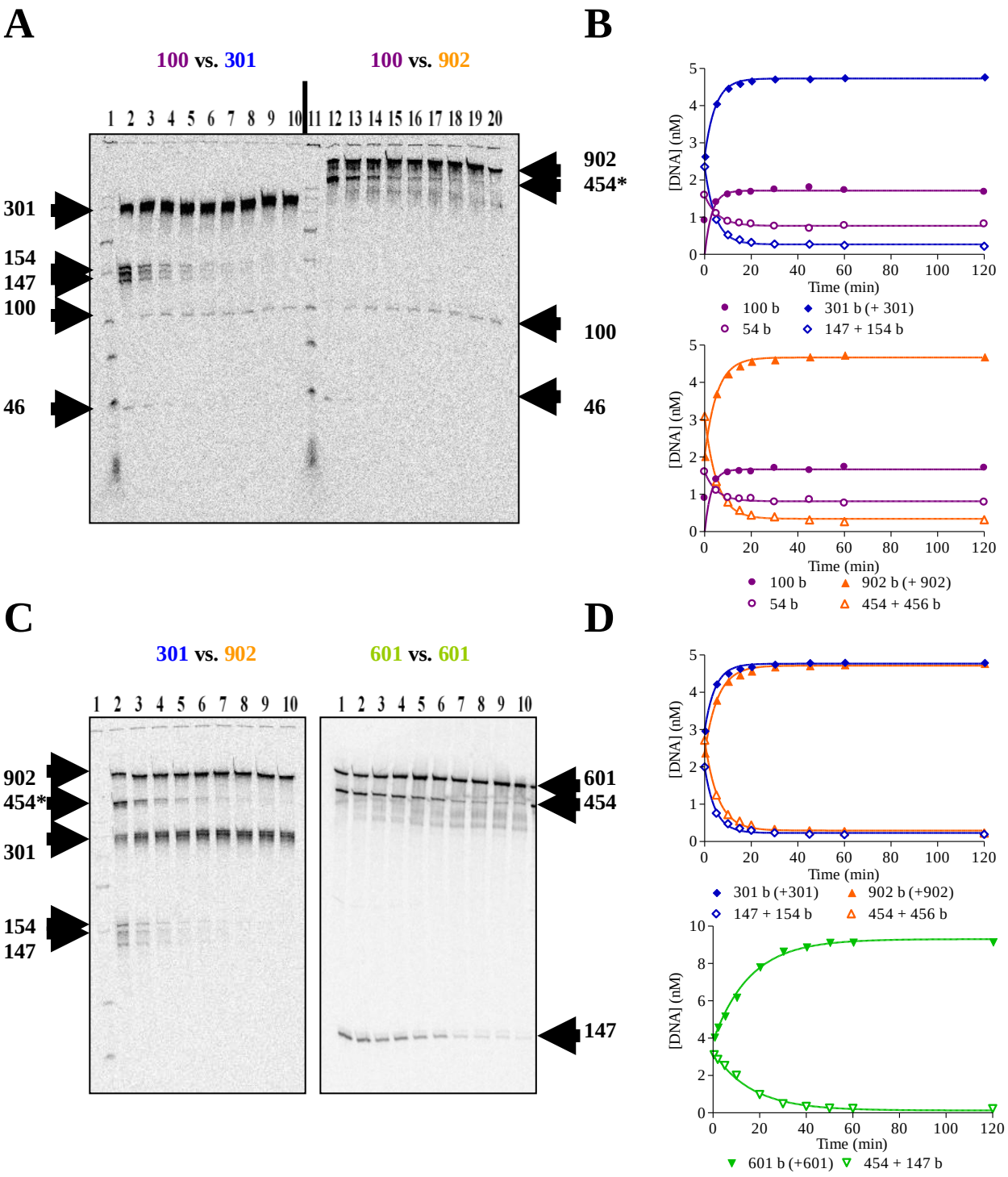


**Figure 4.6 Competition results**

**Panel A**. 5% polyacrylamide gel showing timecourse for 100 vs 301 bp; lanes 2-10, Time points 0, 5, 10, 15, 20, 30, 45, 60 and 120 min and timecourse for 100 vs 902 bp; lanes 11-20, Time points 0, 5, 10, 15, 20, 30, 45, 60 and 120 min. Lanes 1 and 11 are the marker.. Black arrows indicate the position and size of DNA fragments.

**Panel B**. Two graphs showing quantitative results of gels A. The increase in ligation product is shown for 902 b (filled orange triangles), 301 b (filled blue diamonds) and 100 b (filled purple circles).

**Panel C** gel showing timecourse for 301 vs 902 bp; lanes 2-10, and timecourse for 601 vs 601 bp; lanes 11-20. Lanes 1 and 11 are the marker. Timepoints as above.

**Panel D**. Two graphs showing results of gel C. The increase in ligation product is shown for 301 b (filled blue diamonds), 902 b (filled orange triangles) and 601 b (filled green upsidedown triangles).

* indicates two nicked DNA fragments that could not be separated

was also fitted into Prism, as shown in Panel B. Two graphs, the top is 100 (purple circles) vs 301 (blue diamonds), the bottom graph shows the results for 100 bb (purple circles) vs 902 bp (orange triangles). Both the Ligase reactions were completed within 20 min.

Figure 4.6C shows the 5% polyacrylamide gel for 301 vs 902 bp (left, lane 1 marker. lanes 2-10 time intervals were 0, 5, 10, 15, 20, 30, 45, 60 and 120 min; and 601 vs 601 bp (lanes 1-10 time intervals were 0, 5, 10, 15, 20, 30, 45, 60 and 120 min).
The intensity of each band was determined using Image Gauge and the data was transferred into Excel. The DNA concentration was calculated for every band and plotted into a graph (not shown). Initial rates were determined by fitting a linear regression to the first 10% of the Ligase reaction. For 301 vs 902 initial rates were 0.25 $nMmin^{-1}$ for 301 bp, and 0.29 $nMmin^{-1}$ for 902 bp. For 601 vs 601 bp the intial ratewas 0.2 $nMmin^{-1}$. The timecourses were then plotted into graphs using Prism (Panel D). The top graph shows 301 vs 902 bp reaction (301 bp blue diamonds, 902 bp orange triangles). The bottom graph shows the 601 vs 601 bp reaction (green upside down triangles). All the Ligase reactions were completed within 20 min.

Competition experiments were also achieved using 40 bp oligoduplex: 40 vs 40 bp, 40 vs 100 bp, 40 vs 301 bp and 40 vs 902 bp. The results are not shown. However, the initial rates were added to the table in Figure 4.7A which shows the correlated results from all the competition experiments. The tables in Panel A and B are colour coordinated to match the keys used in the Prism graphs as seen in previous figures (Figures 4.4 – 4.6). 40 b substrates are coloured pink for clarity. Figure 4.7A is a table showing the initial rates calculated by fitting a linear regression through the first 10% of every Ligase reaction. The experiment was repeated and the table represents th average of initial rates. The initial rate of the longer DNA was divided by the initial rate of the shorter DNA to give the ratio rates. The ratio of rates was plotted against the ratio of

# A

10% initial rates

| | 40 | 100 | 301 | 601 | 902 | 2707 |
|---|---|---|---|---|---|---|
| 40 | 0.14 | | | | | |
| 100 | 0.02:0.03 | 1.25 | - | - | - | - |
| 301 | 0.04:0.17 | 0.31:0.55 | 0.93 | - | - | - |
| 601 | - | - | - | **0.20** | - | - |
| 902 | 0.06:0.28 | 0.36:0.55 | 0.50:0.50 | - | 1.00 | - |
| 2707 | - | 0.07:0.17 | 0.27:0.30 | - | 0.13:0.14 | 0.50 |

# B

Ratio of DNA lengths (bp)

| | 40 | 100 | 301 | 601 | 902 | 2707 |
|---|---|---|---|---|---|---|
| 40 | 1 | - | - | - | - | - |
| 100 | 2.5 | 1 | - | - | - | - |
| 301 | 7.525 | 3.01 | 1 | - | - | - |
| 601 | - | - | - | 1 | - | - |
| 902 | 22.55 | 9.02 | 3 | - | 1 | - |
| 2707 | - | 27.07 | 9 | - | 3 | 1 |

# C

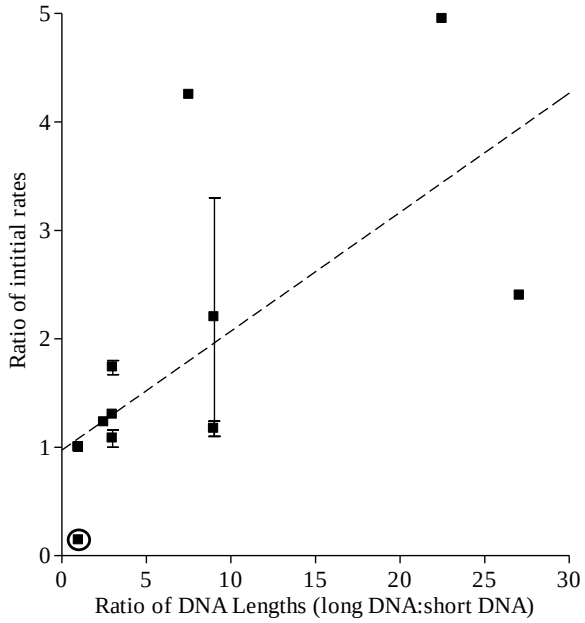


**Figure 4.7. Length Dependency results**

**Panel A**. Table of initial rates in competition experiments. Data represents an average of results. Colour co-ordinated to match the graphical data in previous figures; 40 bp (pink), 100 bp (purple), 301 bp (blue), 601 bp (green), 902 bp (orange) and 2707 bp (red). Competition DNA substrate in black.

**Panel B**. Table of ratio of lengths. The DNA length in the left column was divided by the DNA length in the top row to give ratio of lengths. Colour coordinated as above.

**Panel C.** Graph of ratio of rates against ratio of lengths; data from panel A (the larger DNA length rate divided by shorter DNA length rate) plotted against the ratio of rates in Panel B. 15 data points are plotted, 8 have been repeated and error bars are shown. A single linear fit was added to the data (of the form y=m.x+c) using Prism. The 601 vs 601 bp (data point circled) has an off-centre nick, all other DNA lengths contain a single central nick.

lengths (ratio of lengths shown in Figure 4.7B) to produce the graph in Panel C. A best fit line was fitted to the data (dotted line). This graph shows a rise in the ratio of rates against the ratio of lengths. This data suggests the Ligase uses flanking antenna DNA to locate a nick site, this could mean Ligase uses a method of facilitated diffusion. If ligase reaction rate was not length dependent then there wouldn't be a rise in the initial rate when DNA length was increase.

### 4.4 Discussion

Length dependency experiments were carried out to determine if Ligase was able to bind non-specifically to DNA, and use the flanking sequences as antenna to locate a nick in the DNA backbone. If this were the case the rate at which the nick was located should increase as the length of the flanking DNA increased. Fifteen different length competition experiments were carried out, each containing two different DNA lengths, one longer and one shorter. The overall results shown in Figure 4.7 show two very interesting trends (below).

The first was the relationship between the ratio of rates when the DNA lengths were equal (40 vs 40, 100 vs 100, etc). These revealed no overall increase in the rate of reaction as the length increased (Figure 4.7A, diagonal line of values). This shows that at the concentration of DNA used (5 nM total) the initial random collision rate between Ligase and DNA molecules was about the same for fragment lengths between 40 and 2707 bp.

The second conclusion from the table in Figure 4.7 is that at every pairwise combination of lengths the longer DNA in the pair had a slightly faster rate in every case (as plotted in Panel C). The relationship that is revealed shows a clear increasing trend between the ration of initial rates and the ratio of lengths. The dotted line indicates

this rise, though it is possible to interpret the trend with a rising hyperbola similar to that shown in Modrich (1982). These results could be extended further in future work by exploring more ratios of lengths (perhaps up to 100:1 long to short).

This data is clear evidence that DNA ligase undergoes facilitated diffusion within the domain of a DNA substrate when searching for its nicked target an observation which has not been reported before for a DNA repair enzyme.