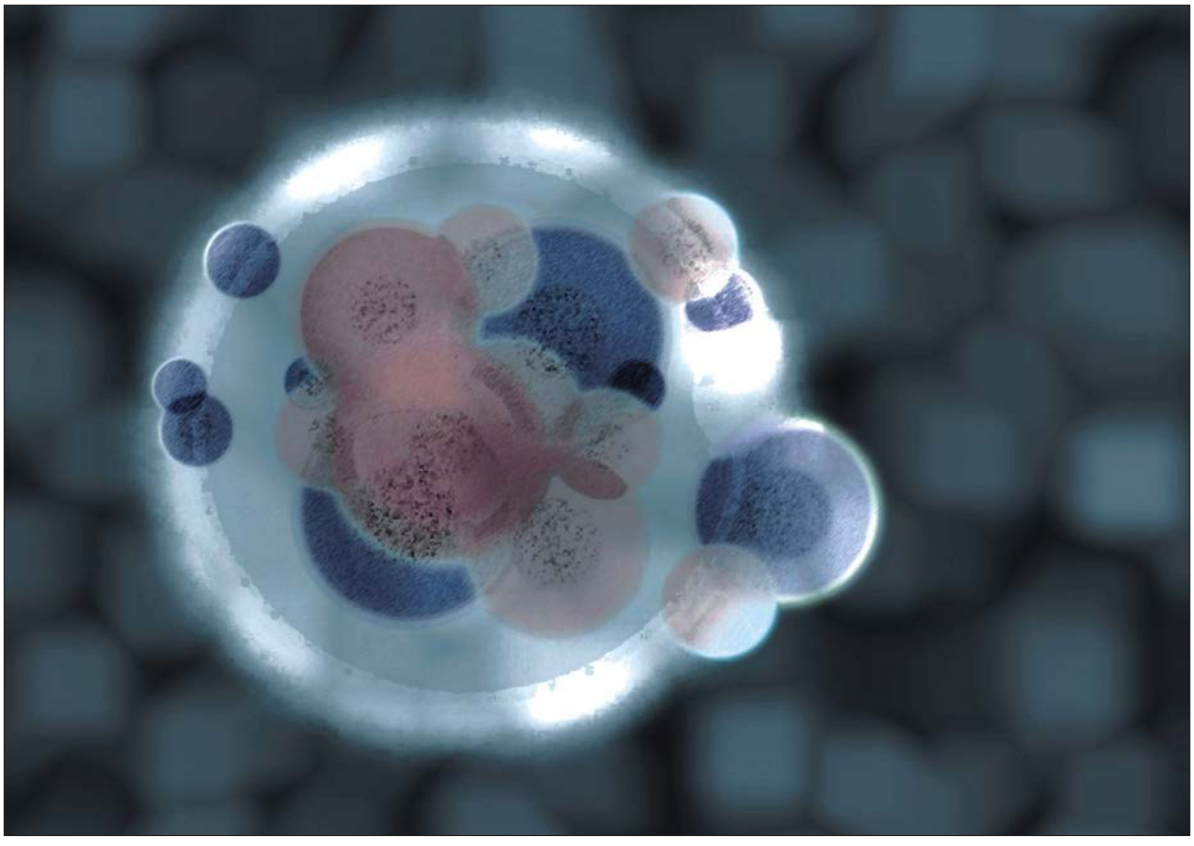

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 5 (3): 274-278, 2005
ISSN 1727-3048

Construction of a New Shuttle Vector for *Acetobacter xylinum* and *Escherichia coli*

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Abstract: Shuttle vector is a DNA molecule (e.g. plasmid) that is able to replicate in two different host organisms and can therefore be used to shuttle or convey genes from one to the other. In this study, a new shuttle vector was constructed that can replicate autonomously in *Acetobacter xylinum* and *Escherichia coli*. The new plasmid shuttle vector, designated pTMU30, was constructed from *E. coli* plasmid pUC18 and origin of replication (ori) of a cryptic plasmid pTMU20, which was originally isolated by us from a naturally occurring cellulose producing *Acetobacter* sp. The resulting shuttle vector, harboured resistance gene for ampicillin and capable of replicating in *Acetobacter xylinum* and *E. coli*. Transformation and replication of shuttle vector confirmed with antibiotic resistant experiments in the transformants bactrium. The vector was successfully transformed in *Acetobacter xylinum* and *E. coli*. This shuttle vector might used as a basis for genetic manipulation studies of *Acetobacter xylinum*.

Key words: *Acetobacter xylinum*, *E. coli*, shuttle vector, construction, plasmid

INTRODUCTION

Cellulose is the most abundant biopolymer in nature. The polysaccharide is synthesized mainly as an essentially component of the cell wall of plants, but cellulose is also synthesized by several procaryotic organisms. *Acetobacter xylinum*, gram negative bacterium, produces a cellulose membrane called bacterial cellulose on the surface of culture medium[1]. *Acetobacter* is also a model system for study of the enzymes and genes involved in cellulose biosynthesis[2]. The bacterial cellulose membrane has superior features to cellulose prepared from pulp such as high mechanical strength and a high degree of purity. Hence, It is expected to be useful as a new industrial materials. Different application have been proposed for the cellulose layer like[3]. Gelatinous membrane of microbial cellulose has numerous applications such as dressing, artificial skin used for temporary covering of wounds and reducing of infection rate in plastic surgery and dental implant. Moreover , it is reported antimicrobial effects about cellulose[4]. The organism also promises to be an important future source for cellulose in the textile, paper and lumber industries, provided its fermentation can be effectively scaled up. However, the bacterial cellulose productivity of *Acetobacter xylinum* is very low and therefore improvement is necessary for practical application[5]. Bacterial cellulose production by *Acetobacter xylinum* reqires many enzyme and substances. Although progress has been made in recent years on several aspects of cellulose biosenthesis, the present knowledge on the formation of this polysaccharide is still limited[6]. Gene manipulation is a way for high production of cellulose by *Acetobacter xylinum*. For the aim, a shuttle vector has been constructed to allow more efficient manipulation of DNA in *Acetobacter xylinum*. Shuttle vector is a DNA molecule that is able to replicate in two different host organisms and can therefore be used to shuttle or convey genes from one to the other. Shuttle vectors consist of double replicons: one half of the plasmid allows replication in a enteric bacterial host, the other half allows replication in any other host from which this replicative region has been derived. This type of vector is used for transfer of recombinant plasmids from *E. coli* into other bacteria and most eukaryotic hosts. Whereas one half of the vector is derived from *E. coli* specific vectors, the second half comes from often small cryptic replicons which were isolated from the individual host strain; they are providing the oriV specific to this host. The two parts are then joined by DNA manipulation. Shuttle vectors with new or improved features were constructed to enable facile genetic manipulations in the procaryotes and eucaryotes. In this study, Base of the new constructed shuttle vector was pUC18 plasmid. pUC18 vector is a

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small, high copy number, *E.coli* plasmids, 2686 bp in length. They are identical except that they contain multiple cloning sites arranged in opposite orientations[7]. pUC18 plasmid contain: (I) the pMB1 replicon rep responsible for the replication of plasmid. The high copy number of pUC18 plasmid is a result of the lack of the rop gene and a single point mutation in rep of pMB1; (ii) bla gene, coding for β-lactamase that confers resistance to ampicillin. It differs from that of pBR322 by two point mutations; (iii) region of *E. coli* operon lac containing CAP protein binding site, promoter Plac, lac repressor binding site and 5'-terminal part of the lacZ gene encoding the N-terminal fragment of beta-galactosidase. This fragment, whose synthesis can be induced by IPTG, is capable of intra-allelic complementation with a defective form of β-galactosidase encoded by host. In the presence of IPTG, bacteria synthesise both fragments of the enzyme and form blue colonies on media with X-Gal. Insertion of DNA into the multiple cloning sites located within the lacZ gene inactivates the N-terminal fragment of β-galactosidase and abolishes α-complementation. Bacteria carrying recombinant plasmids therefore give rise to white colonies[8]. The bla gene nucleotides 2486-2418 code for a signal peptide. The LacZ polypeptide corresponding to wt beta-galactosidase and essential for blue/white screening ends at position 236; another 30 codons in the same reading frame are derived from pBR322. The indicated rep region is sufficient to promote replication. DNA replication initiates at position 866 and proceeds in indicated direction. Plasmids carrying the pMB1 and ColE1 replicons are incompatible, but they are fully compatible with those carrying the p15A replicon[9]. The purpose of the present study was constructing a new shuttle vector in *Acetobacter xylinum* as a genetic tool for the future gene manipulation.

MATERIALS AND METHODS

Strain, media, plasmids and culture conditions: *E. coli* strain DH5α and *Acetobacter xylinum* strain ATCC 23768 were employed in all procedures. *E. coli* cell were grown in Luria Bertani (LB) medium (10 g L^{-1} Bacto tryptone, 5 g L^{-1} Bacto yeast extract and 10 g L^{-1} NaCl) at 37°C on a rotary shaker[10]. *Acetobacter* xylinum strains were grown in HS medium at 28°C[11]. HS medium was composed of 2% (W/V) glucose, 0.5% (W/V) Yeast extract, 0.5% pepone, 0.27% (W/V) Na_2Hpo 4 and 0.115% (W/V) citric acid. The plasmids were used pTMU20, which was originally isolated by us from a naturally occurring cellulose producing *Acetobacter* spp. and pUC18 plasmid.

Enzyme and chemicals: Enzymes BamHI, EcoRI, HindIII, XhoI, KpnI, NdeI, PstI, SacI, SalI, , ScaI, SmaI and Xbal1 were purchased from Roche Company. Bacto yeast extract, bacto trypton, bacto agar, glucose and citric acid were purchased from Merck company.

Antibiotic susceptibility test: *Acetobacter xylinum* and *E. coli* were tested for their sensivity to antibiotic by means of a disc diffusion method. It was investigated using commercial discs (Abtek Biological Ltd). The commercial antibiotic discks were placed on Muller Hinton agar plates previously seeded with 13 h broth culture of the test organisms. The plates were incubated at 37°C for 24 h , after which zone of inhibition were examined and interpreted accordingly[12].

Rapid screening for plasmids: Plasmid was prepared by the alkaline lysis method[13]. Rapid screening for plasmids were prepared as previously described[14]. A small number of cells were picked by touching a colony with a toothpick. They were then inoculated into a microfuge tube containing 300 μL of LB broth. After overnight growth, the cells were pelleted in a microfuge (10,000 rpm, 2 min). The cells were then suspended by vortexing in 20 μL of gel-loading mix (0.25% bromophenol blue and 30% glycerol). Then 40 μL each of chloroform and phenol (saturated with 1.0 M Tris-HCl, pH 8.0) was added. The mixture was vortexed at full speed for 1 min followed by centrifugation for 10 min at 12,000 rpm. Then 10 μL of the aqueous fraction was subjected to electrophoresis on 0.7% agarose minigel (5.2x6.0 cm) with TAE buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM Na_2-EDTA) at 100 volts for 30 min. The gel was stained with ethidium bromide (0.5 μg mL^{-1}) and the DNA bands were visualized under a UV transilluminator.

General DNA techniques: General DNA manipulations were performed with general methods[10]. DNA fragments required for sub cloning were gel-purified using a QiaEx kit (Qiagen). Preparation of bacterial plasmid DNA, transformation of *E. coli* and agarose gel electrophoresis (1%) were carried out as described[10]. Plasmids were transformed into the bacterial cells by electroporation[14].

Shuttle vector construction: Shuttle vector was constructed by the subcloning ori fragment of pTMU20 cryptic plasmid of *Acetobacter xylinum*. pTMU20 was digested with BamHI, EcoRI, HindIII, XhoI, KpnI, NdeI, PstI, SacI, SalI, , ScaI, SmaI and Xbal1 and the fragments cloned in pUC18 plasmid. Recombinant plasmids were transformed to *Acetobacter xylinum*.

Stability test: The stability of plasmids was tested as previously described[14], except that the subculturings were continued for over 20 generations and the antibiotic used was ampicillin.

Incompatibility test: To test for plasmid incompatibility, pTMU30, containing ori fragment of pTMU20 were transformed by electroporation with *Acetobacter xylinum* and *E. coli*[15], selecting for ampicillin resistance conferred by antibiogram test. After verifying the retention of both the resident and the incoming plasmids in the same cells by plasmid extraction, the cells were subcultured by diluting them in fresh LB and HS broth with ampicillin, favoring the retention of the exogenous plasmid. The subculturings were repeated to allow the cells to grow for about 20 generations. Then the cells were spread on LB and HS agar with ampicillin. Two hundred colonies were randomly picked and tested for ampicillin resistance using LB and HS agar plates.

Estimation of copy number: *Acetobacter xylinum* and *E. coli* DH5α containing pTMU30, at mid-log phase, were harvested and diluted with distilled water to obtain several concentrations ranging from 1 to $8x10^8$ cells mL^{-1}, as estimated by reading OD_{550} and OD_{600} for *Acetobacter xylinum* and *E. coli* DH5α, respectively. To achieve better accuracy, each suspension was subjected to plate count on LB and HS agar plate. The rapid-screening method was followed for plasmid extraction, gel electrophoresis and gel staining. The bands were scanned with Foto Analyst II (Fotodyne, Wisconsin, USA). pUC18 extracted from a similar amount of cells was employed as the standard for comparison.

RESULTS AND DISSCUSION

Acetobacter xylinum is a important industrial microorganisms used in the production of bacterial cellulose. New and higher product varieties could potentially be developed by using *Acetobacter xylinum* that have been genetically engineered to produce high cellulose[5]. Plasmids are used to transfer new genes into *Acetobacter xylinum*[2]. A marker gene must also be carried on the vector to allow for identification and selection of the genetically modified organisms. The commonly used marker genes produce biological activity that inactivates antibiotics[10]. We have use ampicillin resistant marker for the new vector. Acetobacter xylinum contains a complex system of plasmid DNA molecules. Plasmids of molecular weights or copy numbers different from the original wild-type, are found in different types of mutants in *Acetobacter xylinum*[16]. Some plasmids and host genotypes have been developed for recombinant protein production in *Acetobacter xylinum* but they have some limitation[4]. The present study, describes the development of a new shuttle vector, pTMU30, by insertion of pTMU20 ori fragment in pUC18 plasmid. *Acetobacter xylinum*, carrying pTMU20, could not grow in LB broth containing ampicillin, indicating that plasmid pTMU20 does not confer resistance to the antibiotic. In addition, no difference was observed between *Acetobacter xylinum* and the *Acetobacter xylinum* carrying pTMU20 in colony morphology, pigmentation, growth in SH and LB broth medium and amounts of exopolysaccharide produced. Therefore, pTMU20 is a cryptic plasmid. The construction strategy was dictated by the following consideration: (i) the nessesity to preserve the function regionals of pUC18, (ii) the requirement for suitable selective marker for experresion in *Acetobacer xylinum* and *E. coli*, (iii) the need to make the vector size as small as possible to decrease the probability of restriction- modification sites occurring. The most suitable candidate to combine with pTMU20 cryptic plasmid appeared to pUC18, a high copy number *E. coli* cloning vector based on the ColE1 ori site. Cells of *Acetobacter xylinum* produce large amounts of exopolysaccharide, which interferes with the extraction of plasmid DNA. *Acetobacter xylinum* do not produce cellulose in HS medium with shaking or in LB broth. A simple and rapid method for screening plasmids has been developed[14]. This method can be achieved in one step simply by phenol/chloroform treatment of the cells pelleted from a volume as small as 300 μL. The experiment from growing cells to the completion of plasmid extraction can be carried out using the same microfuge tube. The whole procedure takes about 40 min from harvesting the bacterial cultures to visualization of the DNA bands in agarose gel. Using this method, we have detected plasmids in *Acetobacter xylinum* and *E.coli*. To construct a physical map, pTMU20 DNA was digested with different restriction endonucleases and subjected to agarose gel electrophoresis (Fig. 1). The fragments cloned in pUC18 and the recombinant plasmid transformed into Acetobacter xylinum and *E. coli*. The results showed that one Xball fragment of pTMU20 (1 kb) is good candide for ori of pTMU30. Ampicillin resistant of *Acetobacter xylinum* and *E.coli* were shown transformation and replication of pTMU30 in the bacterium.To test for host range, pTMU30 was transformed into each of the gram-negative bacteria , selecting for ampicillin resistance. To ensure the efficiency of electroporation, experiments were repeated five times with triplicate samples in each repeat. The plasmids were then extracted from the transformants, digested with Xball, subjected to agarose gel

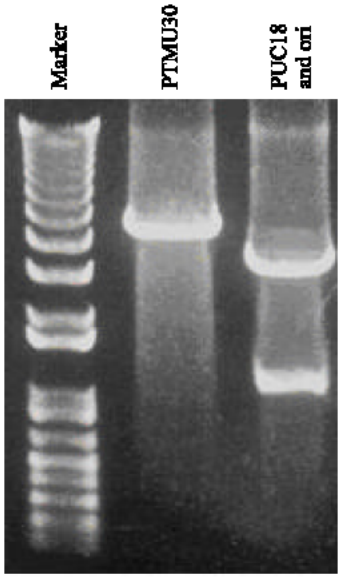

Fig. 1: Agarose gel electrophoresis of pTMU30 plasmid, pTMU30 digested with EcoRI (lane 2) and pTMU30 digested with XbaII (lane 3)

electrophoresis. The results showed that the plasmid was maintained without change in all *Acetobacter xylinum* and *E. coli* tested. Since no ampicillin resistant transformants of other bacteria were obtained, pTMU30 appeared to be a narrow-host-range plasmid.Stability tests on pTMU30 in *Acetobacter xylinum* and *E. coli* showed that about 96% of the colonies of both hosts retained the plasmid after 20 generations of growth without the selective pressure of ampicillin, indicating good stability in both *Acetobacter xylinum* and *E. coli*. The results of copy number estimation showed that the band of pTMU30 extracted from $4.92x10^8$ cells of *Acetobacter xylinum* had an intensity slightly stronger than that of pUC18 extracted from $2.34x10^8$ cells of *E. coli* DH5α. Since the *Acetobacter xylinum* cell number was about twice that of the DH5α, it seems safe to claim that pTMU30 had a copy number of 200 to 350 per cell, which was about half of 400 to 700, the copy number estimated for pUC plasmids[17]. pTMU30 was found to be compatible with other plasmids in both *Acetobacter xylinum* and *E. coli*. In both hosts, after 20 generations of growth, 98% of the cells retained pTMU30 and other native plasmids. To test for transformation efficiency of pTMU30, the plasmid was isolated from the bacterium and transformed by electroporation into *Acetobacter xylinum* and *E.coli*. Data obtained from four independent experiments showed that the efficiency ranged from $2.7x10^5$ to $8.0x10^6$ transformants/µg DNA. The results indicate that the *Acetobacter xylinum* strains tested here can be used as cloning hosts and electroporation is a feasible method to deliver pTMU30 derivatives into the bacteria. A good cloning vector should be small in size, multiple in copy number, stably maintained and bear unique restriction sites for commonly used enzymes, especially inside the antibiotic resistance genes. This study has shown that pTMU30 possesses most of these properties. It is very small and maintained stably at a high copy number without the selective pressure of antibiotic. With its high copy number, pTMU30 has the advantage of exerting gene dosage effects, so that high level expression of cloned genes can be obtained. In addition, pTMU30 is compatible with the commonly used cloning vector. pTMU30 is small plasmid (2.6 kb) compared to the previously reported plasmids[1]. pTMU30 was found to be maintained at very high copy number in the hosts. Since it is cryptic, the significance of its existence at a high copy number remains to be elucidated. In this study, success in construction of pTMU30 has shown that the shuttle vector allows insertion of foreign genes, demonstrating the potential of pTMU30 being developed into a vector for gene cloning in *Acetobacter xylinum*. In addition, we have shown that *Acetobacter xylinum* strain can be used in conjunction with pTMU30 to constitute vector/host systems in which recombinant plasmid can be delivered by means of electroporation.

ACKNOWLEDGMENT

This research was supported by Tarbiat Modarres University grant.

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