Turkish Journal of Biology

Volume 25 | Number 1 Article 4

1-1-2001

Expression of a Fungal Cellulase Gene by \beta-glucanase Promoter of Streptococcus bovis

MEHMET SAİT EKİNCİ

Turk J Biol
25 (2001) 37-44

Expression of a Fungal Cellulase Gene by ß-glucanase Promoter of *Streptococcus bovis*[1]

Mehmet Sait EKİNCİ
Kahramanmaraş Sütçü İmam University, Faculty of Agriculture, Department of Animal Science, Kahramanmaraş - TURKEY

Received: 06.05.1999

Abstract : The promoter region of the ß-glucanase gene was identified using a transcriptional fusion between the upstream region of the *Streptococcus bovis* ß-glucanase gene and the celA gene. Using the transcriptional and protein localisation signals of the *S. bovis* ß-glucanase gene, an in-frame tarnslational fusion between the end of the ß-glucanase signal sequence and the ATG of the *Neocallimastix patriciarum* celA gene was constructed. The ß-glucanase promoter-celA fusion was expressed in both *E. coli* and *S. bovis.* The activity of the protein produced was found to be cell-associated in *E. coli*, it but localised to the supernatant fraction of harvested cells of *S. bovis.* In this study for the first time we have demonstrated that the promoter of the gene from rumen bacteria can express a fungal gene.

Key Words: fungal cellulase, *Streptococcus bovis,* promoter, gene expression.

Introduction

The forage plant cell wall is a complex and a fascinating biological structure. It is made up of polymers of cellulose and other noncellulosic polysaccharides, such as xylans and pectins in association with lignin, proteins, ions, and water (1-5). Cellulose hydrolysis is a complex process thought to be carried out by the combined action of different enzymatic activities including endoglucanase, cellobiosidase and ß-glucosidase (6-8). In recent years, the molecular biology of bacterial cellulases has received considerable attention (9). There have been many reports of genes being cloned from many of the predominant species of ruminal bacteria, but only recently from the ruminal anaerobic fungi, by Gilbert et al., (10) and Xue et al., (11,12). Although the importance of establishing the structure of rumen bacterial promoters is well documented (13-16), there is presently an insufficiency of information regarding the structure of native promoters in rumen bacteria. For some genes isolated from rumen bacteria there is evidence that *E. coli* recognises promoters different from those used in the original host organism. This has been shown for a cellulase gene from *P. ruminicola* (17), but may also apply to genes from

[1] This work was supported by Turkish High Educational Council and KSU and performed at the Rowett Research Institute, Aberdeen, UK.

other species. Expression of some *P. ruminicola* genes occurs in *E. coli* in the absence of any obvious ribosome binding site, and ribosome binding may be dependent on secondary structure (17,18). Ribosome binding sites are generally observed in the expected position for genes isolated from ruminococci, *B. fibrisolvens* and *F. succinogenes* (19), but internal translational start points and proteolysis can complicate interpretation of cloned gene products from *R. flavefaciens* expressed in *E. coli* (20). However, the importance of confirming promoter identity empirically has been demonstrated by studies in which transcriptional sites operating within *E. coli* differed significantly from those that were active within the native bacterium (17,21). Proven promoter sequences have shown similarity to the consensus sequences of *E. coli* (17,22), but other consensus sequences that function in *E. coli* have been shown to be non-functional in the rumen bacterium. Furthermore, any product normally glycosylated in Gram-positive will not be so modified in *E. coli.* Therefore, it is of interest to examine expression of cloned genes in Gram-positive species (17,22).

The importance of rumen bacteria in the digestive processes of ruminants makes them a logical target for genetic manipulation to improve nutritional efficiency. Thus, introducing cellulolytic functions into non-cellulolytic rumen bacteria such as *P. ruminicola* (23-25), and *B. fibrisolvens* (26) has been suggested, and *S. bovis* is another potential recipient for expression of genes encoding cellulolytic functions. These species display more acid tolerance than the predominant rumen cellulolytic bacteria (27) and some strains are moderately cellulolytic. In particular this provides an approach to achieving cellulolysis at rumen pH levels below those tolerated by the existing rumen cellulolytic population, and also could improve fibre digestion when ruuminants are fed on cereal-based diets (23), since the predominant cellulolytic rumen bacteria, *R. albus, R. flavefaciens,* and *F. succinogenes,* will not persist in rumen pH below 6 (27,28). Such a genetic modification may reduce N loss, which occurs when feeding ruminants with silage-based diets, due to the imbalance of ammonia release and energy generated by the metabolism of plant structural polysaccharides (29).

In Gram-positive bacteria, a variety of promoters and vectors have been developed to allow quantitative modulation of gene expression over a range of levels. Unfortunately, many of these systems do not function in Gram-positive organisms, probably because the requirements for promoter usage are more stringent in these bacteria than in *E. coli* (30). Only a few regulated promoters have been described for Gram-positive bacteria, and some of these, such as *Bacillus subtilis* sporulation promoters, require factors unique to the native species that are not present in the heterologous systems, like specific sigma factors and their regulators (31).

Materials and Methods

1. Strains, Plasmids and Growth Conditions

Gram-positive shuttle vector pTRW10, which is a modification of pVA838 (32), containing a multiple cloning site (33), was supplied together with *E. coli* and *S. bovis* JBl by T.R.

Whitehead (USDA, Peoria, USA). *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 50 µg/ml ampicillin for selection. *E. coli* V850 (32) was used as a host for pTRW10 vectors, with 150 µg/ml erythromycin for selection. Selection in Gram-positive hosts was with 5 µg/ml erythromycin. *S. bovis* JBl was routinely maintained anaerobically in a rumen fluid-based medium (34) containing 0.2% soluble starch, 0.2% glucose and 0.2% cellobiose as energy sources. For enzyme determinations *S. bovis* was also grown in M17 Medium (Oxoid Ltd, London, UK).

2. Molecular Biology Procedures

Restriction enzymes were obtained from Pharmacia LKB Biotechnology (Central Milton Keynes, UK). T4 DNA ligase and alkaline phosphatase were obtained from Boehringer (Mannheim, Germany). The restriction and ligation reactions were carried out as described by Maniatis et al., (35). *E. coli* cells were prepared for transformation by the method of Hanahan (36). Transformed cells were plated onto LB agar plates in a overlay of LB medium containing 0.5% agar, 50 µg/ml ampicillin and 0.1% CMC (Carboxymethylcellulose). The transformation method for *S. bovis* JBl followed published procedures (18). Oligonucleotide primers used for amplification of ß-glucanase promoter and celA gene were obtained from Cruachem Ltd. (Paisley, UK).

3. Enzyme Activity Determination

Reducing sugar release from polysaccharide substrates was determined by the method of Lever (37). Plate tests for CMCase (Carboxymethylcellulase), in which plates carrying substrate overlays were stained with Congo red (38), were performed as described previously (39). Detection of endoglucanase activity following SDS-PAGE (zymogram) was as described previously (20).

Results and Discussion

The following strategy was used to obtain a fusion of ß-glucanase promoter with celA gene into pTRW10 shuttle vector. First a 1.1-kb fragment of pL1Hc (40) was amplified with M13 forward and ß-glu reverse primers (Figure 1), which encodes ß-glucanase promoter. *Sal*I and *Bgl*II recognition sequences were synthesised at the 3' end of the ß-glu reverse primer to obtain suitable cloning sites into plasmids and also to obtain frame fusion with the celA gene. Amplified PCR product was then digested with *Pst*I/*Sal*I endonucleases and the digested 0.6kb *Pst*I/*Sal*I fragment purified from agarose gel. The purified fragment was then cloned into the same sites of pUC18 plasmid, a new recombinant plasmid named BgPps. Similarly 1.2 kb celA fragment was amplified by celAF and celAR primers (Figure 1). Both BgPps construct and amplified celA fragments were cut by *Bgl*II and *Eco*RI enzymes. Digested celA fragment was inserted into the same site of BgPps recombinant plasmid. The ligated DNA was then transformed into *E. coli* DH5α selected on LB-ampicillin plates containing CMC substrate, named pBpC. To introduce the

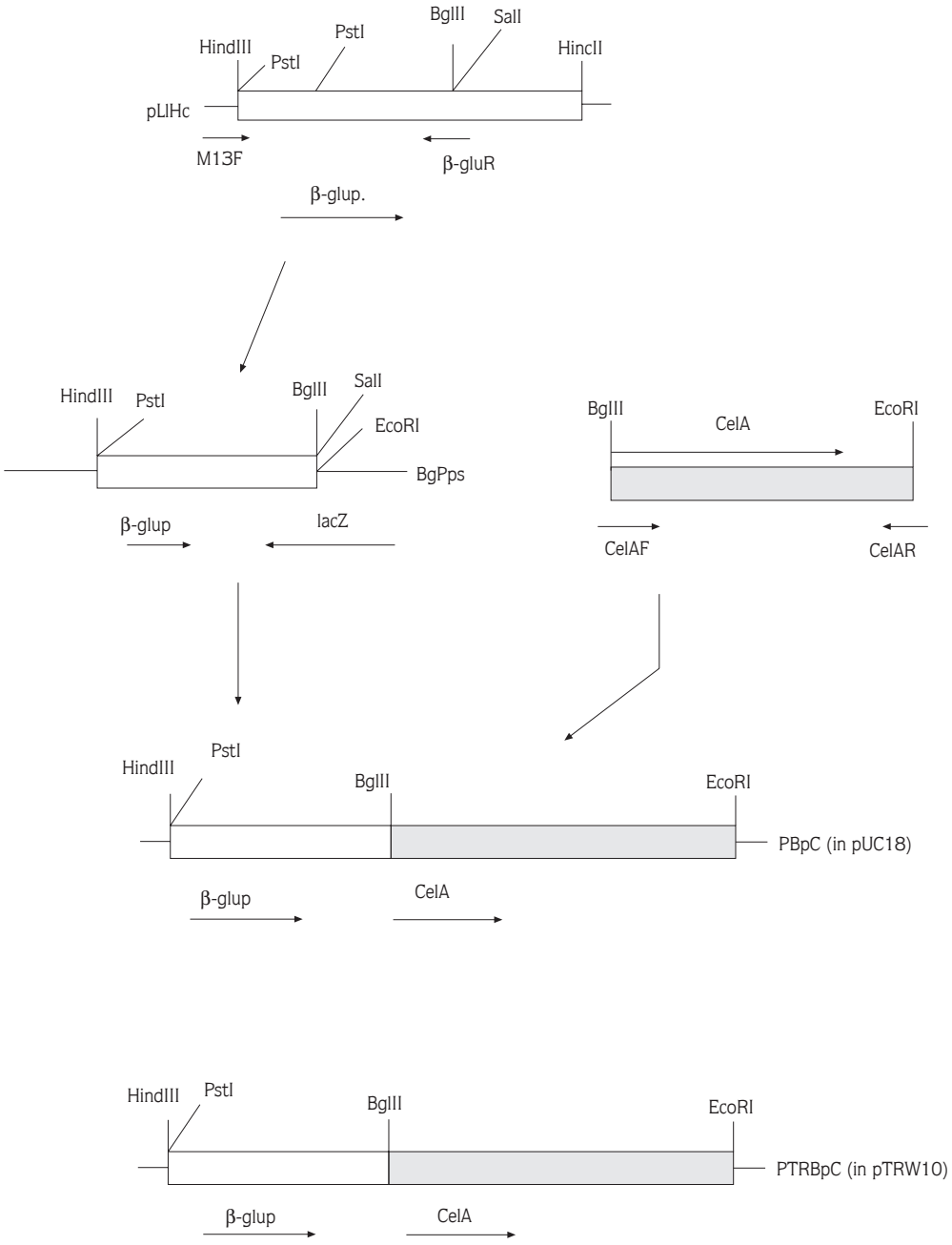

Figure 1. Construction of pTRBpC clone.

construct into Gram-positive species a vector that can be replicated in that species is needed. Therefore an *E. coli/Streptococcus* shuttle vector pTRW10 was used and the following strategy performed. To fulfil the above purpose a 1.8-kb fragment of pBpC encoding ß-glucanase promoter and celA fusion was excised by cutting with *Hind*III and *Eco*RI enzymes and the fragment purified from agarose gel. The purified fragment was then inserted into the same site of pTRW10 shuttle vector. The ligated DNA was transformed into *E. coli* V850 and recombinant colonies were selected on LB erythromycin plates containing CMC substrate overlay. CMCase-positive colonies were identified by clearing zones around the colonies. The recombinant plasmid was termed pTRBpC (Figure 1). The plasmid pTRBpC DNA was then introduced into the *S. bovis* JBI by electroporation (39) for further analysis.

In this study it was demonstrated for the first time that a fungal cellulase gene can be expressed by the promoter of ruminal Gram-positive bacterium *S. bovis* ß-glucanase gene in both *E. coli* and Gram-positive rumen bacterium *S. bovis.* Detectable cell-associated CMCase activity in both *E. coli* and *S. bovis* was found to be 16.2 and 8.3 nmol min^{-1} (mg protein)$^{-1}$ respectively. While supernatant activities of both *E. coli* and *S. bovis* were found to be 12.2 and 28.6 nmol min^{-1}(ml culture)$^{-1}$ respectively. This supports the observation that CMCase transported out of cell in *S. bovis* while in *E. coli* it was mostly cell-associated. pH optimum and stability of celA were found to be similar in *S. bovis* as reported previously (12). Approximately 45kDa active protein band was detected from *E. coli*/pTRBpC and *S. bovis*/pTRBpC extracts by SDS-PAGE zymogram analysis (Figure 2). According to zymogram analysis only one single active protein band was detected, suggesting that celA gene product in both *E. coli* and *S. bovis* was not affected by proteases.

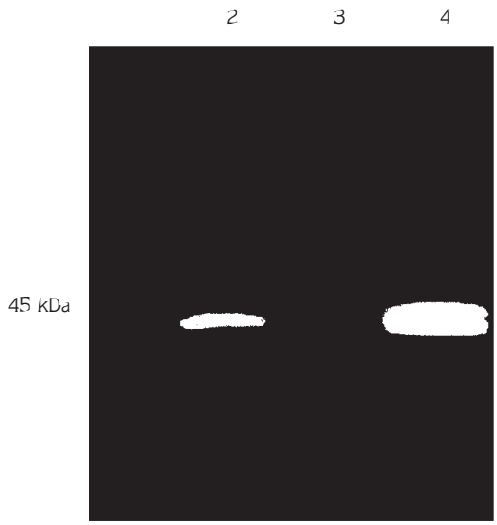

Figure 2. Detection of active polypeptide of pTRBpC from*E. coli* and from *S. bovis* JBI ond SDS-PAGE zymogram. Polypeptides from sonicated cells were separated on an SDS-polyacrylamide gel. After renaturation the gel was incubated between agarose gels containing CMC (carboxymethylcellulose), which were then stained to reveal activity (see Materials and Methods) Lane 1: *E. coli;* Lane 2: *E. coli*/pTRBpC; Lane 3: *S. bovis* JBI; Lane 4: *S. bovis* JBI/pTRBpC

The use of transcription and localisation signals for heterologous gene expression in *S. bovis* is at an early stage of investigation compared with other systems. *S. bovis* is a potentially useful host for heterologous expression, especially for the expression of enzymes such as ß-glucanase. *S. bovis* is a rumen organism that produces and exports a large number of complex-carbohydrate-degrading enzymes, and the use of the expression and localisation signals from these enzymes for the production of similar enzymes from other sources is potentially powerful. ß-glucanase from *N. patriciarum* is an example. The pH stable activity of this enzyme makes it well suited for processes such as plant cell wall polysaccharide degradation, but production of the enzyme from its host, an obligate anaerobe, would be difficult at best. Our investigation of the expression of this ß-glucanase in *S. bovis* clearly shows that the enzyme is expressed and localised and that the activity is pH tolerant. Our efforts to optimise the system for high-level production, using the promoter region of *S. bovis* ß-glucanase resulted in an increase in celA activity.

Finally, the construct that was made in this study seems to have potential for silage inoculation.

References

1. Demeyer, D. I. Rumen microbes and digestion of plant cell walls. Agr. Environ. 6: 295-337, 1981.
2. Carpita, N., Gibeaut, D. M. Structural models of primary cell walls of flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. 3: 1-30, 1993.
3. Mueller-Harvey, I., Hartley, R. D. Linkage of p-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydrate Res. 148: 71-85, 1986.
4. Iiyama, K., Lam, T.B.T., Stone, B. A. Covalent cross-links in the cell wall. Plant Physiol. 104: 315-320, 1994.
5. Bacic, A., Harris, P.J., Stone, B. A. Structure and function of plant cell walls. In "The Biochemistry of Plants" ed. P. K. Stumpf, and E. E. Conn. pp. 297-371. Academic Press, New York, 1988.
6. McNeil, M., Darvill, A.G., Fry, S.C., Albersheim, P. Structure and function of the primary cell walls of plants. Ann. Rev. Biochem. 53: 625-663, 1984.
7. Coughlan, M.P., Ljungdahl, L. G. Comparative biochemistry of fungal and bacterial cellulolytic enzyme systems. pp: 11-30. In: "Biochemistry and Genetics of Cellulose Degradation". Aubert, J. P., Beugin, P., Millet, L. (ed.). London: Academic Press, 1988.
8. Chesson, A., Forsberg, C.W. Polysaccharide degradation by rumen micro-organisms. pp: 251-284. In "The Rumen Microbial Ecosystem". (P.N. Hobson ed.). Elsevier Applied Science, London, 1997.
9. Béguin, P., Aubert, J.P. Biological degradation of cellulose. FEMS Microbiol. Rev. 13: 25-28, 1994.
10. Gilbert, H.J.,Hazlewood, G.P., Laurie, I., Orpin, G.C., Xue, G.P. Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. Mol. Microbiol. 6: 2065-2072, 1992.
11. Que, G-P., Gobius, K. S., Orpin, C.G. A novel polysaccharide hydrolyse cDNA (*celD)* from *Neocallimastix patriciarum* encoding three multi functional catalytic domains with high endoglucanase, cellobiohdrolase and xylanase activities. J. Gen. Microbiol. 138: 2397-2403, 1992a.

12. Xue, G-P., Orpin, C.G., Gobius, K. S., Aylward, J.H., Simpson, C.D. Cloning and expression of multiple cellulase cDNAs from the anaerobic fungus *Neocallimastix patriciarum* in *E. coli*. J. Gen. Microbiol. 138: 1413-1420, 1992b.

13. Vercoe, P.E., Gregg, K. Sequence and transcriptional analysis of an endoglucanase from *Ruminococcus albus* AR67. Anim. Biotechnol. 6: 59-71, 1995.

14. Vercoe, P.E., Finks, J.L., White, B.A. DNA sequence and transcriptional characterisation of a ß-glucanase gene (*celB*) from *Ruminococcus flavefacies* FD-1. Can. J. Microbiol. 41: 869-876, 1995a.

15. Vercoe, P.E., Spight, D. H., White, B.A. Nucleotide sequence and transcriptional analysis of the *celD* ß-glucanase gene from *Ruminococcus flavefacies* FD-1. Can. J. Microbiol. 41: 27-34, 1995b.

16. Chen, C-C., Westpheling, J. Partial characterisation of the *Streptomyces lividans xln*B promoter and its use for expression of a thermostable xylanase from *Thermotoga maritima*. Appl. Environ. Microbiol. 64: 4217-4225, 1998.

17. Vercoe, P. E., Gregg, K. DNA sequence and transcription of an endoglucanase gene from *Bacteroides (Prevotella) ruminicola* AR20. Mol. Gen. Genet. 233: 284-292, 1992.

18. Whitehead, T.R. Analyses of the gene and amino acid sequence of the *Prevotella (Bacteroides) ruminicola* 23 xylanase reveals unexpected homology with endoglucanasesfrom other genera of bacteria. Curr. Microbiol., 27: 27-34, 1993.

19. Flint, H.J. Molecular genetics of obligate anaerobes from the rumen. FEMS Microbiol. Lett. 121: 259-268, 1994.

20. Flint, H.J., Martin, J., McPherson, C.A., Daniel, A.S., Zhang, J.-X. A bifunctional enzyme, with separate xylanase and ß(1,3-1,4)-glucanase domains, encoded by the *xyn*D gene of *Ruminococcus flavefaciens*. J. Bacteriol. 175: 2943-2951, 1993.

21. Matsushita, O., Russell, J.B., Wilson, D.B.A *Bacteroides ruminicola* 1,4-ß-D-endoglucanase is encoded two reading frames. J. Bacteriol. 173: 6919-6926, 1991.

22. Ohmiya, K., Kajino, T., Kato, A., Shimizu, S. Structure of a *Ruminococcus albus* endo-1,4-ß-glucanase gene. J. Bacteriol., 171: 6771-6775, 1989.

23. Russell, J.B., Wilson, D.B. Potential opportunities and problems for genetically altered rumen microorganisms. J. Nutr. 118: 271-279, 1988.

24. Forsberg, C.W., Crosby, B., Thomas, D.Y. Potential for manipulation of the rumen fermentation through the use of recombinant DN Techniques. J. Anim. Sci. 63: 310-325, 1986.

25. Hespell, R.B. Biotechnology and modification of the rumen microbial ecosystem. Proc. Nutr. Soc. 46: 407-413, 1987.

26. Weimer, P.J. Why don't ruminal bacteria digest cellulose faster? J. Dairy Sci. 79: 1496-1502, 1996.

27. Russell, J.B., Dombrowski, D.B. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. Appl. Environ. Microbiol. 39: 604-610, 1980.

28. Stewart, C. S. Factors affecting the cellulolytic activity of rumen contents. Appl. Environ. Microbiol. 33: 497-502, 1977.

29. Johnson, R.R. Influence of carbohydrate solubility on non-protein nitrogen utilization in the ruminant. J. Animal. Sci. 43: 184-191, 1976.

30. Eichenbaum, Z., Federle, M.J., Marra, D., de Vos, W.M., Kuipers, O.P., Kleerebezem, M., Scott, J. R. Use of the Lactococcal nisA promoter to regulate gene expression in Gram-positive bacteria: comparison of induction level and promoter strength. Appl. Environ. Microbiol. 64: 2763-2769, 1998.

31. Haldenwang, W.G. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. 59: 1-30, 1995.

32. Macrina, F. L., Tobian, J. A., Jones, K.R., Evans, R. P., Clewell, D. B. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene. 19: 345-353, 1982.

33. Wyckoff, H. A., Whitehead, T.R. Improved electroporation protocol and vectors for *Streptococcus bovis*. World J. Microbiol. Boitechnol. 58: 1213-132, 1997.

34. Hobson, P. N. Rumen bacteria. In "Methods in microbiology" 3rd ed. Norris, J.R. and D.W. Ribbons. pp133-149. London, Academic Press, 1969.

35. Maniatis, T., Fritsch, E. F. and Sambrook, J. Molecular cloning: a laboratory manual, Cold SPring Harbor Laboratory, Cold Spring Harbour, N.Y., 1982.

36. Hanahan, D. Techniques for transformation of *Escherichia coli* DNA cloning. A Practical Approach. (Glover, D. M. Eds.) pp. 121-127, IRL Press Inc., Oxford, 1985.

37. Lever, M. Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAHBAH): effect of bismut on the reaction. Anal. Biochem. 81: 21-27, 1977.

38. Teather, R. M., Wood, P. J. Use of Congo Red-polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43: 777-780, 1982.

39. Flint, H. J., McPherson, C. A., Martin, J. Expression of two xylanase genes fromthe rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 cloned in pUC13. J. Gen. Microbiol. 137: 123-129, 1991.

40. Ekinci, M.S., McCrae, S.I., Flint, H.J. Isolation and overexpression of a gene encoding an extracellular ß-(1,3-1,4)-glucanase from *Streptococcus bovis* JBl. Appl. Environ. Microbiol. 63: 3752-3756, 1997.