ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (1): 106-111, 2008
ISSN 1682-296X
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Construction of New Generation Shuttle Vectors Harboring Bacteriophage T4 Lysozyme Gene (*gene e*)

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Abstract: The aim of this study was to construct new generation shuttle vectors harboring bacteriophage T4 lysozyme gene (*gene e*). T4 lysozyme gene was inserted into pMK3 and pNW33N shuttle vectors of *Escherichia coli-Bacillus* sp. to construct pMK3L and pNW33NL, respectively. pMK3L and pNW33NL recombinant plasmids were then transferred into *Bacillus subtilis* by electrotansformation. *Escherichia coli-Saccharomyces cerevisiae* shuttle vector bearing *gene e*, pRS416L, constructed in the previous study was also used for directly transformation into *Saccharomyces cerevisiae* in this study. *Escherichia coli* plasmid vectors bearing *gene e* could facilitate the isolation of plasmid DNA without using cell wall and cell membrane disruptive agents such as lysozyme and Etyhlene Diamine Tetra Acetic Acid (EDTA). Therefore, these lysates prepared by using Tris-EDTA (TE) buffer or distilled water of *Escherichia coli* including *gene e* could directly be used for transformation into target *Bacillus subtilis* or *Saccharomyces cerevisiae* without plasmid purification procedures.

Key words: Lysozyme, shuttle vector, transformation

INTRODUCTION

Lysozyme (1,4-β-N-acetylmurmidase, E.C.3.2.1.17) belongs to the class of enzymes that lyse the cell walls of bacteria as the bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine of the peptidoglycan is cleaved (Schindler *et al*., 1977). Lysozymes are commonly found in the major taxa of prokaryotes and eukaryotes, including the bacteria themselves and also in bacteriophages, but they may have different roles in different organisms. For example, animal and plant lysozymes are important players in the defense against bacterial invaders (Hancock and Scott, 2000; Beintema and Scheltinga, 1996; Ellison and Giehl, 1991; Ohno and Morrison, 1989; Taylor, 1983), phage lysozymes play a role in phage penetration into and/or release from the host cell (Rydman and Bamford, 2002; Young, 1992) while some bacterial lysozymes, called autolysins, allow controlled hydrolysis of the cell wall at sites of cell growth or cell division (Höltje, 1995; Shockman and Höltje, 1994).

T4 phage lysozyme is an enzyme produced in cells of *Escherichia coli* after infection with bacteriophage T4. The lysozyme encoded by bacteriophage T4 is a globular monomeric protein of 164 amino acid residues. The molecular mass of the enzyme is 18700 Da and the amino acid sequence has been determined (Tsugita and Inouye, 1968). It has an essential function in the life cycle of the phage: digestion of the host cell's peptidoglycan wall, resulting in lysis and the release of infectious virus particles (Weaver and Matthews, 1987). T4 lysozyme has also been the object of extensive genetic research.

Gene e encoding lysozyme E cloned from bacteriophage T4 has been expressed in *E. coli* (Perry *et al*., 1985; Owen *et al*., 1983) and then its nucleotide sequence has been determined (Owen *et al*., 1983). The phage T4 gene coding for lysozyme has been cloned into a plasmid under control of the (trp/lac) hybrid tac promoter and expressed in *E. coli* with no significant toxic effect to actively growing cells and produced active T4 lysozyme was at levels up to 2% of the cellular protein (Perry *et al*., 1985). Recombinant *E. coli* cells harboring pUC18L and pRS16L, which both expressing *gene e*, are grown in L and LB medium without lysis but burst out easily in hypotonic environment such as distilled water or 10 mM Tris-HCl, pH 8 buffer (Güzel *et al*., 2002).

In the present study, we aimed to create new generation shuttle vectors by using lysozyme gene to facilitate the isolation of plasmid DNA without external lysozyme application to obtain clear cell lysate used for transformation.

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MATERIALS AND METHODS

Microorganism strains, plasmids and growth media: *E. coli Bacillus* sp. shuttle vectors (pMK3 and pNW33N) and host bacterium *Bacillus subtilis* BR151 strain were obtained from BGSC (*Bacillus* Genetic Stock Centre, USA). The host bacterium *E. coli* DH5α was purchased from Stratagene (USA). Yeast *S. cerevisiae* (uracil gene mutated strain) was kindly provided by Jan Walker (Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, United Kingdom). Recombinant plasmid pRS416L (carrying uracil gene) bearing T4 lysozyme gene was created in previous study in Biotechnology and Genetic Laboratories, Department of Animal Science, Faculty of Agriculture, University of Çukurova (Güzel *et al*., 2002) (Fig. 1). *E. coli* and *B. subtilis* cells were cultured in LB (Luria Bertani) at 37°C. Growth media were supplemented with 40 μg mL^{-1} X-Gal plus 100 μg mL^{-1} ampicillin (Amp) and 10 μg mL^{-1} kanamycine (Km) to select recombinant *E. coli* and *B. subtilis* cells bearing pMK3L, respectively. 25 and 5 μg mL^{-1} choloramphenicol (Cm) were used to select recombinant *E. coli* and *B. subtilis* cells bearing pNW33NL. Recombinant pRS416L bearing *S. cerevisiae* cells were selected on yeast minimal medium without uracil (yeast nitrogen base without amino acids (6.7 g L^{-1}), yeast synthetic drop-out media supplemented without uracil (1.92 g L^{-1}) after autoclaving add glucose (50% w v^{-1}) 40 ml L^{-1}) at 30°C (Fig. 1).

Recombinant DNA techniques: All recombinant techniques were performed according to Sambrook *et al.* (1989) if otherwise is stated.

Construction of recombinant vectors: The lysozyme gene was inserted into *E. coli-S. cerevisiae* replicative shuttle vector pRS416 to construct pRS416L in the previous study (Güzel *et al*., 2002). The recombinant plasmid pRS416L was cut in BamHI restriction site to isolate the coding sequences DNA of Bacteriophage T4 lysozyme (*gene e*). To construct pMK3L, the *gene e* was directly inserted into BamHI restriction site in *lacZ* gene of the pMK3 vector. Lysozyme gene (*gene e*) was also inserted into BamHI restriction site in MCS region of the pNW33N vector in order to construct pNW33NL.

Isolation and preparation of transforming DNA

Isolation of plasmid DNA: Plasmid DNA was isolated according to alkaline denaturation method from *E. coli* and *B. subtilis*. RNase treatment was used to obtain purified plasmid DNA (Birnboim and Doly, 1979; Hardy, 1985). pRS416L plasmid from *S. cerevisiae* was isolated by using the glass bead method (Sobanski and Dickinson, 1995). Purified pMK3L, pNW33NL and pRS416L from *E. coli cells* were used for direct electrotransformation into either bacteria or yeast.

Preparation of lysate-1: The recombinant *E. coli* cells carrying pMK3L, pNW33NL or pRS416L were cultured and then harvested by centrifugation. The pelleted cells were washed with TNE (10 mM Tris-100 mM NaCl-1 mM EDTA, pH: 8.0) buffer. The pellets were again resuspended with 150 μL TE (10 mM Tris-1 mM EDTA, pH 7.6) or distilled water instead of using 150 μL external lysozyme (10 mg mL^{-1}) and then incubate 30 min at room temperature to digest the cell wall. The cell lysate were obtained by centrifugation and the total nucleic acids in

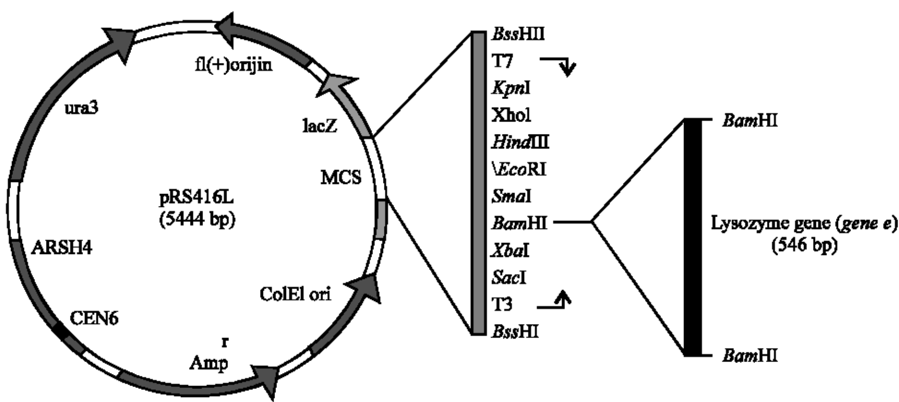

Fig. 1: Structure of pRS416L (5444 bp; pRS416 (4898 bp) plus lysozyme gene (546 bp))

the lysates were measured by spectrophotometer. The lysate-1 was then directly used for electrotransformation into bacteria or yeast cells.

Preparation of lysate-2: To prepare lysate-2, 200 μL SDS-NaOH (1% SDS (Sodium Dodecyl Sulphate), 0.2 M NaOH) solution was added into the lysate-1 and mixed well. Then 150 μL Na-acetate (3 M, pH 4.8) solution was added. After incubating 30 min on ice, the lysate was recovered by centrifugation. The total nucleic acids in the lysate-2 were measured in spectrophotometer and the lysate-2 was then directly used for electrotransformation into bacteria or yeast cells.

PCR detection of lysozyme gene (*gene e*): To amplify the *gene e* coding sequence, PCR reactions were carried out by using original pMK3, pNW33N, pRS416 and the recombinant pMK3L, pNW33NL and pRS416L from *E. coli*, *B. subtilis* and *S. cerevisiae* cells as the template in a 50 μL mixture containing 0.5 U of Taq DNA Polymerase, 1X standard PCR buffer, deoxynucleotide triphosphate (dNTP) at a concentration of 1 mM and 100 ng of each primer with the following cycling conditions: 94°C for 1 min, 59°C for 1 min and 72°C for 1 min for 35 cycles in thermal cycler. The following oligonucleotide primers were used: forward primer 5` CCTTCTATAAATACTTA 3` and reverse primer 5` CCAAGAGAAAGTAAACA 3`.

Transformation and statistical analysis: The pMK3L and pNW33NL constructs were transformed into *E. coli* (Hanahan, 1983) and *B. subtilis* (Vehmaanpera, 1989) by $CaCl_2$ method and electrotransformation, respectively. pRS416L was transformed only into *S. cerevisiae* by electrotransformation (Anonymous, 1997). Three microgram purified plasmid DNA or total nucleic acid was used for transformation. The recombinant colonies were counted and cfu μg^{-1} DNA or total nucleic acid was calculated. All experiments were conducted independently three times and H-Kruscall Wallis statistical analysis was applied to interpret the results.

RESULTS

To construct pMK3L and pNW33NL plasmid vectors, 546 bp BamHI cut DNA fragment of pRS416L bearing bacteriophage T4 lysozyme gene (*gene e*) was transferred into BamHI site of pMK3 and pNW33N shuttle vectors, respectively (Fig. 2, 3). Lac promoter regulates the *gene e* in all the recombinant vectors.

Ligation mixtures were used to introduce plasmids into *E. coli* cells and resulting *E. coli*/pMK3L or *E. coli*/pNW33NL transformants were grown with non-recombinant pMK3 or pNW33N in LB-Agar medium including 40 μg mL^{-1} X-Gal plus 100 μg mL^{-1} Amp and 25 μg mL^{-1} Cm, respectively. To select recombinant *E. coli* cells bearing pMK3L against *E. coli*/pMK3 cells, white colonies grown on solid growth media were directly collected and the plasmid was isolated from each random colony separately with using modified protocol (equivalent volume of TE or distilled water instead of external 150 μL lysozyme (10 mg mL^{-1})). To select recombinant *E. coli* cells harboring pNW33NL against *E. coli*/pNW33N cells, growth media was supplemented with 25 μg mL^{-1} choloramphenicol (Cm). All colonies bearing either pNW33N or pNW33NL grown on solid LB-Cm were collected and the plasmid was isolated from each random colony separately with using only TE or distilled water. Recombinant plasmid DNA isolated from *E. coli* cells that were easily burst without any lysozyme application was obtained pNW33NL.

Recombinant *E. coli* cells bearing either pMK3L or pNW33NL were grown in liquid growth media and then centrifugated. Hypotonic medium, either TE or distilled

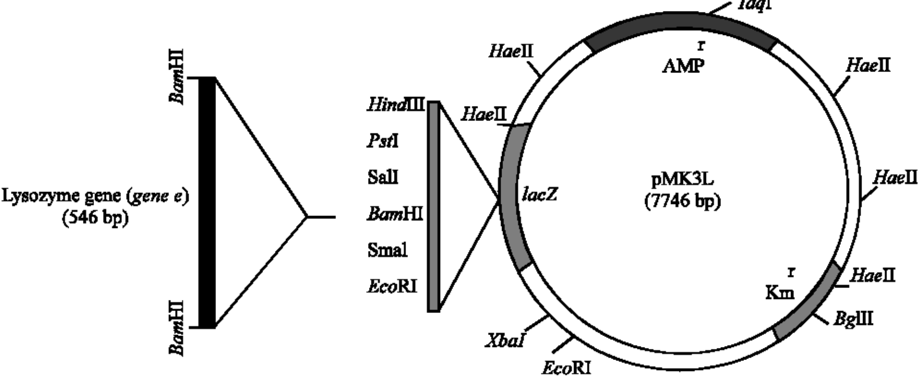

Fig. 2: Structure of pMK3L (7746 bp; pMK3 (7200 bp) plus lysozyme gene (546 bp))

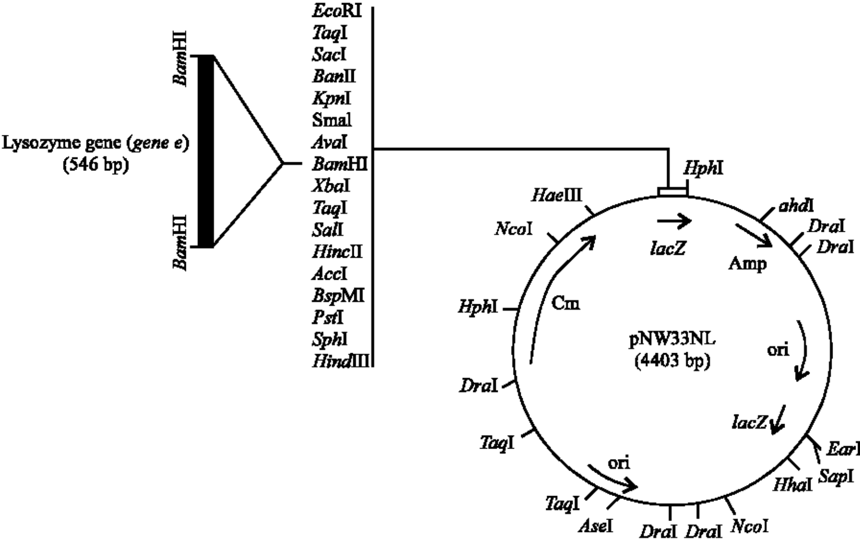

Fig. 3: Structure of pNW33NL (4403 bp; pNW33N (3857 bp) plus lysozyme gene (546 bp))

Table 1: Transformation frequency of lysozyme gene bearing recombinant plasmids

	Plasmids					
	pRS416L total nucleic acid (cfu μg^{-1})		pMK3L total nucleic acid (cfu μg^{-1})		pNW33NL total nucleic acid (cfu μg^{-1})	
Micro-organisms	Purified control DNA	Lysate-1	Purified control DNA	Lysate-1	Purified control DNA	Lysate-1
B. subtilis			286	166	27	3
	NA	NA	310	153	18	2
			295	159	25	3
S. cerevisiae	222	97				
	208	102	NA	NA	NA	NA
	217	84				

NA: Not Applied

water, was used to lyse of the bacteria to obtain clear lysate-1. It was shown that, recombinant *E. coli* cells harboring pMK3L and pNW33NL actively expressed T4 lysozyme gene to produce lysozyme E.

The original (pMK3, pNW33N and pRS416) and recombinant shuttle vectors (pMK3L, pNW33NL and pRS416L) isolated from *E. coli* cells were subject to insert analysis on 0.8% agarose gel electrophoresis. Lysozyme band (546 bp) was determined from the all recombinant shuttle vectors. The purified recombinant shuttle vectors either pMK3L or pNW33NL isolated from *E. coli* colonies were then electrotransformed into *B. subtilis* cells separately. Recombinant *B. subtilis* cells bearing pMK3L or pNW33NL were selected by adding 10 $\mu g\ mL^{-1}$ Km and 5 $\mu g\ mL^{-1}$ Cm antibiotics in LB growth medium, respectively. The recombinant plasmid pRS416L isolated from *E. coli* cells was electrotransformed into *S. cerevisiae* and all grown cells on yeast minimal medium without uracil were determined as recombinant *S. cerevisiae* cells bearing pRS416L. The result of the PCR amplification, all recombinant plasmids (pMK3L, pNW33NL and pRS416L) isolated either from *B. subtilis* or *S. cerevisiae* cells yielded 546 bp fragment of DNA carrying the gene encoding T4 lysozyme gene to determine *gene e* (data not shown). The results were confirmed that the recombinant colonies grown on selective media were all true transformants.

The recombinant shuttle vectors isolated from *E. coli* could be easily electrotransformed into their host cells by using lysate-1. When lysate-1 containing pMK3L or pRS416L was used for electrotransformation into *B. subtilis* and *S. cerevisiae*, respectively, transformation frequency was approximately half of purified DNA samples. Lysate-1 including pNW33NL was transformed into *B. subtilis* with the lowest transformation frequency among three recombinant vectors reported here (Table 1). No colony was detected for all recombinant vectors when the lysate-2 was used for electrotransformation.

The results of statistical analysis showed that differences between the application of pure plasmid DNAs and lysate-1 for all recombinant vectors are significant ($p<0.05$) (Table 2).

Table 2: H-Kruskal-Wallis statistical analysis results of recombinant colonies counted after electrotransformation

Plasmids	H-Kruscal-wallis statistical analysis	p
pMK3L	3.86	0.050
pNW33NL	3.97	0.046
pRS416L	3.86	0.050

DISCUSSION

Bacteriophage T4 lysozyme gene (*gene e*) was inserted into *E. coli-Bacillus* sp. shuttle vectors called pMK3 and pNW33N to construct new generation vectors named pMK3L and pNW33NL, respectively.

On the other hands, *gene e* was cloned and expressed in *E. coli* with pUC18 and pRS416 vectors via PCR amplification in our previous study (Güzel *et al*., 2002). *E. coli* cells harboring recombinant plasmids pUC18L and pRS416L were grown well in L and LB medium without lysis. The phage T4 gene coding for lysozyme has been cloned into a plasmid and expressed in *E. coli* with no significant toxic effect to actively growing cells (Perry *et al*., 1985). This lysozyme destroys the wall of its host, *E. coli*, at the end of infection to release progeny particles. Phage T4 contains two additional lysozymes encoded by *gene 5* that facilitate penetration of the baseplates into host cell walls during adsorption (Mosig *et al*., 1989). Lysozyme E weakens the bacterial cell wall but the weakening cell wall together with cell membrane and outer membrane resists osmotic pressure because L or LB growth medium contains enough osmotic stabilizers for *E. coli* cells such as NaCl. These results clearly showed that any *E. coli* plasmid vectors bearing *gene e* may facilitate the isolation of plasmid or chromosomal DNA without any cell wall and cell membrane disruptive agents such as EDTA, lysozyme and SDS (Güzel *et al*., 2002).

These studies also showed that new generation vectors can be constructed and even new transformation techniques can be developed for the recombinant shuttle vectors. Plasmid DNA is traditionally isolated by application of final concentration 10 μg mL^{-1} external lysozyme to disrupt cell wall. However, in this study, plasmid DNA can be isolated from *E. coli* cells harboring pMK3L, pNW33NL or pRS416L without any external lysozyme and EDTA applications. Moreover, various genes can be cloned with the recombinant vectors bearing T4 lysozyme gene and then transferred by electroporation without application of time consuming plasmid DNA purification procedures.

In conclusion, lysate-1 of *E. coli* cells harboring pMK3L, pNW33NL or pRS416L was compared with purified plasmids as shown in Table 1 and it was found that plasmid isolated by lysate-1 method had lower transformation frequency if compared with purified control plasmids in all experiments. Plasmid concentration in lysate-1 was not calculated correctly because of contaminating chromosomal DNA and RNA. Therefore, total nucleic acid was measured instead of plasmid DNA. If the data were analyzed by H-Kruscall Wallis statistical method, it was shown that there are significant differences for transformation frequencies between lysate-1 and control plasmid DNA ($p<0.05$) (Table 2). Although lysate-1 samples yield less transformants than purified control plasmids, it is still important for bacterial gene transfer experiments because of its simplicity and cost. To increase transformation frequency, further attempt had been made by adding SDS-NaOH and Na-acetate solutions to obtain lysate-2. However, no transformed colony had been obtained as shown in Table 1. It was probably because SDS might be toxic to the transformed host cells.

It was the first report according to our knowledge that new generation shuttle vectors were developed here by incorporating *gene e* to existing shuttle vectors. Also, lysate-1 gave satisfactory transformation frequency with new generation shuttle vectors developed in this study.

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