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Comparison of Four Template Preparation Methods and Optimization of BOX A1R PCR for DNA Fingerprinting of *Escherichia coli* Isolates

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The cycling parameters for BOX A1R PCR were modified and the primer-specific annealing temperature was optimized. Using Trout Lake *Escherichia coli* isolates, four different template preparation methods were compared. The cell washing, heat lysis, and DNA isolation methods all gave clear and bright band patterns with good reproducibility. The cell washing template preparation method for direct use in PCR seems the most promising, as the procedure was simple and the level of detail obtained in band patterns was comparable to that of the DNA isolation method. It was found that the growth stage of the cells did not affect the outcome, and thus showed that the DNA fingerprinting method was robust in this aspect. The alkaline lysis method appeared to be a poor template preparation method; the method had poor reproducibility and poor output signal. The study that investigated the effect of added NaOH to PCR reactions suggested that NaOH was inhibitory to PCR.

Various types of *Escherichia coli* stains are associated with increased risk of certain diseases; thus, the environmental spread of *E. coli* via fecal contamination is of major concern (10, 11). To determine the type of control strategies necessary, it is important to first identify the source of fecal pollution, for instance, whether it is from human origin or non-human origin.

For this purpose, rep-PCR genomic fingerprinting can be implemented, which involves the amplification of repetitive elements in the bacterial genome followed by separation of amplified products with gel electrophoresis (2). An advantage of the rep-PCR genomic fingerprinting method is no prior knowledge of the genome is required and there is no need to design and test potential primers, as the primer used in this method, BOX A1R, works for a wide range of Gram-negative and Gram-positive bacteria (12).

This approach of DNA fingerprinting has been evaluated in various literature articles (1, 4, 5). Prior to this project, Lee and Wong (3) had attempted to reproduce and optimize the literature method, but obtained unsatisfactory outcomes. The band patterns they obtained were faint and the methods they used did not always give reproducible results. It was suspected the source of problem that gave unsatisfactory results was associated with the quality of template used. There might have been inhibitory components in the heat lysate Lee and Wong (3) used for PCR that impaired the quality of DNA or interfered with DNA amplification. For instance, large amount of RNA can chelate Mg^{2+} and decrease PCR yield; also, presence of polymerase inhibitors can reduce PCR reaction efficiency (9).

Thus, in this project, different template preparation methods were investigated. The heat lysis template preparation method described by Lee and Wong (3) was compared to three other template preparation methods suggested in the literature by using the same PCR set-up. The other template preparation methods suggested in the literature included whole cell suspensions washed for direct use in PCR, alkaline lysis cell suspensions, and purified genomic DNA. In addition, the reproducibility of the cell washing and alkaline lysis methods was validated, and the effects of growth stage of cells as well as amount of template on PCR outcome were investigated. In addition, as the original set of PCR cycling parameters was not optimal, a new set of PCR cycling parameters was used and the annealing temperature was optimized for the primer.

In this project, pure liquid culture was used for all analysis. Both pure liquid culture and colonies from plate culture were used by Lee and Wong (3). In their project, single colonies from plate culture gave better results compared to the liquid culture, which either gave faint bands with smearing or no band at all. Only pure liquid cultures were used in this project for the following reasons. The band patterns obtained were expected to be the same, regardless whether pure liquid cultures or single colonies from plate culture were used.

Also, the experiment could be better controlled if liquid culture was used, as the amount of cells used could be known from turbidity measurements.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* isolates A5-23, which had been obtained from a Trout Lake Glaucous winged gull, and A5-82, which had been obtained from a Trout Lake Mallard duck, were used for the optimization studies. *E. coli* stocks stored at -80°C were thawed and first streaked onto LB agar plate for overnight incubation at 37°C. Then, to prepare a starter culture, a single colony of each strain was inoculated into Luria Bertani broth with pH 7.5, prepared by dissolving 1.0 g of tryptone, 0.5 g of yeast extract (Difco, Cat no. 0127-01), and 1.0 g of NaCl (Fisher Chemicals, Cat no. S271-3) in 100 ml distilled water. The inoculated culture (4 ml) was incubated at 37°C overnight with shaking.

A portion of the starter culture was used to inoculate fresh LB broth (4 ml). The culture was grown either to exponential phase or stationary phase. To grow the culture to exponential phase, the turbidity of starter culture was measured and the approximate time required to reach an OD_{600} between 0.65 and 0.95 was calculated, assuming the doubling time was 40 minutes. To grow the culture to stationary phase, the starter culture was diluted 1/500 into fresh LB broth (4 ml) and incubated overnight, after which the turbidity was measured and each culture was diluted to an OD_{600} between 0.65 and 0.95. All culture was grown at 37°C in New Brunswick Scientific Excella E24 Incubator Shaker.

Template preparation for PCR. Four template preparation methods were performed: cell washing method for direct use in PCR, alkaline lysis method, heat lysis method, and DNA isolation method as described below. All templates prepared were stored at -20°C

Cell washing method for direct use in PCR. 1.5 ml of E. coli culture with an OD_{600} between 0.65 and 0.95 was harvested by centrifugation at 6000 x g for 5 minutes. The supernatant was removed, and the cell pellet was washed twice with 0.5 ml of 1M NaCl (Fisher Chemicals, Cat no. S271-3) (8). Then, the pellet was washed once with 0.5 ml of sterile water. The washing step with sterile water was not included in the method suggested in the literature; it was added to eliminate potential carry-over of NaCl. The cell pellet was resuspended in 50 µl of sterile water. 1 µl of cell suspension was used per PCR reaction.

Alkaline lysis method. 10 µl of E. coli culture with an OD_{600} between 0.65 and 0.95 was added to 100 µl of 0.05 M NaOH (Fisher Chemicals, Cat no. 318-1). The mixture was incubated at 95°C for 15 minutes. Three replicates of each were prepared. Prior to PCR, the mixture was centrifuged at maximum speed (16,100 x g) for 2 minutes, and either 1 µl or 2 µl of the supernatant was used per PCR reaction (8).

Heat lysis method. Two variations of this method were performed. Firstly, the entire method was performed strictly according to the procedure described by Lee and Wong (3). 0.5 ml of overnight E. coli culture was centrifuged and the supernatant was removed. The cell pellet was washed once with 0.5 ml of sterile water, resuspended in 100 µl of sterile water, and heated at 95°C for 10 minutes. To allow this method to be directly comparable to the cell washing method, a slight variation of the method was performed as well, as described below. 1.5 ml of overnight E. coli culture was diluted to an OD_{600} between 0.65 and 0.95 then centrifuged. Following removal of the supernatant, the cell pellet was washed once with 0.5 ml of sterile water, resuspended in 50 µl of sterile water, and then heated at 95°C for 10 minutes. 1 µl of cell suspension was used per PCR reaction.

DNA isolation method. Genomic DNA was extracted from 1 ml of E. coli culture using QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit (7). DNA was eluted in 200 µl of elution buffer twice. 1 µl of eluted DNA was used per PCR reaction.

PCR conditions. Each PCR reaction consisted of the following: 1 µl or 2 µl of template, either 2.5 µl of 10X PCR Buffer (Invitrogen, Part no. y02028) plus 1.5 µl of 50 mM MgCl (Invitrogen, Part no. y02016) or 2.5 µl of 10X PCR Buffer containing MgCl (Invitrogen, Part no. 46-0121), either 1 µl of 25 mM dNTPs Mix (Fermentas, Cat no. R1121) or 0.5 µl of 50 mM dNTPs Mix (Invitrogen, Part no. 46-0122), 2.5 µl of 20 µM BOX A1R primer (IDT, Ref no. 39610986), 0.2 µl of 5 U/µl Taq DNA Polymerase (Invitrogen, Cat no. 18038-042), and sterile water to make up to a total volume of 25 µl. When there were many samples, the reagents were often prepared as a premix.

For no template control, sterile water was used instead of template. For positive control, 1 µl of 0.1 µg/µl Control PCR Primers (Invitrogen, Part no. 46-0100) and 1 µl of 0.1 µg/µl Control Template (Invitrogen, Part no. 46-0118) from TOPO TA Cloning Kit pCR 2.1-TOPO Vector (Invitrogen, Part no. 45-0641) were used.

Reproducibility of the cell washing method and alkaline lysis method were evaluated in replicates of three. To analyze the effect of different amounts of starting material, varying dilutions of template prepared using different methods were used for PCR. Specifically, undiluted, 1/10 diluted, and 1/100 diluted samples were compared. For the purposes of testing effect of NaOH on PCR, two different amounts of 0.05 M NaOH, 0.91 µl and 1.82 µl, were added to template prepared using different methods. The two amounts were the same amounts present in 1 µl or 2 µl alkaline lysed samples used for PCR.

PCR was carried out using Biometra T-gradient PCR Thermocycler. Original cycling parameters were initial denaturation 2 min at 95°C; 30 cycles of 3 s at 94°C, 30 s at 92°C, 1 min at 50°C; final elongation of 8 min at 65°C. For the later experiments, PCR cycling parameters were modified to initial denaturation of 2 min at 94°C; 35 cycles of 1 min each of 94°C, 55°C, 72°C; final elongation of 7 min at 72°C. In addition, the annealing step (1 minute at 55°C) of the modified PCR cycling parameters was optimized by studying a temperature range from 49°C to 61°C, set as a temperature gradient

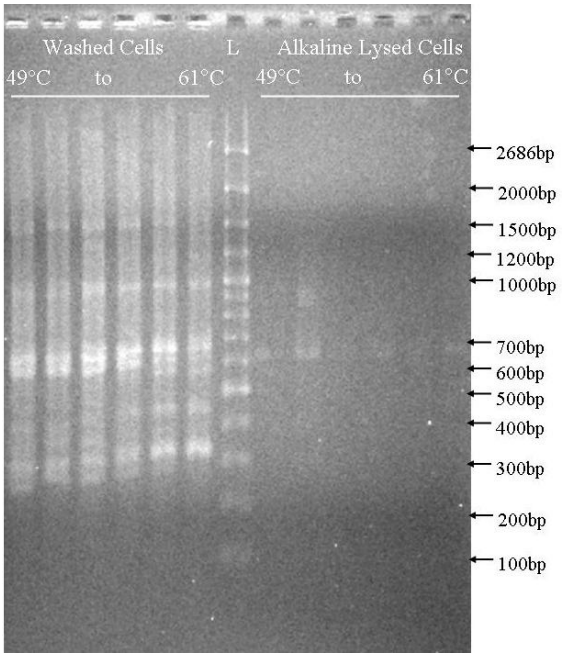

FIG. 1. BOX A1R primer annealing temperature optimization. *E. coli* from gull harvested from exponential growth phase was either washed or alkaline lysed prior to PCR. A temperature range from 49°C to 61°C was tested; neighboring samples have an approximate temperature difference of 2°C. L stands for 100 bp DNA Ladder.

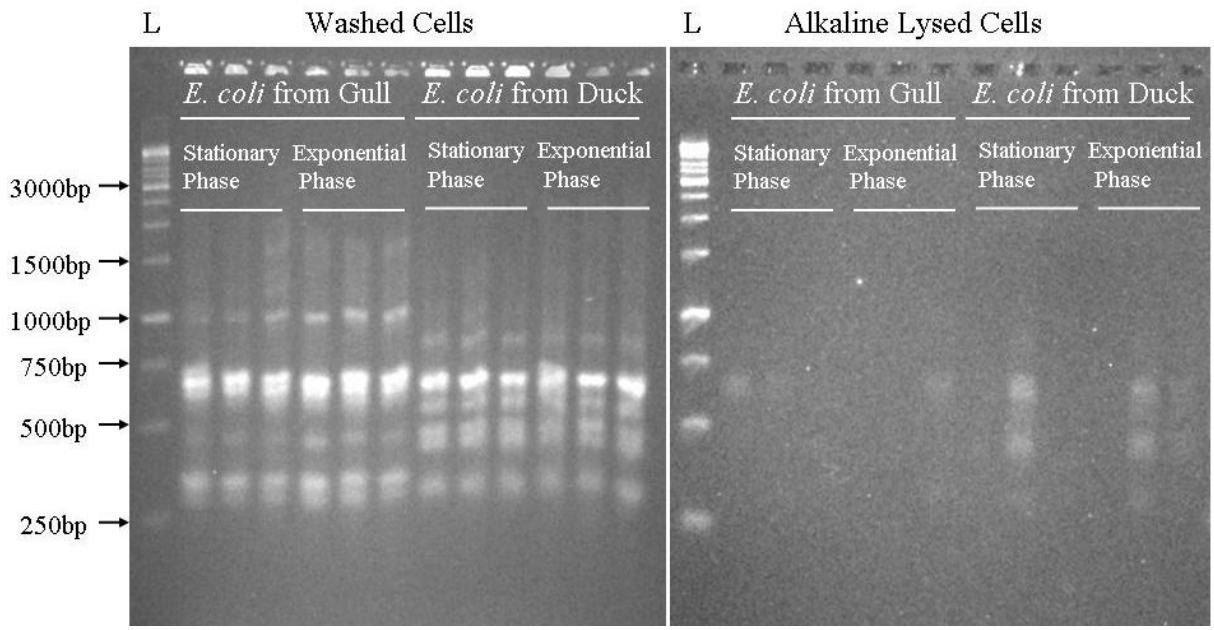

FIG. 2. Comparison of *E. coli* from different growth stages and method reproducibility. *E. coli* from gull and duck that were harvested from stationary and exponential growth phases were either washed or alkaline lysed prior to PCR. Three replicates of each were analyzed. L stands for 1 kb DNA Ladder.

on Biometra T-gradient PCR Thermocycler.

Gel electrophoresis. PCR products were separated on 1.5% agarose gel. Either Certified™ Molecular Biology Agarose (BIO-RAD, Cat no. 161-3101) or TopVision™ Agarose (Fermentas, Cat no. R0491) was used. 5X TBE buffer was prepared using 54.0 g of Tris Base (Fisher Scientific, Cat no. EC201-064-4), 27.5 g of Boric Acid (Sigma, Cat no. B-0252), 3.72 g of EDTA (Fisher Scientific, Cat no. S-311), made up to 1 L with distilled water. 1X TBE buffer was used for both gel preparation and electrophoresis.

10 µl of PCR product was mixed with 2 µl of 6X DNA Loading Dye (Fermentas, Cat no. R0611). Either GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Cat no. SM0321) or GeneRuler™ 1kb DNA Ladder (Fermentas, Cat no. SM0311) was loaded as markers on each gel. After loading, electrophoresis was carried out at room temperature for 1.5 to 2 hours at 70 V and stained for about 20 minutes to several hours in an ethidium bromide solution with a concentration of about 0.5 µg per ml. Gel imaging was obtained with shortwave ultraviolet excitation using the Alpha Imager (Fisher Scientific, Ottawa ON, Canada).

RESULTS

PCR optimization. The original set of cycling parameters worked, but it was not ideal. Thus, considered changes were made to temperature and time at certain PCR stages. Specifically, important changes include increasing denaturation time to 1 minute and adding a 1 minute extension step at 72°C. In addition, the number of PCR cycles was increased from 30 to 35. This new set of PCR cycling parameters was tested, and fairly bright band patterns were obtained.

Then, it was important to optimize this new set of PCR cycling parameters for BOX A1R primer. A range of temperature of 12°C, from 49°C to 61°C, was tested for two types of template, either washed or alkaline lysed cells (Fig. 1). For washed samples, all tested annealing temperatures gave a bright band pattern. It was observed that as annealing temperature increased, certain bright bands appeared fainter and certain faint bands appeared brighter. At the highest temperature, some bands, such as the 1500 bp band, became hardly noticeable.

On the other hand, for alkaline lysed samples, none of the tested annealing temperatures gave an ideal band pattern. Faint band pattern was only observed when the annealing temperature was in the middle of the range. Prior to this experiment, when the annealing temperature was 55°C, a relatively bright band pattern had been observed. A follow up experiment was performed with annealing temperatures 55°C and 58°C. This time, only a faint band pattern was observed when 1 µl of alkaline lysate was used for PCR with the annealing temperature 55°C.

Comparison of *E. coli* harvested at different growth stages and method reproducibility. *E. coli* from gull and duck that were harvested from stationary and exponential growth phases were compared. At the same time, reproducibility of the cell washing method and alkaline lysis method was evaluated. For each *E. coli* strain, three replicates for each of the two growth conditions and for each of the two template preparation methods were prepared (Fig. 2). As shown in the figure, the cell washing template preparation method, again, gave significantly better results than the alkaline lysis

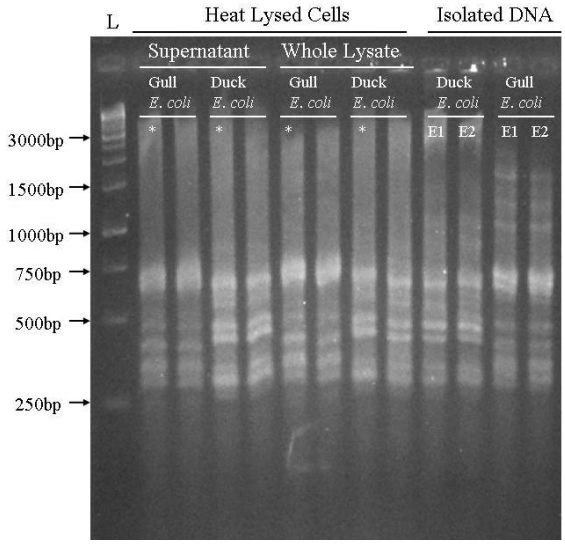

FIG. 3. Heat lysed cells and isolated DNA as template used for PCR. *E. coli* from gull and duck that were harvested from stationary phase were either heat lysed or had DNA extracted prior to PCR.* represent samples that are directly comparable to the cell washing method samples as they have the same number of cells. The unlabeled ones represent samples prepared strictly according to Lee and Wong's method (3). For *E. coli* from gull, samples labeled with * had 3.188X more cells than the neighboring unlabeled samples. For *E. coli* from duck, samples labeled with * had 2.452X more cells than the neighboring unlabeled samples. E1 represents Elution 1; E2 represents Elution 2. L stands for 1 kb DNA Ladder.

template preparation method. The amount of DNA amplified from washed cells was significantly more than the amount of DNA amplified from alkaline lysed cells. When washed cells were used directly for PCR, both *E. coli* from gull and duck gave bright band patterns and *E. coli* from the two species could be easily distinguished. As shown for the washed cells, the band patterns observed for *E. coli* taken from exponential growth phase and stationary growth phase were very similar; this applied to both *E. coli* from gull and duck.

In terms of method reproducibility, the cell washing template preparation method gave fairly consistent results; band pattern and intensity obtained in all triplicates were comparable. On the other hand, the alkaline lysis template preparation method was not reproducible. Significant differences were observed between triplicates; within each set, some showed band patterns while others did not. Although the amplified products from the alkaline lysed samples, if present, were significantly fainter, the band pattern resembled that of the washed sample, except that only the bright bands were visible and the faint bands were not visible.

Comparison of four different template preparation methods for PCR. The previous part of the experiment, which compared *E. coli* from different growth phases and evaluated method reproducibility, examined the cell washing and alkaline lysis methods; next, these methods were compared to the other two methods, namely heat lysis and DNA isolation. For heat lysed cells, both supernatant and whole lysate were analyzed; for isolated DNA, both elutions of each isolate were analyzed in PCR.

Both heat lysis and isolation of DNA template preparation methods worked (Fig. 3). In comparison, the DNA isolation template preparation method seemed superior as the background smear was less and more bands were resolved. For instance, this was especially obvious for *E. coli* from gull DNA Elutions 1 and 2, which showed distinct bands at molecular weights higher than 1000 bp (Fig. 3). In addition, different amounts of starting material used gave the same outcome, as shown by heat lysed samples identified in the lanes with and without the * label, as well as DNA elutions with different concentrations. The same samples with different concentration of starting material looked like replicates (Fig. 3). An interesting observation applicable to the heat lysis method was that no noticeable difference in the outcome was observed regardless whether supernatant or whole lysate was used.

Effect of varying amounts of template. Stationary phase *E. coli* samples that had been processed using the four different methods, either washed, alkaline lysed, heat lysed, or had DNA extracted prior to PCR, were serial diluted to 1/10 and 1/100. Recall from previous

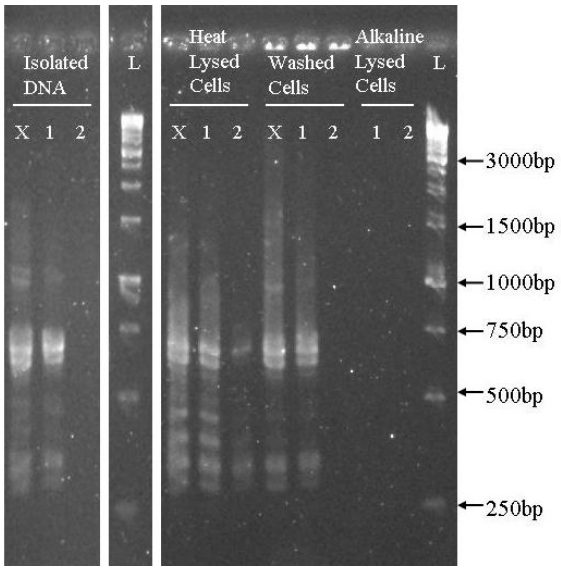

FIG. 5. Effect of NaOH on PCR. Samples of *E. coli* from gull that were harvested from stationary phase were used. X denotes original samples without NaOH added. 1 represents samples containing 0.91 µl 0.05 M NaOH. 2 represents samples containing 1.82 µl 0.05 M NaOH. L stands for 1 kb DNA Ladder.

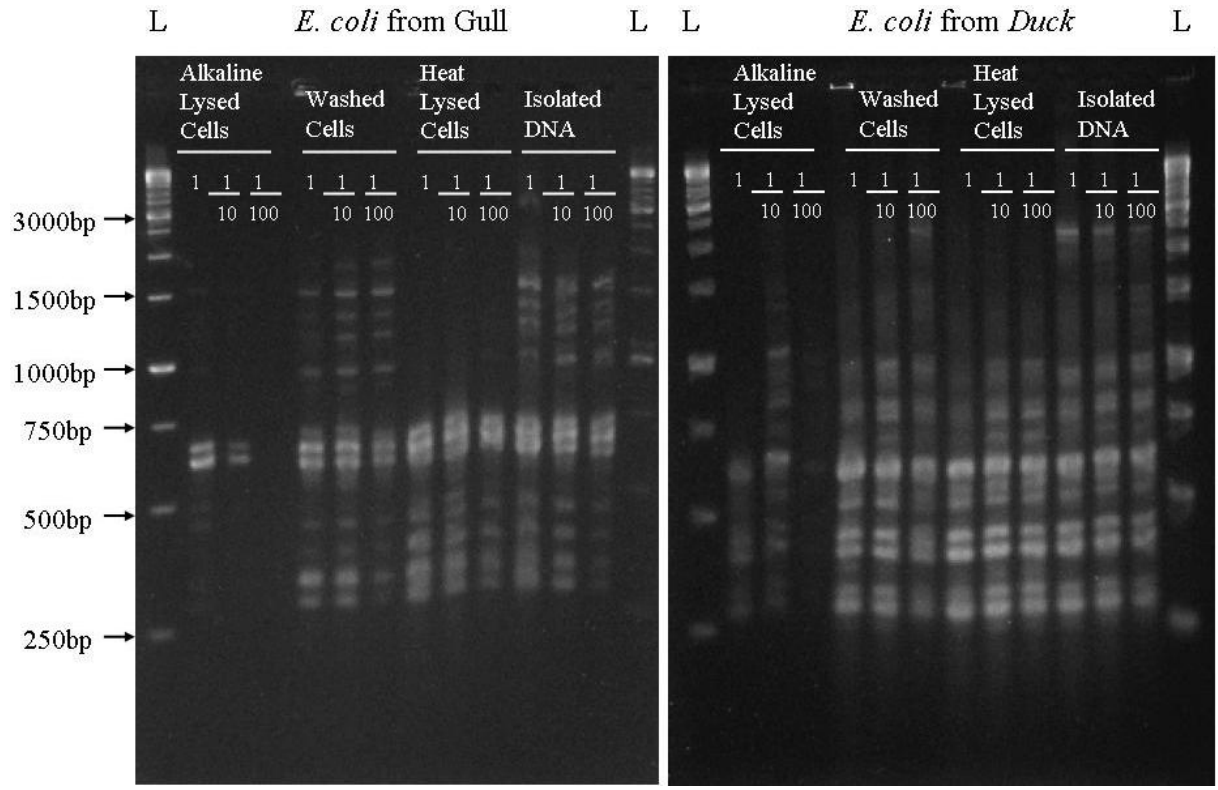

FIG. 4. Effect of undiluted, 1/10 diluted, and 1/100 diluted template prepared using the four different methods. Samples of *E. coli* from gull and duck that were harvested from stationary phase were used. L stands for 1 kb DNA Ladder.

results that the alkaline lysis method did not appear to be a good template preparation method, as sometimes no band pattern was obtained; thus, in order to study the effect of varying amounts of template, a replicate which gave positive result in the previous experiments was deliberatively chosen. For the other three methods, a replicate was chosen randomly. The same set of experiments was performed using *E. coli* from both gull and duck, with the intention to obtain confirmation and unbiased results (Fig.4).

For all template preparation methods except alkaline lysis, there was no major difference between the band patterns and intensities obtained with different dilutions of template. One interesting but not obvious observation was, for washed cells, the intensity of certain faint high molecular weight bands increased when the template was further diluted. For alkaline lysed *E. coli* from gull, band intensity decreased with an increase in dilution. On the other hand, for alkaline lysed *E. coli* from duck, band intensity increased and then decreased with an increase in dilution.

Regardless of which template preparation method was used, the band patterns obtained for each *E. coli* strain were similar, which was expected as the same source of *E. coli* was used and they were just processed differently. For the alkaline lysis method, only the brightest bands were observed. The band pattern obtained with cell washing method and DNA isolation method were very similar; it was apparent that the cell washing method was not inferior to the DNA isolation method in terms of obtaining high molecular weight bands (Fig. 4). In addition, in the case of the heat lysis method, the high molecular weight bands seemed missing in Figure 4. This was consistent with Figure 3, in which the heat lysed samples only showed intense background and no bands in the high molecular weight region. These two figures appeared different due to differences in staining and exposure time when the picture was taken, which resulted in the intensity of the background smear being different.

Effect of NaOH on PCR. The effect of NaOH on PCR was investigated by adding NaOH to the templates prepared using cell washing, heat lysis, and alkaline lysis methods at two levels that were equivalent to the amounts of NaOH present in the templates prepared using the alkaline lysis method. PCR was performed on the original alkaline lysis samples and samples with 0.05 M NaOH added at two levels for direct comparison (Fig. 5). In this experiment, only *E. coli* from gull was used, but the results should be transferable to *E. coli* from other sources. As shown in Fig. 5, for isolated DNA, heat lysed cells and washed

cells, added NaOH had a negative effect on the fingerprinting pattern. The effect was not significant when NaOH was added at the lower level. However, when NaOH was added at the higher level, no band pattern was observed for isolated DNA and washed cells, and only a faint band pattern was observed for heat lysed cells. Neither of the alkaline lysed samples gave an observable band pattern (Fig. 5).

DISCUSSION

Before the template preparation methods could be evaluated, it was important to have a set of PCR cycling parameters that worked well. The modified set of PCR cycling parameters ensured that sufficient time was allocated to the different stages of PCR and that the extension step was carried out at the optimal temperature for Taq polymerase. In addition, the number of PCR cycles was increased with the intention to increase the intensity of band patterns, which is important especially if the starting template copy number is low.

As part of the PCR cycling parameters optimization, the next step was to select the best annealing temperature. It was best to use higher annealing temperature as this increased the specificity. However, at the highest temperature, some band information seemed to be lost; thus, based on the results of the washed cells, the second highest temperature (58°C) seemed most ideal. However, this temperature was not chosen due to the results obtained with the alkaline lysed samples. For the alkaline lysed samples, the higher temperature did not give obvious bands for unknown reasons. It could be that the template material used had poor quality or the alkaline lysis template preparation method was not reproducible. Another possibility is this particular template preparation was not robust and was vulnerable to variations in temperatures set by the gradient. Nonetheless, when the annealing temperature was set at 55°C, observable band pattern had been produced in two other experiments. Thus, to be safe, it was decided to use 55°C as the annealing temperature for all future analysis.

It was uncertain how robust the PCR method was when cells from different stages of growth were used. For instance, cells harvested at different growth phases may differ in their ability to lyse, which would affect the template preparation methods that involve cell lysis. Also, overaging of the culture could lead to DNA degradation (6). Thus, it was important to compare cells taken from different growth phases, namely the exponential growth phase and the stationary growth phase, and determine whether this contributes to variability. The results showed that the band patterns observed for *E. coli* taken from different growth phases were very similar and thus showed the methods were robust in this aspect.

As the amount of cells lysed each time may be different, it was important that reproducibility of each literature template preparation method that involves cell lysis was validated; otherwise the comparison of different methods would not be meaningful. The results showed that the cell washing template preparation method was reproducible, which suggests the amount of cells lysed and the amount of template DNA released from cells during early PCR cycles was consistent. However, the alkaline lysis template preparation method was not reproducible, which likely suggests the amount of cells to begin with or the amount of cells lysed and the amount of template DNA available for PCR in each case was different. The poor results, characterized by either very faint band or no band at all, could be due to a lack of cells used for the alkaline lysis template preparation method or the presence of components in alkaline lysate that was inhibitory to PCR.

Then, the four template preparation methods, including heat lysis described by Lee and Wong (3), whole-cell washing, alkaline lysis, and isolation of DNA, were directly compared for quality of fingerprinting patterns obtained. The whole-cell washing method suggested by Rademaker and de Bruijn (8) has been adopted in many journal articles, one of which being the work of Dombek *et al.* on rep-PCR DNA fingerprinting technique (1). This method is ideal, as researchers can save time as well as resources if whole cells can be used directly in rep-PCR amplification reactions. As shown in the results, this method produced bright and distinct band patterns and it was also very reproducible.

Alkaline lysis was another template preparation method described in the article by Rademaker and de Bruijn (8). They suggested using this method for microbial species that are difficult to lyse and do not release DNA during the early PCR cycles (8). Compared to the heat lysis method Lee and Wong (3) used, the main difference is that NaOH is added prior to incubation at 95°C, and a minor difference is that incubation is longer by 5 minutes. As discussed, this method was not reproducible, and it only produced faint band patterns if it worked at all. Thus, this method is the least recommended.

In order to obtain purified genomic DNA for use in PCR, genomic DNA can be isolated in many different ways. The original plan was to perform the method described by Rademaker and de Bruijn (8), but it was not performed for the following reasons. The procedure suggested in the literature, which required numerous raw reagents, was long and cumbersome. Also, a high yield was not needed, as PCR could amplify very low number of template. Therefore, Qiagen kit was used

instead for this purpose. While this method gave distinct bands at molecular weights higher than 1000 bp, it is important to consider whether this advantage is worth the extra time and resources invested. For the purpose of fingerprinting, it may not be necessary to obtain all the bands for comparison; as long as the current fingerprinting pattern allows the distinction of *E. coli* isolates from different sources, the objective is fulfilled. In addition, the advantage was not obvious for the *E. coli* isolate from duck, which means there may be other similar cases had more strains been analyzed. Furthermore, if a great number of *E. coli* need to be screened, this method would not be practical, considering the time and resources required. Thus, this method is not recommended.

Interestingly, the heat lysis method also gave clear and reproducible results. This was unexpected because Lee and Wong had little success in their project when this template preparation method was used and might have been caused by the limited optimization of the PCR process. Therefore, their source of problem may be unrelated to the template preparation method used. The heat lysis method is also fairly simple, but it does not have advantage over the cell washing method, which is even simpler. In addition, from the results obtained, it seemed that less high molecular weight bands were resolved compared to the cell washing method and the DNA isolation method.

As different ways of preparing the template will yield different amount of DNA available for PCR, it is important to know whether the difference in amount of available DNA is causing any observed difference. Therefore, the effect of varying dilutions of templates prepared using the different methods was investigated. There was no major difference in terms of band pattern and intensity between different dilutions of template for all methods except alkaline lysis. This could be because even the most diluted sample had sufficient template. Provided the reagents were limiting, the final amount of template amplified would be similar even if the template amount differed by one hundred fold, which again shows the robustness of the method. For the washed cells, the intensity of certain faint high molecular weight bands increased with further dilution of template could be due to the fact that certain inhibitory components were diluted. For the alkaline lysed cells, significant difference was observed between different dilutions. A possible explanation for the trend of decreased intensity with increased dilution, as observed for samples of *E. coli* from gull, is that the amount of available template was very low in the undiluted sample, and further dilution made available template even more limiting. A possible explanation for the increased and then decreased intensity with increased dilution, as observed for samples of *E. coli* from duck, is that the most concentrated sample had high concentration of an inhibitor. Recall that as the alkaline lysis method had poor reproducibility, the observation made from samples of *E. coli* from duck might not be significant.

In earlier experiments, the PCR product of the alkaline lysed sample was significantly fainter compared to the samples prepared using the other methods. Thus, with the hope to increase the output signal, the volume of template used in PCR was increased from 1 µl to 2 µl. Interestingly, for the alkaline lysis method, it was found repeatedly that a lower amount of template material used for PCR gave better results. 1 µl of starting material had given faint band pattern, but 2 µl of starting material had never given any visible band. This suggested excess NaOH or other material present in the alkaline lysate might be inhibiting PCR, and thus led to the investigation of effect of NaOH on PCR.

The answer could be found by just adding NaOH to purified DNA, and comparing PCR performed on purified DNA without NaOH and purified DNA with NaOH added. It was decided to go one step further and investigate the effect of NaOH added at two different amounts to templates prepared using all other methods. No band pattern was observed for neither of the alkaline lysed samples which had either 1 µl or 2 µl of template (FIG. 5); this was not surprising as this method had not been reproducible in the earlier experiments. The fact that isolated DNA, washed cells, and heat lysed cells gave bright band pattern without NaOH but gave no or very faint band pattern with NaOH added at the higher level suggests that presence of NaOH is inhibitory to PCR reaction.

In conclusion, the cell washing method for direct use in PCR, alkaline lysis method, heat lysis method, and DNA isolation method were compared. The cell washing method for direct use in PCR is recommended to be used, as this method saves time and resources and gives reproducible results characterized by bright and distinct band patterns. *E. coli* harvested from different growth phases did not result in different PCR outcome; thus, for simplicity, overnight culture can be used. The only template preparation method that gave unsatisfactory and non-reproducible results was the alkaline lysis method. In addition, it was found that addition of NaOH had an inhibitory effect on PCR reactions.

FUTURE EXPERIMENTS

To further evaluate the robustness of the method, stability of template stored over a period of time that is frozen and thawed repeatedly can be monitored, as it is possible that the material can degrade over long storage or the DNA quality can impaired by multiple freezing and thawing cycles. In addition, more studies can be

done on alkaline lysis method to investigate the cause of poor reproducibility and poor band patterns obtained with this method. One potential direction is to modify the original literature method by increasing the amount of cells lysed, as insufficient available template could be a contributing factor. Furthermore, if needed, the method can be applied to colony PCR and the robustness can be investigated.

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