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DNA Sequence of a *Fibrobacter succinogenes* Mixed-Linkage β-Glucanase (1,3-1,4-β-D-Glucan 4-Glucanohydrolase) Gene†

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The DNA sequence of a mixed-linkage β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase [EC 3.2.1.73]) gene from *Fibrobacter succinogenes* cloned in *Escherichia coli* was determined. The general features of this gene are very similar to the consensus features for other gram-negative bacterial genes. The gene product was processed for export in *E. coli*. There is a high level of sequence homology between the structure of this glucanase and the structure of a mixed-linkage β-glucanase from *Bacillus subtilis*. The nonhomologous region of the amino acid sequence includes a serine-rich region containing five repeats of the sequence Pro-Xxx-Ser-Ser-Ser-Ser-(Ala or Val) which may be functionally related to the serine-rich region observed in *Pseudomonas fluorescens* cellulase and the serine- and/or threonine-rich regions observed in *Cellulomonas fimi* endoglucanase and exoglucanase, in *Clostridium thermocellum* endoglucanases A and B, and in *Trichoderma reesei* cellobiohydrolase I, cellobiohydrolase II, and endoglucanase I.

Fibrobacter succinogenes (known until recently as *Bacteroides succinogenes* [30]), plays a major role in plant fiber degradation in the rumen (10, 17, 31, 41). This organism has been shown to synthesize a number of β-1,4-glucanases (37). Several of these enzymes have been isolated and characterized, including a cellodextrinase (23), two endoglucanases (28), and a chloride-stimulated cellobiosidase (24).

Gene cloning experiments have shown that the number of glucanases produced by this organism is greater than suggested by the biochemical studies. For example, six distinct genes were defined among 14 clones expressing β-1,4-glucanase activity in *Escherichia coli* (9). A xylanase (40), a 1,3-1,4-β-D-glucanohydrolase (25), a cellodextrinase (15), and the *cel-3* gene (29) have also been cloned and expressed in *E. coli*.

The existence of a distinct 1,3-1,4-β-D-glucanohydrolase had not been demonstrated prior to its cloning. The cloned 1,3-1,4-β-D-glucanohydrolase has been isolated and characterized biochemically (12). The cloned gene is expressed from its own promoter in *E. coli*, and part of the activity appears in the periplasmic fraction, indicating that the gene codes for a signal sequence which is recognized in *E. coli*. The failure to identify this enzyme activity in cultures of *F. succinogenes* by using biochemical techniques can be explained by the similarity of its physical properties to those of one of the 1,4-β-D-glucanohydrolases. The enzymes are not readily separable biochemically, and since the 1,3-1,4-β-D-glucan substrate is readily hydrolyzed by 1,4-β-D-glucanohydrolases, the presence of the mixed-linkage specific enzyme was not suspected.

There is only limited information available on the structure of the glucanases or on the structure of the regions regulating the expression and secretion of enzymes in *F. succinogenes* (29). We have therefore determined the DNA sequence of the cloned 1,3-1,4-β-D-glucanohydrolase gene. We have also determined the amino acid sequence of the amino terminus of the mature enzyme purified from the *E. coli* clone to confirm the coding region and to help in defining the signal peptide structure.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains OR95 (*E. coli* HB101 carrying plasmid pJI5) and OR156 (*E. coli* HB101 carrying plasmid pJI10, a derivative of pJI5) have been described previously (25). Media and growth conditions were as previously described (25).

DNA sequence determination. A series of deletions of pJI10 was prepared by using the ExoIII-mung bean nuclease technique (22) and cloned into *E. coli* HB101. Plasmid DNA was isolated from cleared lysates by the CsCl-ethidium bromide density gradient method (8) as previously modified (43) or from 5-ml cultures by the alkaline extraction method of Birnboim (4). The double-stranded plasmid DNA was sequenced by using 17-base-pair forward and reverse primers (Boehringer Mannheim, Dorval, Quebec, Canada), a 17-base-pair −40 primer (Pharmacia, Baie d'Urfé, Quebec, Canada), and both Klenow polymerase (Boehringer Mannheim) and T7 DNA polymerase (Pharmacia) under the reaction conditions specified by the suppliers. Extension of the sequence into pJI5 and determination of the sequence of the second strand were accomplished by using 21-base-pair primers with a sequence based on the sequence of the first strand. 5′-[α-^{35}S]dATP (500 Ci/mmol) was obtained from Du Pont Co. (Markham, Ontario, Canada).

Purification of mixed-linkage glucanase. All procedures were carried out at 4°C. Washed, freeze-dried cells (38.4 g) of *E. coli* OR95 were stirred with 500 ml of bis-Tris-propane (BTP) buffer (pH 6.8) containing 1 mM dithiothreitol for 30 min. The extract was centrifuged at 27,000 × *g* for 30 min. The extraction was repeated twice, and the combined supernatants were concentrated in a Minitan tangential-flow filtration apparatus (Millipore Corp., Mississauga, Ontario, Canada) with a 10,000-dalton molecular mass exclusion membrane. The extract was concentrated to about 25 ml and then dialyzed against 1.5 liters of buffer in the Minitan. The extract was chromatographed on a Q-Sepharose column (Pharmacia), as previously described (12). Fractions containing mixed-linkage β-glucanase activity were concentrated and dialyzed with the Minitan and then chromatographed on a high-resolution hydroxylapatite column (Behring Diagnostics, La Jolla, Calif.), as previously described (12). Final purification was performed on a Waters

Protein Pak DEAE 5PW column (21.5 by 150 mm) (Millipore Corp.). Proteins were eluted with 200 ml of 25 mM BTP buffer (pH 6.8) containing 1 mM dithiothreitol, followed by a 0 to 0.12 M lithium perchlorate gradient in 25 mM BTP buffer (pH 6.8) containing 1 mM dithiothreitol. Total gradient volume was 400 ml. The flow rate was 3 ml/min.

Fractions were assayed for activity by a reducing sugar assay with oat glucan as a substrate (12), and protein was determined by the Coomassie blue assay (5). Fractions with maximum specific activity were combined and concentrated in a 10-ml stirred cell (Amicon Corp., Oakville, Ontario, Canada) with a YM5 membrane. The preparation was exhaustively dialyzed (three times, 24 h each time versus 500 volumes) in 1-cm dialysis tubing against distilled water and then lyophilized. The purity of the final preparation was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26).

Estimation of kinetic parameters. The kinetic parameters of enzymes produced by deletion mutants were estimated by using centrifuged crude cell lysates and the Congo red agar diffusion assay (47).

Amino acid sequence determination. The N-terminal amino acid sequence of the mixed-linkage glucanase was determined with a gas phase sequencer system model 475 (Applied Biosystems).

Data processing. Sequence assembly, analysis, comparisons, and preparation for publication were carried out by using versions 5 and 6.1 (GCG 5 and GCG 6.1) of the sequence analysis software package of the Genetics Computer Group of the University of Wisconsin (11).

RESULTS AND DISCUSSION

The purified enzyme had a specific activity of 906 μmol/min per mg and was obtained at about a 5% yield from the initial freeze-dried cell extract. Coomassie blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed a single protein band. The amino acid sequence of the N terminus of the purified enzyme is underlined in Fig. 1.

The sequence of the cloned DNA coding for the mixed-linkage glucanase is shown in Fig. 1. The sequence contains a single major open reading frame of 1,047 bases, coding for a 349-amino-acid peptide. Analysis of the amino acid sequence of the N terminus of the mixed-linkage glucanase isolated from *E. coli* indicated that the peptide is processed in *E. coli*, with a 27-amino-acid signal sequence being removed to give a mature active enzyme of 322 amino acids. The DNA sequence predicts a mature peptide molecular mass of 35,168 daltons. This conforms closely to the experimentally determined values of 35,200 to 37,200 daltons (12). The predicted peptide has a calculated isoelectric point of pH 6.42. There were no cysteine residues. Codon usage (Table 1) was unusual in two respects. The dominant bacterial lysine codon, AAA (16, 19), was used rarely (2 of 21 residues), and the dominant bacterial leucine codon, CTG, was not used at all (0 of 18 residues).

The 27-amino-acid presumptive signal sequence deduced from the DNA sequence contains all of the features normally associated with a bacterial signal sequence (45), including a positively charged amino-terminal n region (lysine residues at amino acid positions −24, −23, and −19) and a strongly hydrophobic h region from amino acid residues −17 through −7 (amino acid positions are given relative to the first residue of the mature peptide). As is typical for peptides cleaved by signal peptidase I, the border between the h and c regions falls between amino acid residues −6 and −7. The c region conforms to the "−3,−1 rule", with valine at −3 and alanine at −1. The residue at +2 (aspartic acid) is negatively charged, conforming to the bacterial consensus for secreted proteins.

The noncoding portion of the sequence contains a number of readily identifiable features. There is a −35 sequence ($^{-83}$TTGGTT^{-78}) and a −10 sequence ($^{-60}$TATCTT^{-55}) spaced 17 bases apart (Fig. 1), conforming closely to the consensus for transcription initiation sites dependent on *E. coli* σ^{70} or *Bacillus subtilis* σ^{43} (21, 27). The sequence also shows the AT-rich region upstream of the promoter typical for strong *B. subtilis* promoters (36). There is a potential ribosome-binding site (Shine-Dalgarno sequence), $^{-13}$ATG GAG^{-8}, which has five matches of six residues with the 3′ end of *E. coli* 16S RNA and is spaced appropriately from the presumptive ATG initiation codon (14, 38, 42).

Both the leading and trailing regions have a very high AT content (70 and 71%, respectively, compared with 46% AT in the gene itself). The leading region contains three inverted repeats (Fig. 1), which were identified as potential rho-independent transcription termination sites by the method of Brendel and Trifonov (6). The trailing region contains five such sequences, including two inverted repeats (10 and 8 bases) immediately preceding a run of T residues. This structure is typical for bacterial rho-independent transcription termination regions (35). It is therefore highly improbable that the cloned gene is part of an operon. This is in keeping with the observation that six distinct glucanase genes cloned into *E. coli* had no DNA sequence homology, suggesting that the genes involved in cellulose degradation in this organism are widely scattered on the chromosome (9). In agreement with this conclusion, none of the clones expressing polysaccharide-degrading enzymes from this organism have included other genes expressing related enzyme activities (15, 25, 29, 40).

In general, the sequences in this gene which affect the regulation of transcription, translation, and processing all fit the established consensus patterns for gram-negative bacteria, a not-too-surprising finding in view of the ease with which genes from *F. succinogenes* are cloned and expressed in *E. coli*. It is therefore probable that genes from other organisms will be readily expressed in *F. succinogenes*.

The structural region of the gene contains a unique and striking feature, consisting of five repeats of the sequence Pro-Xxx-Ser-Ser-Ser-Ser (Fig. 1) between amino acid residues 243 and 279 (bases 810 to 917), with each repeat separated by one to three hydrophobic amino acids and a hydrophobic amino acid preceding and following the region containing the five repeats. Xxx is an uncharged residue (glutamine, alanine, or proline). Deletion of the carboxy-terminal region of the gene up to and including base 923 (leaving the repeats intact) reduced the activity of the enzyme in the culture to about 0.16% of the original value. Extending the deletion to include base 796 (removing the repeats) reduced the activity an additional 16-fold (residual activity, 0.01%). Extending the deletion to include base 718 (212 amino acids remaining) left no detectable enzyme activity. The apparent K_m of those deleted proteins which retained enzyme activity was approximately the same as that of the native protein, in the range of 0.5 to 1 mg of oat glucan per ml. (In the deletion mutants studied, the deleted peptides were expressed as fusion proteins, with the carboxy terminus continued through the *Pst*I site in the multiple-cloning site of pUC8 into the *lacZ* gene. The size of the fusion

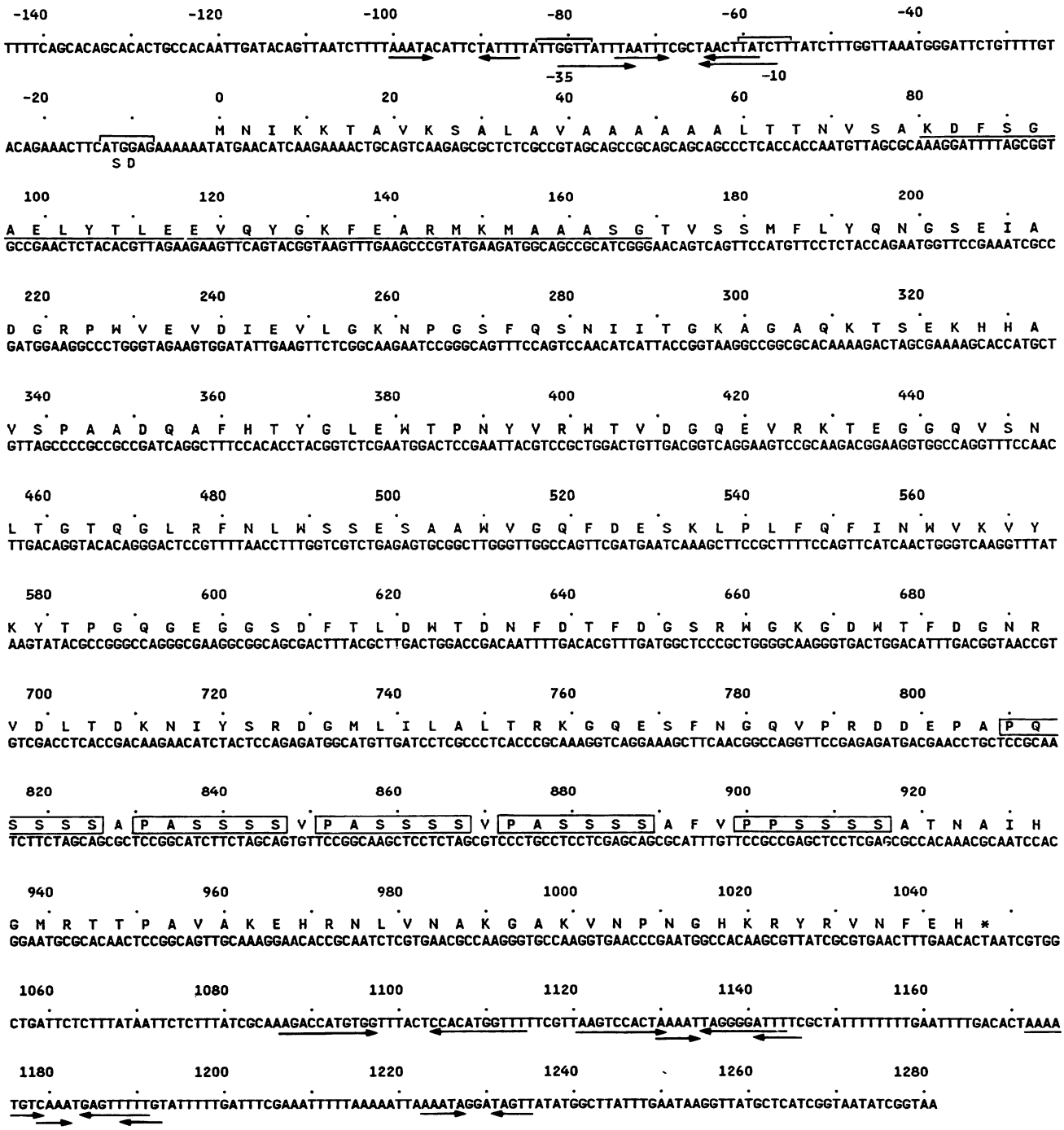

FIG. 1. DNA and amino acid sequences of mixed-linkage β-glucanase from *F. succinogenes*. Arrows underlining the nucleic acid sequence show the inverted repeats associated with potential rho-independent termination sites. The −35, −10, and Shine-Dalgarno (SD) sequences are bracketed. The amino-terminal amino acid sequence determined from the purified mixed-linkage β-glucanase is underlined. Five repeats of the sequence Pro-Xxx-Ser-Ser-Ser-Ser are boxed. The nucleotide sequence data reported in this figure will appear in the EMBL, Genbank, and DDBJ nucleotide sequence databases under accession number M33 676.

proteins varied with the reading frame imposed by the deletion site.)

Serine- and/or threonine-rich regions are a common feature of microbial glucanases, appearing in two repeats in *Pseudomonas fluorescens* cellulase (20), in the serine- and/or threonine-rich regions observed in *Cellulomonas fimi* endoglucanase (46) and exoglucanase (33), in *Clostridium thermocellum* endoglucanases A (1) and B (18), and in *Trichoderma reesei* cellobiohydrolase I (39), cellobiohydrolase II (44), and endoglucanase I (34). No specific function has been assigned to these regions, but it has been shown that the Ser-Thr domain in *T. reesei* cellobiohydrolase I is a site of extensive O glycosylation (for a review, see reference 2).

In the present case, secondary-structure predictions based on computer analysis of the amino acid sequence by the

TABLE 1. Codon usage in the mixed-linkage glucanase gene

Amino acid	Codon	No. of times used	Amino acid	Codon	No. of times used
Gly	GGG	0	Glu	GAG	1
	GGA	4		GAA	17
	GGT	12	Asp	GAT	8
	GGC	14		GAC	10
Val	GTG	4	Ala	GCG	1
	GTA	2		GCA	15
	GTT	12		GCT	6
	GTC	7		GCC	15
Arg	AGG	1	Lys	AAG	19
	AGA	2		AAA	2
Ser	AGT	4	Asn	AAT	7
	AGC	16		AAC	12
Met	ATG	6	Thr	ACG	5
Ile	ATA	0		ACA	6
	ATT	2		ACT	5
	ATC	7		ACC	7
Trp	TGG	9	End	TAG	0
End	TGA	0		TAA	1
Cys	TGT	0	Tyr	TAT	6
	TGC	0		TAC	6
Leu	TTG	2	Ser	TCG	4
	TTA	1		TCA	1
Phe	TTT	9		TCT	6
	TTC	7		TCC	9
Arg	CGG	0	Gln	CAG	12
	CGA	0		CAA	2
	CGT	4	His	CAT	1
	CGC	7		CAC	6
Leu	CTG	0	Pro	CCG	12
	CTA	0		CCA	0
	CTT	4		CCT	2
	CTC	11		CCC	2

methods of Chou and Fasman (7) and Garnier et al. (13), as implemented in the program Peptidestructure in GCG 6.1 (11), suggest that the region containing the repeats could form five repeats of a turn-alpha helix-turn structure. The helical repeat distance corresponded roughly to the repeat size of the polysaccharide, suggesting a possible role for the serine repeats in the interaction of the enzyme with the substrate. A structurally related sequence in the endonuclease gene (*end-1*) from *Butyrivibrio fibrisolvens*, consisting of five direct repeats of the amino acid sequence Pro-Asp-Pro-Thr-Pro-Val-Asp, has been reported (3). However, while deletion of the serine repeats did cause a large reduction in enzyme activity (about 16-fold, relative to a deletion which removed the carboxy terminus of the peptide up to but not including the serine repeats), the protein did retain some enzyme activity and the apparent K_m of the enzyme did not change. This suggests that the serine repeats participate in stabilizing the active conformation of the protein but do not participate directly either in forming the substrate-binding site or in catalysis.

There is little published information available on protein or DNA sequences for polysaccharidase genes from rumen bacteria with which this sequence could be compared. A recent study on the structure of endoglucanase 2 of *F. succinogenes* has shown that the protein has distinct catalytic and substrate-binding domains which can be isolated after trypsin digestion, an organization similar to that found in other fungal and bacterial cellulases (M. McGavin and C. W. Forsberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K-80, p. 258). The *cel-3* gene cloned in *E. coli*, which codes for an enzyme (endoglucanase 3) which exhibits both endoglucanase and cellobiosidase activity, has been sequenced. The native gene product has an apparent molecular weight greater than that predicted from the DNA sequence, and the protein may therefore be glycosylated in *F. succinogenes* (it is partially degraded in *E. coli*) (29). The DNA sequence shows homology with the *celC* gene from *C. thermocellum* (29). The DNA sequence possesses typical (for *E. coli*) consensus promoter, ribosomal-binding, and rho-independent termination sites (29).

The mixed-linkage glucanase showed no significant homology with the *cel-3* gene from *F. succinogenes* or any other β-1,4-glucanase but a very high level of homology with the β-1,3-1,4-endoglucanase from *B. subtilis* (32). For the deduced amino acid sequence, the percent identity for amino acids 1 to 193 of the *F. succinogenes* glucanase versus amino acids 9 to 241 of the *B. subtilis* glucanase is 36% and percent similarity is 57%; for amino acids 43 to 140 of the *F. succinogenes* glucanase versus amino acids 100 to 191 of the *B. subtilis* glucanase, percent identity is 48% and percent similarity is 63%.

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