APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3752–3756 Vol. 63, No. 10
0099-2240/97/$04.00+0

Isolation and Overexpression of a Gene Encoding an Extracellular β-(1,3-1,4)-Glucanase from *Streptococcus bovis* JB1

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Received 23 December 1996/Accepted 16 July 1997

Streptococcus bovis **JB1 was found to produce a 25-kDa extracellular enzyme active against β-(1,3-1,4)-glucans. A gene was isolated encoding a specific β-(1,3-1,4)-glucanase that corresponds to this size and belongs to glycoside hydrolase family 16. A 4- to 10-fold increase in supernatant β-glucanase activity was obtained when the cloned β-glucanase gene was reintroduced into *S. bovis* JB1 by use of constructs based on the plasmid vector pTRW10 or pIL253. The β-(1,3-1,4)-glucanase gene was also expressed upon introduction of the pTRW10 construct pTRWL1R into *Lactococcus lactis* IL2661 and *Enterococcus faecalis* JH2-SS, although extracellular activity was 8- to 50-fold lower than that in *S. bovis* JB1. The β-(1,3-1,4)-glucanase purified from the culture supernatant of *S. bovis* JB1 carrying pTRWL1R showed a K_m of 2.8 mg per ml and a V_{max} of 338 μmol of glucose equivalents per min per mg of protein with barley β-glucan as the substrate. The *S. bovis* β-(1,3-1,4)-glucanase may contribute to the ability of this bacterium to utilize starch by degrading structural polysaccharides present in endosperm cell walls.**

Streptococcus bovis is a facultatively anaerobic member of the rumen microflora which is normally present in relatively low numbers but which can become abundant in animals fed high-grain diets, when starch is readily available (18). *S. bovis* is a lactate producer, and under some conditions its proliferation is associated with the development of low pH and of the potentially serious digestive disorder lactic acidosis (25, 30). There has also been interest in the potential for application of *S. bovis* as a silage inoculant because of its ability to effect rapid reductions in pH (19) and as a possible vehicle for manipulation of rumen function and for enzyme production and fermentation processes (12, 33, 35, 36). For these reasons, work has begun on the genetics of this species and several genes have now been isolated. These include operons concerned with proline synthesis (4) and lactose utilization (12) and genes encoding intracellular (5, 6, 28) and extracellular (28) amylases. The intracellular α-amylase gene has also been used as a probe to detect *S. bovis* strains from the human intestinal tract (34). Progress has also been made with the introduction of vectors based on the plasmid pVA838 into *S. bovis* by electroporation (33) and with the expression in this species of a cellulase gene from the rumen bacterium *Ruminococcus flavefaciens* (35).

The high starch-degrading activity of *S. bovis* (22) is partly attributable to its amylase enzymes, which have been investigated previously (11, 28). On the other hand, the utilization of starch from plant material also requires the degradation of polysaccharide and proteinaceous material that coats starch grains; endosperm cell walls from cereals such as barley are particularly rich in β-(1,3-1,4)-glucans and arabinoxylans (7). We describe here the isolation of a gene coding for an extracellular β-(1,3-1,4)-glucanase from *S. bovis* JB1 and the expression of the gene product in *S. bovis* and in related gram-positive bacteria.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The *Escherichia coli*, gram-positive shuttle vector pTRW10 is a modification of pVA838 (21) containing a multiple-cloning site (36) and was supplied together with the *S. bovis* strain JB1 (24) by T. R. Whitehead (U.S. Department of Agriculture, Peoria, Ill.). The high-copy-number pAMβ1 derivative pIL253 (29) and *Lactococcus lactis* IL2661 were from Y. Duval Iflah (INRA, Jouy-en-Josas, France). The low-copy-number *E. coli* vector pMTL6000 was constructed by N. P. Minton (CAMR, Porton, United Kingdom) by modification of PMTL1000 (3). Bacteriophage lambda EMBL3 clones were propagated in *E. coli* P2392, as described previously (9). *E. coli* strains were routinely grown in LB medium. *E. coli* DH5α was used as a host for pUC-based constructs, with 50 μg of ampicillin per ml for selection. *E. coli* V850 (21) was used as a host for pTRW10 vectors, with 150 μg of erythromycin per ml for selection. Selection in gram-positive hosts was with 5 μg of erythromycin per ml. *Enterococcus faecalis* JH2-SS is a derivative of *E. faecalis* JH2-2 that carries mutations conferring resistance to streptomycin and spectinomycin. *S. bovis* JB1 was routinely maintained anaerobically in a rumen fluid-based medium (17) 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 also grown in M17 medium (Oxoid Ltd., London, United Kingdom) modified by the addition of 0.1 M MOPS (morpholine propanesulfonic acid) buffer (pH 6.8) and the inclusion of the appropriate carbohydrate substrate, unless otherwise stated, at 0.2% (wt/vol).

Molecular biology procedures. Chromosomal DNA was prepared from *S. bovis* by a modification of the method of Saito and Miura (26, 28). A gene library was constructed by ligation of size-fractionated *Sau*3AI fragments (10 to 22 kb) in bacteriophage lambda EMBL3 as described previously (9). Screening for β-(1,3-1,4)-glucanase-producing clones was by Congo red staining of plates overlaid with 0.4% agarose containing 0.1% lichenan (9). Phage DNA was recovered from plate lysates of purified recombinant phages by use of Qiagen (Dorking, United Kingdom) kits and used for subcloning in plasmid vectors. Restriction enzymes were used as described in the manufacturer's instructions. Southern transfers were performed by capillary blotting onto positively charged nylon membranes (Boehringer GmbH, Mannheim, Germany). ^{32}P-labelled probe DNA was prepared by the random priming method (27). Transformation methods for *S. bovis* JB1 followed published procedures (33). DNA sequencing was performed on both strands with an ABI373 automated sequencer in conjunction with the ABI Prism kit. M13 primers or internal oligonucleotide primers, obtained from Cruachem Ltd. (Paisley, United Kingdom) were used as appropriate, and template DNA was derived from the pL1Hc subclone. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group software available through the Seqnet facility (Daresbury, United Kingdom).

Enzyme determinations. Reducing sugar release from polysaccharide substrates was determined by the method of Lever (20). Laminarin was washed extensively before use as a substrate to remove soluble sugars. Incubation conditions, assays for *p*-nitrophenyl release, and procedures for the detection of β-glucanase activity following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were as described previously (10). Culture supernatants were assayed directly, while cell pellets were resuspended in 50 mM Na phosphate buffer (pH 6.5), subjected to two cycles of freeze-thawing, and soni-

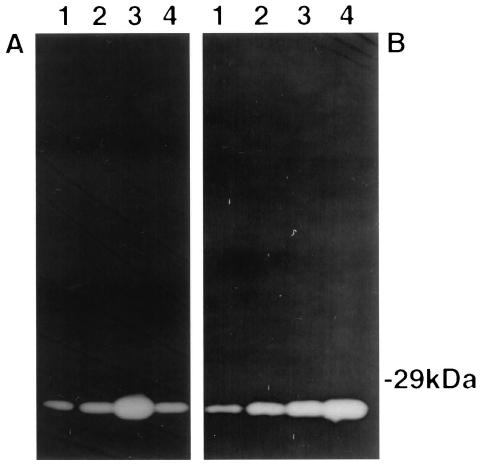

FIG. 1. Expression of native and cloned *S. bovis* β-glucanase, detected by SDS-PAGE zymograms. (A) Expression of the cloned *S. bovis* JB1 β-glucanase gene in *E. coli*. Lanes: 1 and 2, plate lysates from two independent EMBL3 phage clones, JBL2 and JBL1, respectively (8 μl of sixfold-concentrated phage lysate); 3 and 4, cell sonicate (10 μg of protein) and unconcentrated culture supernatant (10 μl) from a 16-h culture of *E. coli* DH5α carrying the plasmid clone pTRWL1R. (B) *S. bovis* JB1 carrying the uninserted vector pVA838 after 4 (lane 1) or 18 (lane 2) h of growth or carrying pTRWL1R, with the cloned β-glucanase gene, after 4 (lane 3) or 18 (lane 4) h of growth. To ensure detection of the native enzyme, lanes 1 and 2 received 10 μl of fivefold-concentrated culture supernatant, while lanes 3 and 4 received 10 μl of unconcentrated supernatant. Growth of *S. bovis* was in M17 medium with 0.1% lichenan and 5 μg of erythromycin per ml. Panels A and B are from different gels.

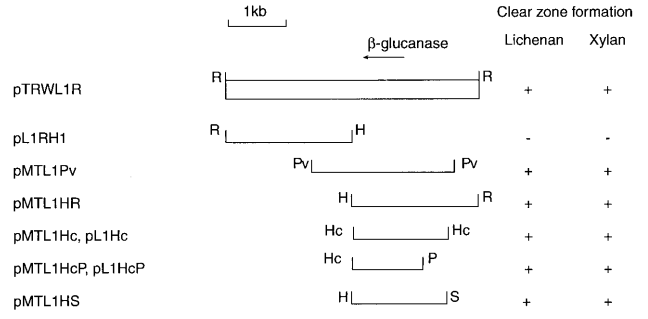

FIG. 2. Identification of the β-(1,3-1,4)-glucanase-encoding region in *S. bovis* DNA by subcloning. Fragments of the 3.6-kb *Eco*RI fragment recovered in plasmid pTRWL1R were subcloned in pUC18 (pL1RH1, pL1Hc, and pL1HcP) or in pMTL6000 (pMTL) as indicated. Activities against lichenan and xylanase are based on the formation of clear zones in overlay plates (see Materials and Methods). Pv, *Pvu*II; P, *Pst*I; H, *Hin*dIII; R, *Eco*RI; Hc, *Hin*cII; S, *Sph*I.

cated extensively (5 times for 1 min; MSE Soniprep, maximum setting) before assay. The pH dependence of the *S. bovis* supernatant enzyme was determined by preparing 50 mM Na phosphate assay buffers ranging from pH 8.0 to 3.0, with pH values between 6.5 and 3 being achieved by the addition of DL-lactic acid. To determine pH stability, incubations were performed without substrate for 6 h at 38°C, followed by adjustment to pH 6.5 by the addition of predetermined volumes of NaOH and incubation for a further 20 min before measurement of reducing sugar release.

β-(1,3-1,4)-Glucanase purification. *S. bovis* JB1/pTRWL1R was grown overnight in anaerobic M17 medium supplemented with 0.05% glucose and 0.05% lichenan and containing 5 μg of erythromycin per ml. Cells were removed by centrifugation (3,000 × *g*, 10 min), and proteins were precipitated from the culture supernatant by the addition of $(NH_4)_2SO_4$ to 85% saturation at 1°C. Precipitated proteins were desalted on a NAP-5 column and separated by fast protein liquid chromatography on a Mono Q HR5/5 column (Pharmacia) with a 0 to 1 M NaCl gradient in 50 mM phosphate (pH 6.5). The β-glucanase, which was frontally eluted, was then applied to a phenyl-Superose HR5/5 column (Pharmacia) and eluted with a decreasing 1.7 to 0 M gradient of $(NH_4)_2SO_4$. The protein composition of the β-glucanase peak was analyzed by SDS-PAGE on a Pharmacia Phastsystem, with detection by silver staining, and revealed a single protein band of approximately 25 kDa.

RESULTS AND DISCUSSION

Isolation of a β-(1,3-1,4)-glucanase gene from *S. bovis* JB1. A major band of β-(1,3-1,4)-glucanase activity of approximately 25 kDa was detected in *S. bovis* JB1 cultures by SDS zymograms (Fig. 1B, lanes 1 and 2). The same band was evident in cell pellets and supernatants from cultures grown with lichenan, glucose, or starch as the substrate (results not shown). A gene encoding this activity was isolated following construction of a genomic library of *Sau*3A1 fragments in bacteriophage lambda EMBL3 and screening for plaques that form clear zones in lichenan overlays detectable by Congo red staining (9). Two active clones were isolated from 6,000 plaques screened; these were found by restriction analysis to have overlapping inserts, and both encoded enzymes with a size similar to that of the native β-(1,3-1,4)-glucanase (Fig. 1). Subcloning of a 3.6-kb *Eco*RI fragment in pUC18 gave high levels of β-glucanase activity in *E. coli*, but the recombinant plasmid was thought to be unstable. Subcloning of the same fragment in the *E. coli*, gram-positive shuttle vector pTRW10 and subcloning of smaller fragments into the *E. coli* vectors pMTL6000 and pUC18 gave rise to stable recombinant plasmids (Fig. 2). pTRW10 is a smaller derivative of pVA838 which carries the pACYC replicon and has a lower copy number than pUC plasmids in *E. coli* cells (36). Southern blot analysis confirmed the presence of a single *Eco*RI fragment of 3.6 kb in chromosomal DNA from *S. bovis* JB1, suggesting that the gene is present as a single copy (Fig. 3).

The nucleotide sequence of the *S. bovis* β-glucanase gene was determined. The predicted translation product is a 237-amino-acid polypeptide with an obvious N-terminal signal peptide (32) followed by sequences that have close similarity to those of β-glucanases of family 16 (14, 15) (Fig. 4). The predicted subunit molecular size for the translation product is 26,943 Da, and that for the mature protein is 24,200 Da. The

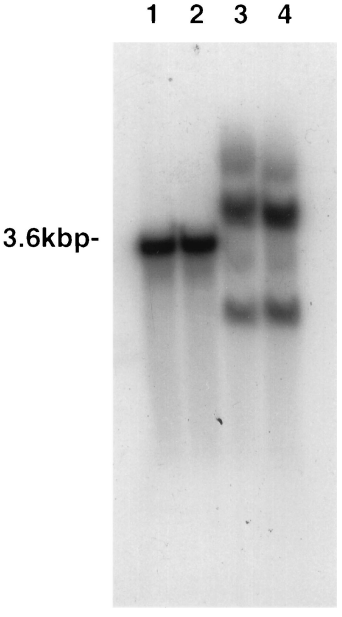

FIG. 3. Southern hybridization analysis of *S. bovis*. Chromosomal DNA from *S. bovis* JB1 was cleaved with *Eco*RI (lanes 1 and 2) or *Sau*3A1 (lanes 3 and 4), transferred to a nylon filter, and probed with the ^{32}P-labelled 3.6-kb insert from plasmid pTRWL1R that carries the β-glucanase gene.

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TTTGAAGTAACAGTTACTGAAAAGAAAAAGTGAATTTAACTACGATTGATTAGGCGTTTT    60
CAATCTGTAGTAGAGGAGATTAGTTCCTTTTTCTGACAGAACTCCTTTTTAAAATGATTA   120
AAATTGAGGTATTATTATGTGGAAAAAAGTTAGTTGTTTAATTGTTCTCTTATTGGCTTT   180
                M  W  K  K  V  S  C  L  I  V  L  L  L  A  F     15
CTTTGGTTTTCAACAAGGTGTTGATGCGCAAAGCAAATACCACTATAGTCAAGAACTAAA   240
 F  G  F  Q  Q  G  V  D  A▼Q  S  K  Y  H  Y  S  Q  E  L  N     35
TTACTATAATGGTAATGCCATGGAACTTCGTAATGGTTCTAATGGTGGTATGTTTAACTG   300
 Y  Y  N  G  N  A  M  E  L  R  N  G  S  N  G  G  M  F  N  C     55
TAATTTTGTCCCTGGAAATGTCGGCTTTAATAACGGCTTGATGAGTCTTAAAATTGACAG   360
 N  F  V  P  G  N  V  G  F  N  N  G  L  M  S  L  K  I  D  S     75
TGATGGTCGTGGCGGCTACACTGGGGGCGAATGGCGTAGTAAAGAACGCTTTGGCTATGG   420
 D  G  R  G  G  Y  T  G  G  E  W  R  S  K  E  R  F  G  Y  G     95
TCTTTTCCAAGTAAACATGAAACCTATTAAAAATCCAGGTGTCGTTTCTTCATTCTTTAC   480
 L  F  Q  V  N  M  K  P  I  K  N  P  G  V  V  S  S  F  F  T    115
TTATACTGGACCAAGTGATGGTACAAAATGGGATGAAATCGACATTGAATTTTTAGGGAA   540
 Y  T  G  P  S  D  G  T  K  W  D  E  I  D  I  E  F  L  G  K    135
AGATACAACAAAAGTTCAATTCAACTACTACACAAGCGGTCAAGGTAATCACGAATACCT   600
 D  T  T  K  V  Q  F  N  Y  Y  T  S  G  Q  G  N  H  E  Y  L    155
TTACAATTTAGGATTTGATGCATCTCAAGGCTTCCACACTTATGGTTTTGATTGGCAAGC   660
 Y  N  L  G  F  D  A  S  Q  G  F  H  T  Y  G  F  D  W  Q  A    175
TGATCACATTACTTGGTATGTTGATGGAAGAGCTGTTTACACTGCTTACAACAATATTCC   720
 D  H  I  T  W  Y  V  D  G  R  A  V  Y  T  A  Y  N  N  I  P    195
TAGCACACCTGGTAAGATTATGATGAATGCTTGGCCTGGAACTCATGAAGTGGATTCTTG   780
 S  T  P  G  K  I  M  M  N  A  W  P  G  T  H  E  V  D  S  W    215
GTTGGGTGCTTACAATGGTCGAACTCCTTTGTATGCATATTATGACTGGATTAGCTACGA   840
 L  G  A  Y  N  G  R  T  P  L  Y  A  Y  Y  D  W  I  S  Y  D    235
CCAATTTTAATAAAGCTAGACAGTAACCAAATAACAATTTGTTTTTACTTAACTGATTAT   900
 Q  F  *                                                       237
AACTACACTCCCCAAATAAATCGGTTAAAATCTCTCTCAACAAAATAACTCTTTAAAATT   960
CTTTCTTTTTAAACGTTGACT  981
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FIG. 4. Sequence and predicted translation of the β-(1,3-1,4)-glucanase gene from *S. bovis* JB1. The predicted signal peptide cleavage site is indicated by an inverted triangle.

primary sequence had 53.3, 55, and 50.7% identity, respectively, with sequences of β-glucanases from *Clostridium thermocellum* and *Bacillus subtilis* and the C-terminal β-glucanase domain of the XynD enzyme of *R. flavefaciens* (Fig. 5). Codon usage is strongly biased in the *S. bovis* gene, and only 4% of codons that involve a fourfold redundancy have a G in the third position (results not shown). CAA and AAA are apparently strongly favored for glutamine and lysine, respectively, in *S. bovis*, whereas CAG and AAG are strongly favored in another gram-positive rumen species, *R. flavefaciens* (37).

Expression of the cloned *S. bovis* β-glucanase in gram-positive bacteria. The construct pTRWL1R carrying the cloned *S. bovis* gene was introduced by electroporation into *S. bovis*, *E. faecalis*, and *L. lactis* cells. Expression in *S. bovis* JB1 resulted in increases of between four- and fivefold in β-glucanase activity relative to that of a control *S. bovis* strain carrying an uninserted pVA838 vector plasmid after 18 h of growth (Table 1; Fig. 1). As for the native enzyme, the majority of the β-glucanase activity was detected in the culture supernatants of the strains carrying pTRWL1R. Thus, both the native and cloned enzymes appear to be efficiently secreted by *S. bovis*. Expression of the cloned enzyme was readily detected by clear-zone formation around *L. lactis* and *E. faecalis* colonies grown on lichenan overlay plates, although assayable activities were much lower than that in *S. bovis* (Table 1). It is not known whether this reflects differences in promoter recognition, translation, or protein export or, alternatively, the lack of a positive activator in these species. A band of β-(1,3-1,4)-glucanase activity was detectable in supernatant from cultures of *L. lactis* carrying pTRWL1R when analyzed by SDS-PAGE zymograms. This band showed the same mobility as the enzyme from *S. bovis* (results not shown).

A second construct carrying the β-glucanase gene was made by fusing the plasmid pL1HcP, which carries a 1.4-kb *Hin*cII-*Pst*I fragment subcloned in pUC18, with the vector pIL253 (Fig. 6). pIL253 is derived from the broad-host-range plasmid pAMβ1, which has been shown to replicate in *S. bovis* (16), and the resulting construct was stably maintained in *S. bovis* JB1. The copy number of pIL253 in *L. lactis* has been estimated at 45 to 85 copies per cell (29), whereas that for pVA838 in *Streptococcus sanguis* is approximately 15 copies per cell (21). This suggests that pILHcP should have a higher copy number than that of pTRWL1R in *S. bovis*, but this was not reflected in

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GUB_BACLI       QTGGSFYEPFNNYNTGLWQKADGYSNGNMFNCTWRANNVSMTSLGEMRLSLTSP--SYN-
GUB_BACMA       LAGSVFWEPLSYFNRSTWEKADGYSNGGVFNCTWRANNVNFTNDGKLKLGLTSS--AYN-
GUB_BACSU       QTGGSFFDPFNGYNSGFWQKADGYSNGNMFNCTWRANNVSMTSLGEMRLALTSP--AYN-
GUB_CLOTM       VVNTPFVAVFSNFDSSQWEKAD-WANGSVFNCVWKPSQVTFSN-GKMILTLDREYGGSY-
RF              NTSATMISDFRTGKAGDFFASDGWTNGKPFDC-WWYKRNAVINDGCLQLSIDQKWTNDKN
SBV             QSKYHYSQELNYYNGNAMELRNG-SNGGMFNCNFVPGNVGFNN-GLMSLKIDSD--GRG-
                          .           .  .**  *.*          . * . * .

GUB_BACLI       -----KFDCGENRSVQTYGYGLYEVNMKPAKNVGIVSSFFTYTGPTDGTPWDEIDIEFLG
GUB_BACMA       -----KFDCAEYRSTNIYGYGLYEVSMKPAKNTGIVSSFFTYTGPAHGTQWDEIDIEFLG
GUB_BACSU       -----KFDCGENRSVQTYGYGLYEVRMKPAKNTGIVSSFFTYTGPTDGTPWDEIDIEFLG
GUB_CLOTM       -----PYKSGEYRTKSFFGYGYYEVRMKAAKNVGIVSSFFTYTGPSDNNPWDEIDIEFLG
RF              PDWDPRYSGGEFRTNNFYHYGYYECSMQAMKNDGVVSSFFTYTGPSDDNPWDEIDIEILG
SBV             -----GYTGGEWRSKERFGYGLFQVNMKPIKNPGVVSSFFTYTGPSDGTKWDEIDIEFLG
                      .  .* *.   . ** ..  *.. ** *.**********..   *******.**

GUB_BACLI       KDTTKVQFNYYTNGVGNHEKIVNLGFDAANSYHTYAFDWQPNSIKWYVDGQLKHTATTQI
GUB_BACMA       KDTTKVQFNYYTNGVGGHEKVISLGFDASKGFHTYAFDWQPGYIKWYVDGVLKHTATANI
GUB_BACSU       KDTTKVQFNYYTNGAGNHEKIVDLGFDAANAYHTYAFDWQPNSIKWYVDGQLKHTATNQI
GUB_CLOTM       KDTTKVQFNWYKNGVGGNEYLHNLGFDASQDFHTYGFEWRPDYIDFYVDGKKVYRGTRNI
RF              KNTTQVQFNYYTNGQGKHEKLYDLGFDSSEAYHTYGFDWQPNYIAWYVDGREVYRATQDI
SBV             KDTTKVQFNYYTSGQGNHEYLYNLGFDASQGFHTYGFDWQADHITWYVDGRAVYTAYNNI
                *.**.**** * .* * .* .  ****..  .***.*.*..  *  ****     .  .*

GUB_BACLI       PQTPGKIMMNLWNGA-GVDEWLGSYNGVTPLSRSLHWVRYTKR
GUB_BACMA       PSTPGKIMMNLWNGT-GVDDWLGSYNGANPLYAEYDWVKYTSN
GUB_BACSU       PTTPGKIMMNLWNGT-GVDEWLGSYNGVNPLYAHYDWVRYTKK
GUB_CLOTM       PVTPGKIMMNLWPGI-GVDEWLGRYDGRTPLQAEYEYVKY--Y
RF              PKTPGKIMMNAWPGL-TVDDWLKAFNGRTPLTAHYQWVTY---
SBV             PSTPGKIMMNAWPGTHEVDSWLGAYNGRTPLYAYYDWISYDQF
                * ******** * *   ** **  ..*  **    . . *
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FIG. 5. Multiple alignment of *S. bovis* β-glucanase with other enzymes of glycoside hydrolase family 16. SBV, *S. bovis* (EMBL, GenBank accession no. Z92911); RF, *R. flavefaciens* XynD (C-terminal domain; accession no. A36910); CLOTM, *C. thermocellum* (accession no. P29716); BACSU, *B. subtilis* (accession no. P04957); BACMA, *Bacillus macerans* (accession no. P23904); BACLI, *Bacillus licheniformis* (accession no. P27051).

TABLE 1. β-(1,3-1,4)-glucanase (lichenase) activity in *S. bovis* JB1, *L. lactis* IL2661, and *E. faecalis* JH2-SS carrying recombinant plasmids containing the β-(1,3-1,4)-glucanase gene[a]

Species	Plasmid	Medium[b]	Lichenase activity (nmol/min/ml of culture)		Cell protein (mg/ml of culture)
			Cell	Supernatant	
S. bovis	pVA838	M17G	8	88	0.30
		M17L	10	143	0.27
		M2S	5	107	0.33
	pTRWL1R	M17G	17	638	0.45
		M17L	40	792	0.36
		M2S	10	649	0.45
	pILHcP	M17G	26	687	0.30
		M17L	48	909	0.27
		M2S	8	682	0.35
E. faecalis	pTRWL1R[c]	M17G	15	82	0.72
L. lactis	pTRWL1R[c]	M17G	5	14	0.82

[a] Cultures were grown anaerobically to the stationary phase (18 h) in the media indicated. The plasmids pTRWL1R and pILHcP both carry the cloned gene, while the vector plasmid pVA838 does not (see text).

[b] M17G, M17 medium with 0.2% glucose; M17L, M17 medium with 0.2% lichenan; M2S, M2 (rumen fluid-based) medium with 0.2% soluble starch as the only added energy source. All media contained 5 μg of erythromycin per ml.

[c] No β-glucanase activity was detected in *E. faecalis* or *L. lactis* without the pTRWL1R construct (data not shown).

the β-glucanase activity produced (Table 1). It is also possible that there is some limitation, perhaps due to regulatory mechanisms, in the maximum level of β-glucanase expression in *S. bovis.*

There was no evidence for any dramatic induction of β-glucanase activity resulting from growth with lichenan or starch as energy sources, either for the native enzyme in *S. bovis* JB1 or for the cloned enzyme in *S. bovis* carrying pTRWL1R or pILHcP, although the highest activities were obtained for lichenan-grown cultures (Table 1).

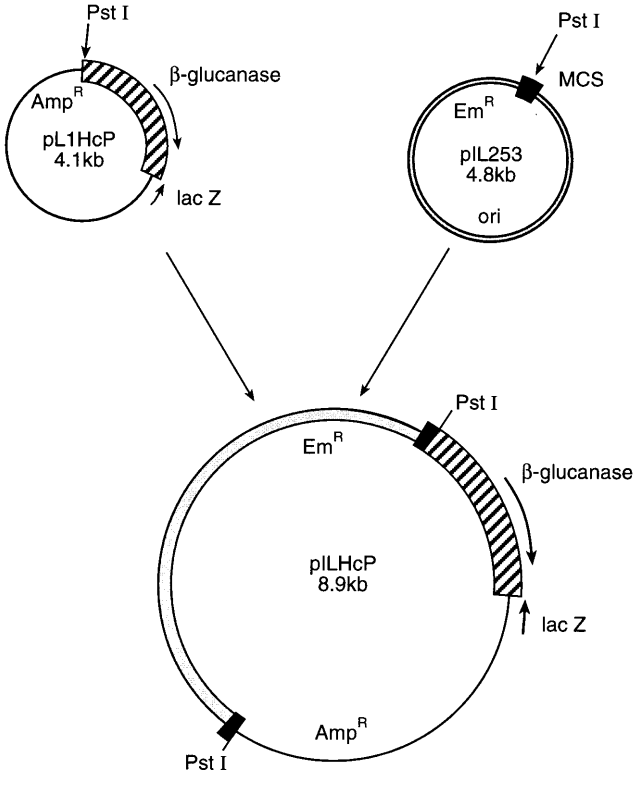

FIG. 6. Construction of an *S. bovis-E. coli* shuttle construct carrying the *S. bovis* β-(1,3-1,4)-glucanase, based on the gram-positive vector pIL253.

TABLE 2. Activities of the cloned *S. bovis* β-(1,3-1,4)-glucanase purified from *S. bovis* JB1/pTRWL1R culture supernatant

Substrate	Relative enzyme activity[a]
Lichenan	89.7
Barley β-glucan	100
Laminarin	7.2
Oat spelt xylan	<0.5
Birchwood xylan	<0.5
Carboxymethyl cellulose	<0.5

[a] Activities were determined for the purified β-(1,3-1,4)-glucanase by reducing sugar release from polysaccharide substrates present at 1% (wt/vol). Activities are expressed as a percentage of the activity against barley β-glucan, for which the V_{max} was 338.3 μmol per min per mg of protein.

Purification and properties of the *S. bovis* JB1 β-(1,3-1,4)-glucanase. The β-(1,3-1,4)-glucanase was purified from the supernatant of cultures of *S. bovis* JB1/pTRWL1R by ion-exchange chromatography and hydrophobic-interaction chromatography, as described in Materials and Methods. Purification resulted in a 228-fold increase in specific activity against lichenan, implying that the enzyme constituted no more than 0.5% of total extracellular protein in these cultures. The purified enzyme gave a single band of approximately 25 kDa by SDS-PAGE and demonstrated a V_{max} of 338.3 μmol per min per mg of protein and a K_m of 2.78 mg per ml with barley β-glucan as the substrate. The pH optimum in 50 mM Na phosphate buffer was 6.5. Enzyme activity was stable for 20 min at 50°C, but 20 min of preincubation at 60°C destroyed 75% of the activity. Table 2 shows the relative activity of the purified enzyme with different polysaccharide substrates and indicates a high degree of specificity for β-(1,3-1,4)-glucans, although the β-(1,3)-glucan laminarin was also hydrolyzed at a much lower rate. Although there was very little release of reducing sugar from xylans (Table 2), the purified enzyme and also the cloned activity expressed in *E. coli* (Fig. 2) gave rise to weak clear zones on plates containing 0.5% oat spelt xylan. It is therefore possible that the enzyme can effect sufficient cleavage of xylans to achieve partial solubilization.

Because *S. bovis* cultures produce much lactic acid, the stability of the crude supernatant β-(1,3-1,4)-glucanase was studied in Na phosphate buffers acidified with DL-lactic acid (see Materials and Methods). Activities at pH 5, 4, and 3 were approximately 69, 23, and 2% of maximal activity, respectively, but the enzyme was able to recover full activity at pH 6.5 following 6 h of exposure at 38°C to pHs as low as 3 (results not shown). Thus, any inactivation of the gene product by accumulation of lactic acid in cultures is expected to be reversible.

Conclusions. The most likely role for the extracellular β-(1,3-1,4)-glucanase of *S. bovis* is in the degradation of β-glucans present in endosperm cell walls from cereals. Plant β-(1,3-1,4)-glucanases have long been thought to play an important role in initiating starch utilization from endosperm reserves in the developing seed and are important in initiating the malting process in brewing (8, 23). The enzyme studied here may therefore fulfill a similar role in allowing *S. bovis* access to stored starch reserves. Sequence analysis of the *S. bovis* enzyme shows that it is related to typical bacterial β-glucanases of glycoside hydrolase family 16, which are distinct from plant β-glucanases belonging to family 17 (14, 15). Genes encoding

specific family 16 β-(1,3-1,4)-glucanases have been reported previously from two cellulolytic ruminal bacterial species, *Fibrobacter succinogenes* (31) and *R. flavefaciens* (10), although in the latter case the activity is encoded by the C-terminal domain of the bifunctional xylanase XynD. In monogastric animals, especially poultry, β-glucans can lead to nutritional problems due to viscosity which can be counteracted by inclusion of β-glucanases in diets (2, 13).

This is the first gene to be isolated from *S. bovis* JB1 that encodes a product that is secreted from the cell, although a gene for an extracellular amylase has been isolated from *S. bovis* 148 (28). It will therefore be of interest in the future to explore the use of the promoter and N-terminal coding regions of this β-glucanase gene for the secretion of foreign gene products by *S. bovis* strains. This work also shows that pIL253-based vectors and the new shuttle vector pTRW10 (36) can both be used to express cloned genes in *S. bovis*, making this species a convenient gram-positive host for the expression of cloned gene products and potentially for the production of extracellular enzymes. Appropriate genetic modification of *S. bovis* strains might enhance their efficacy as silage inoculants, for example, through the introduction of cellulase or xylanase activity (1). Because of its relatively greater tolerance of oxygen, *S. bovis* may offer practical advantages compared with the more-abundant ruminal obligate anaerobes as a target for genetic manipulation aimed at altering rumen function. Finally, it is also worth noting that since β-glucanase activity has not been reported from other lactic acid bacteria, the *S. bovis* β-(1,3-1,4)-glucanase gene could have potential as a convenient reporter gene for many other gram-positive species.

ACKNOWLEDGMENTS

We thank Terry Whitehead for generously providing the pTRW10 vector prior to publication and also for valuable discussions. We are also grateful to Y. Duval-Iflah, F. Macrina, and H. J. Gilbert for supplying the strains and plasmids used in this work.

This work was supported by the Scottish Office Agriculture Environment and Fisheries Department. M.S.E. acknowledges the support of a Ph.D. studentship from the Turkish Government.

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