Transformation of *Streptococcus* sp. by cholera toxin gene

Swadesh R. Biswas, Sukanya Pal, Sukanta K. Sen and Sushil C. Pal

Department of Botany, Visva-Bharati, Santiniketan 731 235, India

Calcium chloride-treated cells prepared from *Streptococcus* sp., a curd-forming bacterium isolated from whey, were transformed by pCVD15 DNA containing the cholera toxin gene. The transformants conferred resistance to ampicillin and chloramphenicol. Transformation efficiency was slightly higher for the competent cells than for the uncompetent cells. The *Streptococcus* cells were found to be naturally competent.

CHOLERA is a life-threatening infectious disease caused by *Vibrio cholerae* colonizing the small bowel and secreting enterotoxin – cholera toxin – that leads to diarrhoeal disease characterized by profuse water loss. Cholera toxin consists of a single A subunit (27 kDa) and 5-B-subunit (11.6 kDa)[1] which binds to the GM ganglioside receptor of intestinal mucosal cells[2,3]. This binding can be prevented by antibody against B-subunit[4,5]. Thus B-subunit of cholera toxin gives protection against *V. cholerae* when antibody is located in the lumen of the intestine[6]. Circulating antibody has been proved to be ineffective in giving protection. However, protection by B-subunit is of short duration (few months). Attempts to administer vaccine orally has been unsuccessful because of lack of colonization ability of the strain[7]. For effective use of B-subunit as vaccine the gene has been cloned in *E. coli*[8], though the extraction and purification costs are very high. Recently trial is being made to inactivate the cholera toxin gene by modifying amino acid residues at positions 7 and 112 of cholera A subunit for use as oral vaccine by exchanging this modified toxin gene to the wild type strain[9].

An effective way has been devised by Haq *et al.*[10] by making transgenic plant as expression and delivery system for oral vaccine. Plants have expressed the B-subunit gene and feeding of such transgenic potatoes to mice induced protection to treated mice against cholera.

In an alternative way we have devised a system for delivery of oral vaccine against diarrhoeal disease through gram-positive lactic acid curd-forming bacterium *Streptococcus* sp. isolated from whey. Since the nontoxicogenic *V. cholerae* with only B-subunit gene incorporated into its chromosome does not colonize permanently in the intestine[7], gram-positive bacteria can be tested for this purpose. Lactic acid curd-forming bacteria are safe to eat through curd. Moreover, no extraction and purification of vaccine is needed. Frequent consumption of curd by this recombinant cultures will maintain the availability of vaccine in the lumen. Also the vaccine can be made at home by making curd, and in our laboratory it has been shown that curd, using this culture, can be made within 4 h. No data, however, is available whether this bacterium does colonize in the intestine.

In this study, *Streptococcus* sp. was transformed with recombinant plasmid pCVD15[7] containing the entire cholera toxin gene[7]. The transformants conferred resistance to ampicillin and chloramphenicol and had the ability to make curd. This result suggests that both the antibiotic genes have been expressed in *Streptococcus* sp. Since this plasmid does not replicate in gram-positive bacteria, it may be assumed that the plasmid has integrated into bacterial chromosome. Plasmid preparation from the transformant colonies in the presence of antibiotics was done and no plasmid of 18 kb (pCVD15) was found. The number of transformants (2×10^4 µg CsCl purified pDNA) indicates that this strain shows high frequency of gene transfer and thus can be manipulated genetically.

The working strain was found to be naturally competent. Transformation frequency of *Streptococcus* cells grown in TGE medium[11] was 1.5×10^4 (Table 1) whereas it is 2×10^4 when cells were made competent (CaCl method)[12]. This observation was verified by transforming both competent and uncompetent cells of *Streptococcus* with pUC19 DNA containing ampicillin resistance gene. The transformation frequency was higher for pUC19 DNA than for pCVD15 (Table 1). The mechanism of genetic exchange was proved to be dependent upon uptake of free DNA since no transformants were observed when 20 µg of pancreatic DNase (in 5 µl of 0.02 M MgCl, 0.02 M maleate, pH 6.5) was added to transforming DNA one hour before transformation. The transformants were destroyed after completion of experiments as safety measure.

Work is in progress to find out whether the toxin

Table 1. Transformants (chromosomal integrations) efficiency in competent and uncompetent cells of *Streptococcus* sp.

Plasmid used	Transformants (µg DNA)
Competent cells	
a) pCVD15, 5 ng	2×10^4
b) pCVD15, 10 ng	1.8×10^4
c) pUC19, 5 ng	2×10^5
d) pUC19, 10 ng	2.03×10^5
Uncompetent cells	
a) pCVD15, 5 ng	1.5×10^4
b) pUC19, 5 ng	8.4×10^4

gene has integrated into bacterial chromosome or it has expressed.

1. Sixmal, T. K., *Nature*, 1991, **351**, 371–377.
2. Vine, H. L. and Cuatrecass, P., *Pharmacol. Ther.*, 1981, **12**, 167–169.
3. Gill, D. M. and Meren, R., *Proc. Natl. Acad. Sci. USA.*, 1978, **75**, 3050–3054.
4. Clements, J. D. and Finkelstein, R. A., *Infect. Immun.*, 1978, **22**, 709–713.
5. Gyles, C. L., *Infect. Immun.*, 1974, **9**, 564–570.
6. Mekalanos, J. J., Collier, R. J. and Roming, W. R., *J. Biol. Chem.*, 1979, **254**, 5855–5861.
7. Kaper, J. B., Lockman, H., Baldini, M. M. and Levine, M. M., *Biotechnology*, 1984, 345–349.
8. Mekalanos, J. J., Swartz, D. J., Pearson, G. D., Harford, N., Groyne, F. and de Wilde, M., *Nature*, 1983, **306**, 551–557.
9. Claudia, C. H., Louise, S. T., Mary, B. F., Vernon, L. M., Burnette, W. N, Haevey, R. K., Linda, A. S. *et al.*, *Infect. Immun.*, 1994, **62**, 3051–3057.
10. Haq, T. A., Mason, H. S., Clements, J. D. and Arntzen, C. J., *Science*, 1995, **268**, 714–716.
11. Biswas, S. R., Ray, P., Johnson, M. C. and Ray, B., *Appl. Environ. Microbiol.*, 1991, **57**, 1265–1267.
12. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, N.Y., 1989.

ACKNOWLEDGEMENT. S. R. Biswas thanks CSIR, New Delhi, for the award of SRA.

Received 29 January 1996; revised accepted 2 September 1996

Occurrence of a 'mariner' element in the silkworm *Bombyx mori*

S. Mathavan, S. Mayilvahanan and A. Jeyaprakash*

Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India
*Department of Entomology and Nematology, University of Florida, Gainesville, Florida, USA

Occurrence of mariner element in *Bombyx mori* is reported here for the first time using degenerate primers in polymerase chain reaction (PCR) technique. The PCR product (Bm.MAR1) has been cloned and sequenced; it is 457 bp in length. DNA and peptide sequences of *Bombyx* mariner show a high degree of similarity with the mariner of the predatory mite, *Metaseiulus occidentalis* and low degree of similarity with the lepidopteran *Hyalophora cecropia*. Since no transcripts could be identified for the mariner of *B. mori* in northern hybridization, it appears to be a transcriptionally defective element.

SINCE McClintock[1] discovered mobile genetic element in maize, presence of such transposable elements has been reported from a number of other eukaryotic genomes[2]. A new transposable element 'mariner' has been identified and cloned from an unstable white mutant of *Drosophila mauritiana*, a sibling species of *D. melanogaster*[3]. The original mariner element is 1286 bp in length having 28 bp inverted repeats and an open reading frame (ORF) coding for a polypeptide of 345 amino acids. It is functional for germline transformation of *Drosophila*[3]. Lidholm *et al.*[5] identified a mariner element in *Hyalophora cecropia* and showed about 40% DNA sequence identity with *Drosophila* mariner element. Using degenerate PCR primers, Robertson[6] has shown the widespread occurrence of mariner elements in insects (17% among 400 species screened). Using the same PCR primers, Jeyaprakash and Hoy[7] cloned a mariner element from a predatory mite *Metaseiulus occidentalis* and showed the presence of functional open reading frame. They constructed a transformation vector containing the mariner and made stable transformation of the predatory mite. Despite the fact that more than 400 species have been screened, the presence of mariner elements in the silkworm *Bombyx mori* has not been detected. This paper is the first report on the occurrence of mariner elements in *Bombyx mori*.

Bombyx mori strains (NB_4D_2, NB_{18}, KA, NB_7) were obtained from the Central Sericultural Research and Training Institute, Mysore. The genomic DNA isolation (from larval fatbody cells) was performed as described by Sambrook *et al.*[8]

The PCR was carried out using degenerate primers [MAR124F-5′-TGGGTNCCNCAYGARYT (17-mer) and MAR276R-5′-GGNGCNARRTCNGGNSWRTA (20-mer)[6]]. The conditions for PCR were: *Taq* polymerase buffer (1x), 2.5 mM $MgCl_2$, 150 µM dNTPs, 800 nM each primer, 0.25 units *Taq* polymerase, and 1–100 ng genomic DNA in a 25 µl total reaction volume[9]. For 35 cycles, amplification conditions were: denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 74°C for 1 min in Perkin-Elmer Thermol cycler. An aliquot from each reaction (10 µl) was electrophoresed on a 2% TBE gel. The amplified PCR product was purified by electro-elution and cloned into 'T' tailed plasmid. The clone was termed as pBm.MAR1.

The recombinant plasmid containing pBm.MAR1 was isolated and purified following standard methods[8]. The recombinant plasmid was transformed into *E. coli* strain DH5α; the plasmid DNA was isolated, purified and sequenced by conventional dideoxy method in an automated sequencer[10]. The sequence of *B. mori* mariner element was translated and compared with the translated sequence of mariner element of other species using the software 'GCG' (Genetic Computer Group, Wisconsin-Madison, USA)[11]. Mariner sequences of other species were recovered from 'EMBL Gene bank' for comparison.

Using degenerate primers (MAR124F and MAR276R)