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MEHMET SAİT EKİNCİ

HARRY J. FLINT

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Research Article

Transformation and Stability of Cloned Polysaccharidase Genes in Gram-Positive Bacteria

Mehmet Sait EKİNCİ
Kahramanmaraş Sütçü İmam University, Faculty of Agriculture, Department of Animal Science, Kahramanmaraş - TURKEY
e-mail: sekinci@ksu.edu.tr

Harry J. FLINT
Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, Scotland, UK.

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Abstract: Polysaccharidase genes from rumen bacteria were transferred to and expressed in ruminal and non-ruminal Gram-positive bacteria. The transformation efficiency and genetic stability of polysaccharidase genes in bacteria from different habitats were investigated. PCR amplification of cloned polysaccharidase genes from *Escherichia coli, Lactococcus lactis, Enterococcus faecalis, Streptococcus sanguis* and *S. bovis* strain 26 showed that rearrangement of plasmid and the gene fragment did not occur. The DNA band sizes from all primers agreed with the expectations of some transformants of *S. bovis* JB1 being rearranged. In spite of the rearrangement in *S. bovis* JB1 polysaccharidase activities were conserved. Growing in the continuous culture proved the plasmid survival and gene stability of the recombinant microorganisms.

Key Words: Polysaccharidase Gene, Stability, Transformation, Enzyme

Klonlanmış Polisakkaridaz Genlerinin Gram-Pozitif Bakterilere Transformasyonu ve Kararlılığı

Özet: Rumen bakterilerine ait polisakkaridaz genleri ruminal ve ruminal olmayan Gram-pozitif bakterilere transfer edilerek eksprese edildi. Polisakkaridaz genlerinin farklı ortamlardan gelen bakterilerdeki transformasyon etkinliği ve genetik kararlılığı incelendi. *Escherichia coli, Lactococcus lactis, Enterococcus faecalis, Streptococcus sanguis* ve *S. bovis* 26 suşundan alınan polisakkaridaz gen klonlarının PZR (Polimeraz zincir reaksiyonu) ile amplifikasyonu *S. bovis* JB1 daki bazı değişmeler dışında plasmid ve genlerin herhangi bir değişikliğe uğramadığı ve elde edilen DNA bantlarının aynı olduğunu gösterdi. *S. bovis* JB1 deki bazı yeniden düzenlenmelere rağmen polisakkaridaz aktiviteleri korundu. Rekombinant mikroorganizmalardaki plasmidlerin yaşamını sürdürmesi ve genlerin kararlılığı devamlı kültürlerde üreme ile ispatlanmıştır.

Anahtar Sözcükler: Polisakkaridaz Geni, Kararlılık, Transformasyon, Enzim

Introduction

Gene transfer systems are powerful tools in genetic analysis, and essential for the construction of recombinant bacteria. Genetic transfer systems have been developed for predominant species of rumen bacteria (1,2) and for other non-ruminant Gram-positive species (3-5). Genetic transformation requires the development of appropriate vectors that permit the manipulation and amplification of genes in vitro and the introduction and expression of those genes in ruminal and Gram-positive bacteria. To study the replication and expression of genes in ruminal and Gram-positive bacteria, appropriate replicon and promoter elements are required (6,7). *Escherichia coli* based antibiotic resistance shuttle plasmids have been largely used to construct recombinant DNA. A variety of techniques have been used to transfer DNA into various species of bacteria including electroporation (8,9), protoplast fusion (10) and conjugation (11).

A number of genes encoding for plant cell wall polysaccharidases have been isolated from both Gram-positive and Gram-negative rumen bacteria, including *Ruminococcus flavefaciens, R. albus, Fibrobacter succinogenes, Butyrivibrio fibrisolvens* and *Prevotella ruminicola* (12). So far such genes have been studied in *E. coli*, where expression is often anomalous because of internal translational start sites and where extensive proteolysis breakdown can occur (13). Furthermore, any

product normally glycosylated in ruminal bacteria will not be so modified in *E. coli* (14). Therefore, it is of interest to examine the expression of cloned genes in Gram-positive species more closely related to ruminal species (8, 15-17).

Thus, in this work we studied the transformation of polysaccharidase genes into ruminal and other Gram-positive bacteria, and their stability in these hosts.

Materials and Methods

Strains, plasmids and growth conditions

The high copy number pAMβ1 derivative pIL253 (18) and *L. lactis* IL2661 were from Y. Duval Iflah (INRA, France). The *E. coli*, Gram-positive shuttle vector pVA838 (19), *Streptococcus sanguis* DL1 and *S. bovis* JB1 were supplied by T.R. Whitehead (USDA, Peoria, USA). *S. bovis* 26 was supplied by the Rowett Research Institute (Scotland, UK), *E. faecalis* JH2-SS is a derivative of *E. faecalis* JH2-2 that carries mutations conferring resistance to streptomycin and spectonomycin. *E. coli* strains were routinely grown in LB medium. *E. coli* DH5α and *E. coli* HB101 were used as hosts for pUC based constructs, with 50µg/ml ampicillin for selection. *E. coli* V850 (19) was used as a host for pVA838 vectors, with 150 µg/ml erythromycin for selection. Selection in Gram-positive hosts was with 5 µg/ml erythromycin. *S. bovis* was routinely maintained anaerobically in a rumen fluid-based medium (20) containing 0.2% soluble starch, 0.2% glucose and 0.2% cellobiose as energy sources (M2GSC medium). M2S refers to the same medium with 0.2% soluble starch as the sole added energy source. For enzyme determinations, *S. bovis* was grown in M17 medium (Oxoid Ltd, London, UK) modified by the addition of 0.1M MOPS buffer pH6.8 and the inclusion of the appropriate carbohydrate substrate, unless otherwise stated, at 0.2% (w/v). Other Gram-positive bacteria were grown in M17 medium.

Molecular biology procedures

The restriction (Pharmacia LKB Biotechnology, Central Milton Keynes, UK) and ligation (Boehringer, Mannheim, Germany) reactions were carried out as described by Maniatis *et al.* (21). *E. coli* cells were prepared for transformation by method of Hanahan (22). Transformed cells were plated onto LB agar plates in an overlay of LB medium containing 0.5% agar, 50mg/ml ampicillin and 0.1% lichenan or 0.5% oat spelt xylan or 0.1% CMC (carboxymethyl cellulose). The transformation method for *S. bovis* JB1, *L. lactis* and *E. faecalis* followed published procedures respectively (8,9,23).

PCR amplification of DNA

Reactions for DNA amplification were carried out based on the guidelines given by Sambrook *et al.* (24). The reactions were run in either the Perkin Elmer 480 (Perkin Elmer, Bucks., UK) or the Techne PHC-3 (Techne, Cambridge, UK) thermal cycler as follows:

Cycle	Denaturation	Primer annealing	Primer extension
1	5 min at 94°C	2 min at 45-60°C	3 min at 72°C
2-29	1 min at 94°C	2 min at 45-60°C	3 min at 72°C
30	1 min at 94°C	2 min at 45-60°C	10 min at 72°C

Enzyme determinations

Plate tests for CMCase, xylanase and lichenase, in which plates carrying substrate overlays were stained with Congo Red, were performed as described previously (25). Reducing sugar release from polysaccharide substrates was determined by the method of Lever (26). Detection of CMCase, xylanase and β-glucanase activities following SDS-PAGE was as described previously (13). Culture supernatants were assayed either directly or concentrated using Amicon filter paper, while cell pellets were resuspended in 50 mM Na phosphate buffer pH 6.5, subjected to two cycles of freeze thawing, and sonicated extensively (5 times 1 min, MSE Soniprep, maximum setting) before assay.

Results

Cloning strategy

The transformation and stability of polysaccharidase genes from the rumen bacterium *R. flavefaciens* strain 17 were investigated. A xylanase, lichenase and cellulase gene of this strain had to be cloned to obtain suitable constructs for genetic analysis. Therefore, the following gene cloning strategies were performed. In previous work a lambda bacteriophage clone, L9, carrying 11.5kbp fragment of *R. flavefaciens* 17 was found to encode xylanase, and lichenase [β-(1,3-1.4)-glucanase] (27), and also a 1.5 kbp fragment encoding only the

lichenase activity was isolated from a *Hind*III partial digest of phage clone L9 by subcloning in pUC13 (L956). In addition, a 2.6 kbp fragment encoding only the xylanase activity was isolated from a *Eco*RI-*Bam*HI partial digest of phage clone L9 by subcloning in pUC13 (L9XRB) (27). Sequencing these fragments showed that both activities are encoded by a single 2.406 kbp open reading frame corresponding to the *xyn*D gene (13). The following strategy was used to obtain suitable cloning sites for cloning the whole *xyn*D gene into pVA838 shuttle vector. First a 0.5-kb *Bam*HI-*Sal*I fragment of L956, which encodes part of the lichenase domain, was ligated into the *Bam*HI-*Sal*I sites of the L9XRB subclone and transformants selected in *E. coli* DH5α on LB-ampicillin plates. This new 3.1-kb fragment encoding lichenase and xylanase activities was named L9XRBS/pUC13. The insert containing the whole *xyn*D gene was then excised from L9XRBS/pUC13 by digestion with *Pst*I and ligated into the same site in the pUC18 vector, and new construct named L9XRBSS/pUC18. The insert containing the *xyn*D gene was then excised from L9XRBSS/pUC18 by digestion with *Sph*I and *Sal*I and ligated into the same sites in the *E. coli*/*Streptococcus* shuttle vector pVA838. The ligated DNA was transformed into *E. coli* DH5α and recombinant colonies were selected on LB-chloramphenicol or erythromycin plates. Colonies were transferred onto LB-chloramphenicol or erythromycin plates containing lichenan or xylan identified as lichenase or xylanase-positive by clearing zones around the colonies. The recombinant plasmid was termed pVAXL. The pSEXL1 construct was also made by fusion of the high copy number pAMβ1-derived vector pIL253 and pUC18/L9XRBS clone. pSEXL1 was made to increase enzyme production.

The carboxymethyl cellulase (CMCase) enzyme encoding gene of *R. flavefaciens* was cut with *Sma*I/*Hind*III and the DNA fragment inserted into pBR322 plasmid cut with EcoRV/*Hind*III restriction enzymes. The insert containing the CMCase gene was then excised from pBR322/CMCP by digestion with *Sph*I and *Sal*I and ligated into the same sites in the shuttle vector pVA838. The ligated DNA was transformed into *E. coli* HB101 and recombinant colonies were selected on LB-chloramphenicol or erythromycin plates. Colonies were transferred onto LB-chloramphenicol or erythromycin plates containing carboxymethyl cellulose identified as CMCase-positive by clearing zones around the colonies. The recombinant plasmid was termed pVACMCI. Transformable and transformation efficiency of all constructs to the Gram-positive hosts (Table) was then investigated.

Table. Shuttle and Recombinant Plasmid Used for Transformation by Electroporation into Gram-Positive Bacteria and Their Transformation Frequencies.

Bacteria	Construct	Frequency μg^{-1} DNA	Pulse time (msc)	Resistance Ω
S. bovis JB1	pVAXL	$2x10^2$	8.8	400
	pVA838	$1.2x10^3$	6.7	400
	pSEXL1	$1.8x10^2$	7.8	400
S. bovis 26	pVAXL	$1x10^2$	6.2	400
	pVA838	$1.3x10^2$	5.8	400
S. sanguis DL1	pVAXL	$8x10^1$	4.8	400
	pVA838	$4.6x10^3$	5.2	400
E. faecalis JH2- SS	pVAXL	$5.4x10^3$	9.8	400
	pVA838	$1.25x10^4$	9.2	400
L. lactis NCIMB 8622	pVAXL	$3x10^4$	4.8	200
	pVA838	$1x10^4$	4.9	200
IL1403	pVAXL	$3x10^4$	6.2	200
	pSEXL1	$1x10^4$	5.6	200
MG1363	pVAXL	$3x10^3$	4.8	200
	pSEXL1	$5x10^2$	4.2	200
IL2661	pSEXL1	$2.1x10^4$	5.2	200

Stability of polysaccharidase genes in Gram-positive hosts

PCR amplification was done to find out whether any rearrangement had occurred in the cloned gene (*xyn*D and CMCase) fragment after transfer into Gram-positive hosts. pVAXL was found to be stably maintained in all the above-mentioned hosts. PCR amplification of *xyn*D from *E. coli*, *L. lactis*, *E. faecalis*, *S. sanguis* and *S. bovis* 26 showed that rearrangement of plasmid and the *xyn*D gene did not occur and the band sizes from all primers agreed with the expectations (Figure 1). However, some transformants of *S. bovis* JB1 were found to be rearranged and two unexpected bands were obtained from rearranged DNA due to multiple primer binding sites (JB1/pVAXL^R), while other transformants were not (JB1/pVAXL) (Figure 1). In spite of the rearrangement in *S. bovis* JB1/pVAXL^R both lichenase and xylanase activity were conserved (Figure 2). The active polypeptides from JB1/pVAXL^R detected were approximately 66 and 60 kDa. Both showed xylanase and lichenase activity (data not shown). These bands were unexpected since deletions at the N or C terminus of *xyn*D are expected to remove one of the two activities. *S. bovis* JB1/pVAXL showed different activity bands from *S. bovis* JB1/pVAXL^R. Full-size *xyn*D product was detected from *L. lactis*, *E. faecalis* and *S. sanguis* containing pVAXL on both lichenan and xylan containing SDS-PAGE (28). Same rearrangement of cloned CMCase gene was also observed in some *S. bovis*/pVACMCI cells while no rearrangement was

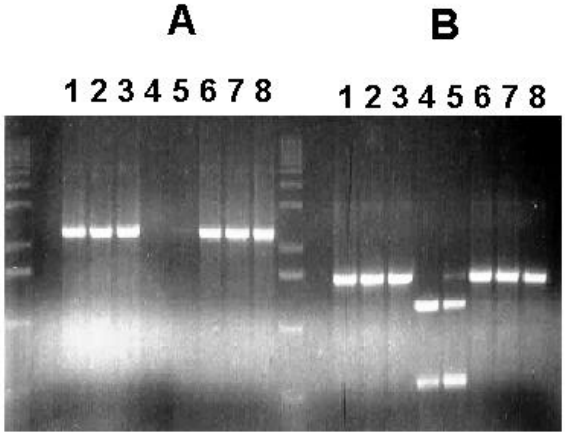

Figure 1. PCR Amplification of *xyn*D Gene from Different Hosts Using Two Different Primers. Unnumbered lines show the molecular weight marker. A: Lica11-β-glucanase, B: Lica11-Lica3 primers. Lane 1: *E. coli*/pVAXL, lane2: *S. bovis* 26/pVAXL, lane 3: *S. bovis* JB1/pVAXL, lane 4-5: *S. bovis* JB1/pVAXL^R, lane 6: *L. lactis*/pVAXL, lane 7: *E. faecalis*/pVAXL, lane 8: *S. sanguis*/pVAXL.

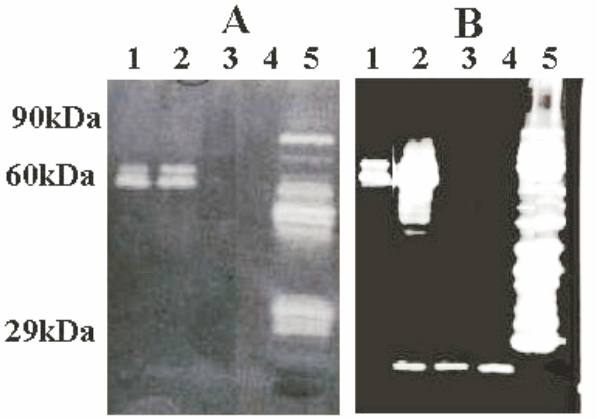

Figure 2. Detection of A: xylanase and B: lichenase activity of cloned genes from *S. bovis* JB1 on SDS-PAGE. Lane 1-2: cell and supernatant activity of *S. bovis* JB1/pVAXL^R grown on M2S medium; lane 3-4: cell and supernatant activity *S. bovis* JB1 grown on M2S medium; lane 5: *E. coli*/pVAXL.

observed in other Gram-positive bacteria. According to restriction enzyme analysis, the rearranged pVACMCI clone did not cut with *Sph*I endonuclease enzyme, which was previously used for insertion of the CMCase gene fragment into the same site of the pVA838 vector.

Plasmid survival and gene stability was proved by growing the *S. bovis* JB1/pVAXL, *S. bovis* JB1/pVACMCI and *S. bovis* JB1/pVAXL^R under anaerobic conditions and plating out under aerobic conditions as described below. *S. bovis* JB1/pVAXL and *S. bovis* JB1/pVAXL^R were inoculated into M2 broth medium, to test plasmid survival, without antibiotic, and grown until the 160th generation. After the 160th generation 10^{-9} dilution of broth culture was plated out at 37°C for 16 h and then randomly some of the colonies were tested for CMCase, lichenase and xylanase activities on the CMC, xylan and lichenan overlay containing plate in the presence of 5 µg ml^{-1} erythromycin. All of the colonies grew and exhibited CMCase, xylanase and lichenase activities. Although endogenous enzyme activity cannot be distinguished from the clone enzyme activity, growing on antibiotic containing medium confirmed that the plasmid and *xyn*D gene are stably maintained under *in vitro* conditions

Discussion

Polysaccharidase genes containing construct, which were made in this work, were successfully transferred to different Gram-positive hosts by electroporation. The stability of cloned genes was also investigated. PCR

amplification was carried out to determine whether any rearrangement had occurred in the cloned gene (*xyn*D and CMCase) fragment after transfer into Gram-positive hosts.

How the rearrangement happens in some *S. bovis* JB1 cells is not clear. Some possibilities are as follows:

If plasmids carry sequences homologous to those of the host organism they could integrate into the chromosome. Such insertions have been used for generating mutations (29), for mapping unselectable genes (30), for fast analysis of target genes (31) and for cloning specific segments of the host genome (29). By this method α-amylase and endoglucanase genes of *Clostridium thermocellum* were integrated in the *Lactobacillus plantarum* chromosome (32), and heterologous plasmid DNA was integrated into the chromosome of *Streptococcus pneumoniae* (33). The same integration may happen between *S. bovis* JB1 chromosome and *xyn*D and CMCase genes, since both carry β-glucanases and have sequence similarity (2).

For a genetically engineered organism to have an affect on rumen fermentation it must persist at a significant level for a certain time period within the rumen. Having constructed the required genetically engineered rumen bacterium, the organism must be re-introduced into the rumen environment. This appears to be the most problematic step in manipulating rumen fermentation. Many strains of bacteria seem to lose the ability to compete successfully in a mixed culture if they have been maintained in selective medium for long periods. Flint *et al.* (34) have shown that recent isolates of *S. ruminantium* can establish and be maintained on reintroduction to the rumen of sheep. Older isolates of *B. multiacidus* (34) and *B. ruminicola* (35) have been shown not to be maintained when reintroduced into the rumen. In contrast, Miyagi *et al.* (36) have reported that antibiotic resistant transconjugated *R. albus* A3 was found to be constant for 14 days in the rumen of the goat.

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