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# Expression of Bifunctional Genes Encoding Xylanase and β(1,3-1,4)-Glucanase in Gram-Positive Bacteria

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**Abstract:** The xylanase and β(1,3-1,4)-Glucanase activities encoding gene from the cellulolytic rumen anaerobe bacterium was cloned into *Escherichia/Streptococcus* shuttle vector for introduction into Gram-positive bacteria, including the rumen facultative anaerobe bacterium *Streptococcus bovis*. Activities due to the cloned gene decreased in the stationary phase in batch cultures of *S. bovis*, reflecting the sensitivity of the cloned enzymes to inactivation in the presence of accumulated lactic acid. Cloned gene activity was detected in the culture supernatant, indicating recognition of the cloned gene signal peptide by Gram-positive bacteria.

**Key Words:** Gene expression, Gram-positive bacteria, Enzyme

## Ksilanaz ve β(1,3-1,4)-Glukanaz Enzimlerini Kodlayan Çift Fonksiyonlu Genlerin Gram-Pozitif Bakterilerde Ekspresyonu

**Özet:** Rumen anaerop selulolitik bakterisine ait Ksilanaz ve β(1,3-1,4)-Glukanaz aktivitelerini kodlayan gen, fakültatif anaerop *Streptococcus bovis* dahil bazı Gram-pozitif bakterilere transfer edilmesi için *Escherichia/Streptococcus* plasmid vektöre klonlandı. Klonlanmış genden kaynaklanan enzim aktiviteleri *S. bovis*'in sıvı kültürlerinde duraklama fazından itibaren düşüş göstermektedir. Bu düşüşler klonlanmış enzimlerin ortamda bulunan birikmiş laktik aside olan duyarlılıktan kaynaklanmaktadır. Klonlanmış gen aktivitelerinin kültür sıvısı içine salınması klon genin işaret peptidinin Gram-pozitif bakteriler tarafından tanındığını göstermektedir.

**Anahtar Sözcükler:** Gen ekspresyonu, Gram-pozitif bakteriler, Enzim

## Introduction

The ruminal ecosystem consists of diverse microbial species. These microorganisms have complex plant cell wall polysaccharides degrading enzymes. Polysaccharidase encoding genes of rumen microorganisms have been extensively studied for biotechnological application. The predominant ruminal species are oxygen sensitive strict anaerobes, but genes derived from them, especially those concerned with the breakdown of plant cell wall polysaccharides, have potential for exploitation, particularly if they can be expressed in more amenable, oxygen tolerant host species. Previous work has studied the expression of genes from ruminococci in tobacco plants, bacteria and yeast (1-3). The potential of *Streptococcus bovis* and other Gram-positive bacteria as expression hosts for polysaccharidases from anaerobic ruminococci has also been examined (4). Gram-positive bacteria and *S. bovis* particular are of interest as oxygen-tolerant microorganisms which have been considered as an expression host for use in vitro, as well as potential inoculants for silage or for feeding to ruminants (5). Genes for an extracellular amylase (6) and β-glucanase (7) have been cloned and their products are efficiently secreted into the culture supernatant. Several vectors have been shown to be functional in Gram-positive lactic acid bacteria and *S. bovis*, including pVA838 and its derivative pTRW10, and the construct that carries the replicon from pAMβ1 (4, 7-9). *S. bovis* JB1 was recently shown to be capable of natural transformation (10). In the present work, we examine aspects of the expression of cloned polysaccharidases in Gram-positive lactic acid bacteria and in *S. bovis*.

## Materials and Methods

### Strains, plasmids and growth conditions

The *Escherichia coli*, Gram-positive shuttle vectors pVA838 (11), *Streptococcus sanguis* DL1 and *S. bovis* JB1 were supplied by T.R. Whitehead (USDA, Peoria, USA). The high copy number pAMß1 derivative pIL253 (12) and *Lactococcus lactis* IL2661 were from Y. Duval Iflah (INRA, France). *Enterococcus faecalis* JH2-SS is a derivative of *E. faecalis* JH2-2 that carried mutations conferring resistance to streptomycin and spectonomycin. *E. coli* DH5α and *E. coli* HB101 were used as the host for pUC based constructs, with 50mg/ml ampicillin for selection. *E. coli* V850 (11) was used as host for pVA838 vectors, with 150 mg/ml erythromycin for selection. *S bovis* JB1 was routinely maintained anaerobically in a rumen fluid-based medium (13) containing 0.2% (w/v) soluble starch, 0.2% (w/v) glucose and 0.2% (w/v) cellobiose as energy sources (M2GSC medium). M2S refers to the same medium with 0.2% (w/v) soluble starch as the sole added energy source. For enzyme determinations, *S. bovis* was also grown in M17 medium (Oxoid Ltd, London, UK), modified by the addition of 0.1M MOPS buffer pH 6.8 and the inclusion of the appropriate carbohydrate substrate, unless otherwise stated, at 0.2% (w/v).

### Molecular biology procedures

The restriction and ligation (Boehringer, Mannheim, Germany) reactions were carried out as described by Macrina et al. (11). Transformed *E. coli* cells were plated onto LB agar plates in an overlay of LB medium containing 0.5% (w/v) agar, 50 mg/ml ampicillin and 0.1% (w/v) lichenan or 0.5% (w/v) oat spelt xylan. The transformation method for *S. bovis* JB1, *L. lactis* and *E. faecalis* followed published procedures respectively (14-16).

### Enzyme determinations

Reducing sugar release from polysaccharide substrates was determined by the method of Lever (17). Plate tests for xylanase and for lichenase, in which plates carrying substrate overlays were strained with Congo Red (18), were performed as described previously (7). 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

### Expression of the cloned polysaccharidase gene in *S. bovis* and in Gram-positive bacteria

The *R. flavefaciens* gene encodes a bifunctional polysaccharidase that has a domain with homology to stabilising regions of thermophilic xylanases, a family 11 xylanase domain, a dockerin region and a C terminal family 16 β-glucanase domain (19, 20). The polysaccharidase gene was cloned into the plasmid vector pVA838 as described in the Materials and Methods section to create the construct pVAXL (12.3 kb), which was subsequently introduced into *S. bovis* JB1 and into *L. lactis, E. faecalis* and *S. sanguis* by electroporation.

Relatively little enzyme was detected in *S. bovis* cell material, suggesting that the majority of the cloned product is secreted. The full length gene product (89 kDa) was detected in lichenase (ß 1,3-1,4 glucanase) zymograms from culture supernatants of *S. bovis*/pVAXL together with breakdown products of around 70 and 30 kDa (data not shown). The full length 90 kDa gene product was also detectable in *E. faecalis*, *L. lactis* and *S. sanguis* carrying pVAXL (data not shown). Again there was evidence of proteolysis in all three hosts, although in no case was proteolysis as extensive as in *E. coli*.

### Effect of growth stage upon expression of the cloned gene products in Gram-positive bacteria

Assays were performed on cells and supernatant material from 4h and 16h cultures of *S. bovis*, *E. coli*, *E. faecalis*, *L. lactis* and *S. sanguis*, carrying the construct pVAXL. Because of the background native lichenase activity of the *S. bovis*, the results in the Table are presented only for xylanase. Between 40 and 80% of the total culture xylanase activity attributable to cloned gene was detected in the culture supernatant, confirming secretion of much of the foreign gene product (Table). Activities were substantially lower after 16h than after 4h growth in *S. bovis*, *E. coli*, *E. faecalis*, *L. lactis* and *S. sanguis*, carrying the construct pVAXL, suggesting some inactivation of the product during growth (Table).

Significant inactivation was also noted for the cloned gene product studied when expressed in *S. bovis*, *E. faecalis*, *L. lactis* and *S. sanguis*, and this phenomenon was investigated further. Xylanase and lichanase activities are readily detectable in *S. bovis*, *E. faecalis*, *L. lactis* and

Table Cell Associated and Total % Supernatant Xylanase Activities of *E. coli*, and Other Gram-positive Bacteria, Carrying the Construct pVAXL.

| Constructs | Specific cell activity (nmol min$^{-1}$ (mg protein)$^{-1}$ | % Supernatant activity |
|---|---|---|
| *E. coli* pVAXL-4h | 18.1 | 12 |
| *E. coli* pVAXL -16h | 8.0 | 10 |
| *S. bovis* pVAXL -4h M2GSC | 28.7 | 40 |
| *S. bovis* pVAXL -16h M2GSC | 8.2 | 64 |
| *S. bovis* pVAXL -4h M2S | 28.4 | 58 |
| *S. bovis* pVAXL -16h M2S | 17.2 | 80 |
| *L. lactis* pVAXL -4h M17 | 5.6 | 55 |
| *L. lactis* pVAXL -16h M17 | 2.3 | 78 |
| *S. sanguis* pVAXL -4 h M17 | 8.3 | 63 |
| *S. sanguis* pVAXL -16h M17 | 4.7 | 82 |
| *E. faecalis* pVAXL -4h M17 | 9.6 | 46 |
| *E. faecalis* pVAXL -16h M17 | 5.2 | 75 |

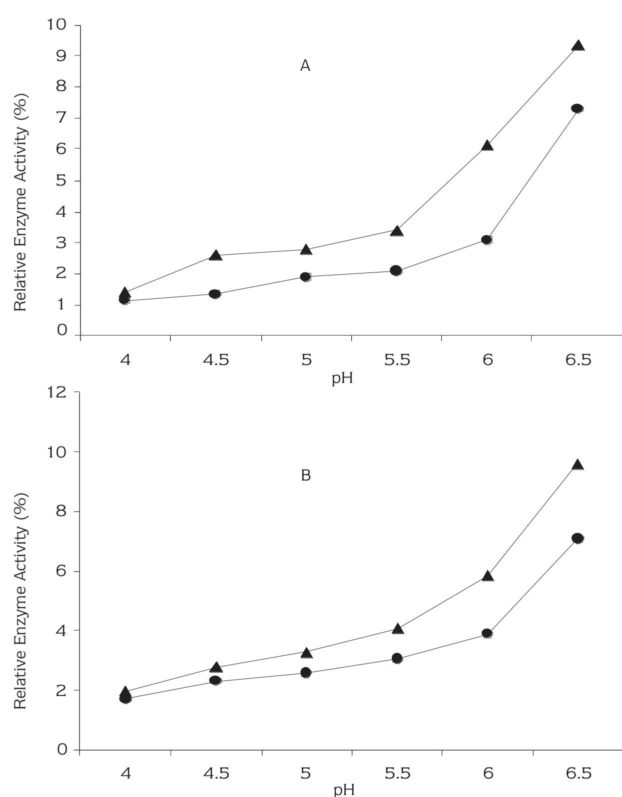


Figure Effect of Lactic Acid on (A) Xylanase and (B) Lichenase activities (nmol min$^{-1}$ (mg cell protein)$^{-1}$) from *S. bovis*/pVAXL. Cell extracts were obtained from *S. bovis*/pVAXL grown for 4 h in M2S medium containing 0.1% starch as energy source and 0.1M MOPS (pH 6.0). Different molarities of lactic acid were used to obtain the different pH values. Cell extracts were preincubated with these lactic acid concentrations at 37ºC for 6 (▲) and 16 (•) hours and then the pH was adjusted to 6.5, and then they were assayed at 37ºC against xylan and lichenan.

*S. sanguis*, carrying the construct pVAXL, by the detection of clear zones in lichenan or xylan-containing agar plates after Congo Red staining. When cultures of *S. bovis*/pVAXL were grown overnight in broth media, however, little or no xylanase and lichenase activities were detectable either by standard reducing sugar assays or by spotting cell extracts onto lichenan or xylan overlay plates. When *S. bovis*/pVAXL were grown in anaerobic M2GSC broth medium the peak of assayable xylanase and lichenase activities, expressed per mg cell protein, was found after 4 hours growth, while xylanase and lichenase activities declined to an undetectable level after 16 hours growth. M2S medium, in which soluble starch is the only added energy source, resulted in the same yield of cell protein, but some activity was still present for cells grown in this medium for 16h. This correlated with a slightly higher final pH in M2S compared with M2GSC cultures, presumably reflecting the difference in availability of readily fermentable sugars between the two media. A likely explanation for the loss of xylanase and lichenase activities in growing cultures of *S. bovis*, *E. faecalis*, *L. lactis* and *S. sanguis*, carrying the construct pVAXL, is therefore inactivation of the cloned gene product by accumulated lactate, and the accompanying pH reduction. This possibility was further investigated by preincubating preparations of the cloned enzyme in lactic acid solutions of different concentrations and pH values. pVAXL xylanase and lichenase activities after preincubation at pH 5 were 24-30% of the values obtained following preincubation at pH 6.5 (Figure).

## Discussion

The bifunctional genes from *R. flavefaciens* were apparently expressed *S. bovis*, *E. faecalis*, *L. lactis* and in *S. sanguis* from their own promoters. In the case of bifunctional genes, an 800bp region of DNA upstream of the start has been used elsewhere to express the gfp gene from *Aequoria victoria* in *S. gordonii* and *L. lactis*, establishing this as an active Gram-positive promoter region (21). Enhanced expression should in future be achievable using alternative promoters, including endogenous *S. bovis* promoters. The use of the amino terminal regions of the native β-glucanase gene from *S. bovis* JB1 (7) to achieve expression and secretion of fusion proteins has also been explored recently.

An apparent limitation of *S. bovis* as an expression host, in common with other lactic acid bacteria, is the inactivation of heterologous enzymes by accumulation of lactic acid in cultures. This is largely a problem of batch culture, however, which should be minimised in continuous culture or in an open ecosystem. The *R. flavefaciens* enzymes studied here have a narrow range of pH tolerance and other cloned products might prove to be more resistant to inactivation by low pH. It is possible that factors in addition to pH tolerance, such as protease activity, may also be involved in inactivation, in which case selection of protease negative strains could be helpful. There was less evidence of proteolytic cleavage in the regions between the catalytic domains of bifunctional gene in *S. bovis* than occurs in *E. coli*. The use of more closely related Gram-positive hosts for expression of genes from *Ruminococcus* spp. may offer further advantages, particularly since there is evidence for glycosylation of extracellular protein both in *Streptococcus* spp. and *Ruminococcus* spp. (22, 23), which might affect the activity or stability of cloned products.

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