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Studies of *Streptomyces reticuli cel-1* (Cellulase) Gene Expression in *Streptomyces* Strains, *Escherichia coli*, and *Bacillus subtilis*

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Various *Streptomyces* strains [*Streptomyces lividans* 66, *Streptomyces vinaceus*, and *Streptomyces coelicolor* A3 (2)] acquired the ability to utilize crystalline cellulose (Avicel) after transformation with a multicopy vector containing the *cel-1* gene from *Streptomyces reticuli*. The expression level in these hosts was two to three times lower than in *S. reticuli*, indicating the absence of positive regulatory elements. Like *S. reticuli*, they processed the Avicelase to its catalytic domain and to an enzymatically inactive part. The *cel-1* gene with its original upstream region was not expressed within *Escherichia coli*. When *cel-1* had been fused in phase with the *lacZ* gene, large quantities of the fusion protein were produced in *E. coli*. However, this protein was enzymatically inactive and proteolytically degraded to a series of truncated forms. As the cellulase (Avicelase) synthesized by *S. reticuli* is not cleaved by the *E. coli* proteases, its posttranslational modification is proposed. With *Bacillus subtilis* as host, the *cel-1* gene was expressed neither under its own promoter nor under the control of a strong *Bacillus* promoter.

Streptomycetes are gram-positive mycelial bacteria which are studied primarily for their ability to produce a large portion of the naturally occurring antibiotics. In soil, they are able to synthesize a wide range of extracellular enzymes which hydrolyze many macromolecules such as chitin, starch, xylans, proteins, nucleic acids, and cellulose (29).

A screening for cellulolytic *Streptomyces* strains revealed (42) that nearly all of the 180 species tested were able to utilize soluble or amorphous cellulose such as hydroxyethyl- or carboxymethylcellulose through the action of endoglucanases (EC 3.2.1.4). These data are supported by the fact that four of five cloned *Streptomyces* cellulase genes code for endoglucanases: *celA* (38) and *celB* (45) from *Streptomyces lividans* 66, *casA* from *Streptomyces* sp. KSM-9 (25), and *celA1* from *Streptomyces halstedii* (10). In contrast, only a few percent of streptomycetes hydrolyze microcrystalline cellulose (Avicel) with the help of cellobiohydrolases (EC 3.2.1.91) (42). Within several cellulolytic organisms (i.e., *Trichoderma reesei* or *Cellulomonas fimi*), endoglucanases, cellobiohydrolases, and β-glucosidases (EC 3.2.1.21) have been shown to act synergistically to convert Avicel to glucose (2, 27, 39).

Owing to its comparatively high efficiency in hydrolyzing Avicel, the cellulolytic system of *Streptomyces reticuli* was studied in detail (42). An unusual mycelium-associated Avicelase (82 kDa) was found to be solely sufficient to degrade crystalline cellulose to cellobiose (34). This to-date-unique *Streptomyces* enzyme can specifically be detected with the substrate analog *para*-nitrophenylcellobioside (pNPC). It is interesting that, during cultivation of *S. reticuli*, the Avicelase (Cel-1) is proteolytically processed to a truncated enzyme (42 kDa) representing the catalytic domain and an inactive protein (40 kDa) which contains the cellulose-binding domain (24, 35). Processing of extracellular enzymes, which strongly interact with their substrate via a specific binding domain, is widely spread among several microorganisms (20, 26, 39).

Recently, the Avicelase-encoding gene (*cel-1*) from *S. reticuli* was cloned and sequenced, and in addition, a transcriptional start site was mapped about 180 bp upstream of the GTG start codon of *cel-1* (35). Comparisons with amino acid sequences deduced from known cellulase genes revealed that the *S. reticuli* Avicelase (35) belongs to the cellulase family E (12).

In this article, we report the introduction of the *S. reticuli cel-1* gene in different Avicelase-negative bacteria in order to answer the following questions. (i) Are various *Streptomyces* species, *Escherichia coli*, and *Bacillus subtilis* able to hydrolyze Avicel after having obtained the *cel-1* gene? (ii) Is the promoter of the *cel-1* gene used and regulated in the different bacteria? (iii) How are the characteristics of the enzyme produced by various hosts?

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM109 (46) served as host for subcloning experiments with both pUC18 and -19 as well as with the bifunctional vectors pZG6 (*Streptomyces* sp.-*E. coli* [9]) and pRB373/374 (*Bacillus* sp.-*E. coli* [5]). The wild-type strain *S. reticuli* TÜ45 was obtained from H. Zähner, Tübingen, Germany, and had already been described earlier (42). *S. lividans* 66, *Streptomyces coelicolor* A3(2) M145 (both obtained from D. A. Hopwood [John Innes Institute, Norwich, England]), and *Streptomyces vinaceus* (obtained from J. Dusart, University of Liège, Liège, Belgium) as well as *B. subtilis* BR151CM1 (*tryC2 metB10 lys-3 spoCM1*; obtained from J. Kreft, University of Würzburg, Würzburg, Germany) and *E. coli* JM109 were used as hosts for the expression of the Avicelase encoded by the *cel-1* gene.

In addition, the *Streptomyces* strains *S. olivaceoviridis* ATCC 11238, *S. venezuelae* ATCC 10712, *S. antibioticus* ETH22014, *S. ambofaciens* ETH6703, *S. parvulus* ATCC 12434, and *S. flavogriseus* ATCC 33331 were needed for hybridization experiments.

pALTER was utilized for site-directed mutagenesis (Promega). pUCel1 (35) is a pUC19 derivative which carries the *cel-1* gene (Avicelase) from *S. reticuli* as a 6.8-kb *Bam*HI fragment (see Fig. 1).

Media and culture conditions. For isolation of plasmid DNA, both *E. coli* and *B. subtilis* were grown in Luria broth (LB) containing 100 μg of ampicillin ml^{-1} or 5 μg of kanamycin ml^{-1} at 37°C overnight. *Streptomyces* strains were cultivated in a complete medium (15) containing 10 μg of thiostrepton ml^{-1} at 30°C for 2 days on a rotary shaker. For the expression studies, 0.2 ml of a fresh overnight culture of *E. coli* was transferred to 50 ml of LB containing 100 μg of ampicillin ml^{-1} and incubated at 37°C on a rotary shaker. IPTG (isopropyl-β-

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D-thiogalactopyranoside; final concentration, 2 mM) was added when A_{600} reached 0.3 *B. subtilis* was grown in LB (with 1.5% agar when necessary), supplemented with 1% hydroxyethylcellulose (HEC) and 5 μg of kanamycin ml^{-1} at 37°C. Spores of the different *Streptomyces* strains were inoculated in minimal media (15) supplemented with 1% microcrystalline cellulose (Avicel). To maintain the selection pressure for maintenance of plasmids, thiostrepton (20 μg/ml) was added to the media. The cultures were grown in conical flasks with indentations at 30°C for 2 to 10 days.

Transformation of strains. *E. coli* was transformed with plasmid DNA by the $CaCl_2$ method (32). The transformation of *B. subtilis* protoplasts was carried out as described by Chang and Cohen (6). Preparation of *Streptomyces* protoplasts and their polyethylene glycol-assisted transformation were performed according to the standard procedure (15). An overlay of 0.4% agarose containing 500 μg of thiostrepton ml^{-1} was used to select transformants.

Isolation of DNA. Plasmids of *E. coli*, *B. subtilis*, and *Streptomyces* strains were isolated by the alkaline method with strain-specific modifications (15, 32, 41). Total DNA of *Streptomyces* strains was released by the neutral lysis after growth for 2 days in a sucrose-containing complete medium (15).

General DNA techniques. Modifications of DNA with nucleases and polymerases as well as ligases were carried out by the standard procedures (32). DNA restriction fragments were resolved by agarose gel electrophoresis.

DNA was sequenced by the dideoxy chain termination method (33) with double-stranded DNA templates and T7 DNA polymerase (Pharmacia sequencing kit). Oligonucleotides were synthesized individually or corresponded to primers of the *lacZ* system.

Base exchanges were performed with the help of a site-directed mutagenesis kit (Promega). To this end, single-stranded DNA was isolated from infected *E. coli* JM109 containing the plasmid pAV2 (see Fig. 1). The following modifications were done with the help of the corresponding oligonucleotides: GTG-ATG, 5′ CGTCGTTTCATGTCTGGGCTC 3′; *Sst*I restriction site, 5′ TCACGTCTG AGCTCCTCGG 3′; *Bam*HI restriction site, 5′ CTGACCTGCTCGGGATCCT CGGCACCG 3′. For hybridization experiments, the transfer of DNA fragments onto nylon membranes was performed as described previously (32). Hybridization and immunodetection were carried out according to the specifications of the DNA labelling and detection kit supplied by Boehringer.

Preparation of proteins and enzyme assays. For the preparation of whole-cell extracts, the washed cells of *E. coli* or *B. subtilis* were broken by sonification for 3 min in 20-s intervals (Branson sonifier B12, 75 W). Cell debris was removed by centrifugation for 10 min at 7,000 × *g*. Protein concentrations were measured according to the established method (4). pNPC served as substrate to test the presence of Avicelase activity (34). Assay mixtures included 100 μl of 50 mM citrate-phosphate buffer (pH 8.0) containing 1 mM pNPC and 50 μl of culture filtrate or 30 μg of proteins of the whole-cell extract. The mixtures were incubated at 37°C, and the release of *p*-nitrophenol was measured at 410 nm. The cellulolytic activity of proteins within 0.1% HEC-containing polyacrylamide (PAA) gels was determined after incubation of the proteins in sodium dodecyl sulfate (SDS) with sample buffer for 10 min at 37°C. After electrophoretic separation, proteins were renatured by washing the gel twice for 30 min in 0.1% Triton X-100 at 30°C and subsequently in 20 mM Tris-HCl, pH 7.5, for 30 min. Cellulolytic activities were detected after incubation at 30°C for 5 h and staining of the gel in 0.1% solution of Congo red in water as described by Schwarz et al. (36).

SDS-PAGE and Western blot (immunoblot) analysis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 10% acrylamide gels in the presence of 0.1% SDS (19). For immunodetection, proteins were transferred onto nylon membranes after SDS-PAGE (Western blotting), and then the filters were incubated in phosphate-buffered saline (PBS) containing a 1:10,000 dilution of anti-Avicelase antibodies (35). The filters were washed twice with PBS and incubated with alkaline-conjugated goat anti-rabbit immunoglobulin G in PBS. Color development was done according to the method of West et al. (44).

Computer analysis. Sequence comparisons were carried out with the help of the FASTA program with the Swissprot and EMBL databases. The sequences were analyzed with the GENMON program (GBF, Braunschweig, Germany), and reading frames were determined with the GCWIND program (D. Shields, Dublin, Ireland) on the basis of the codon usage preferences in *Streptomyces* DNA.

Nucleotide sequence accession number. The nucleotide sequence of ORF1 appears together with that of the *cel-1* gene in the EMBL-GenBank-DDBJ nucleotide sequence data libraries under the updated accession number X65616 SRCEL1G.

RESULTS

Construction of plasmids. The *cel-1* gene encoding the Avicelase from *S. reticuli* was cloned as a 6.8-kb *Bam*HI fragment (Fig. 1) in pUC19 (35). In order to separate the cellulase-encoding region and its regulatory upstream region, an *Apa*I-*Kpn*I fragment was cloned in two orientations in pUC18 (resulting in pUV1 and pUV2) and in pALTER (pAV2). With the help of site-directed mutagenesis, the GTG start codon of *cel-1* was changed to an ATG as required for more efficient translation in *E. coli*. This mutagenized gene was subcloned in both orientations in pUC18 (pUATG1 and pUATG2). None of the above-cited constructs allowed synthesis of a fusion protein consisting of the α-peptide and the Avicelase, as translation stop codons were present in all three reading frames upstream of the *cel-1* gene.

To construct plasmids which were predicted to encode fusion proteins consisting of the NH_2-terminal part of the α-peptide (*lacZ*) and the Avicelase with (pUFus2) or without (pUFus1) the signal peptide, one *Sst*I and one *Bam*HI restriction site were generated (see Materials and Methods). The resulting fragments were cloned in frame in pUC18 and -19, respectively (for details, see Fig. 1). The first amino acids of both predicted fusion proteins are given in Fig. 1.

Moreover, the insert of pUATG2 was subcloned in the *Bacillus-E. coli* shuttle vectors pRB373 and pRB374. The resulting constructs (pRATG1 and pRATG2) are shown in Fig. 1. pRATG2 contains a strong *B. subtilis* promoter upstream of the *cel-1* gene. The efficiency of this *veg*II promoter for the expression of heterologous genes had been demonstrated earlier (30).

In order to test the expression of the Avicelase-encoding gene in *Streptomyces* strains, the inserts of pUV1 and pUV2 were transferred into the *E. coli-Streptomyces* shuttle vector pZG6, which consists of pIJ350 and pUC18. The resulting plasmids were named pZV1 and pZV2.

In order to study the influence on expression, ORF1 (which was originally situated in front of the *cel-1* gene) was subcloned as a 1.75-kb *Sma*I fragment (from pUCel1) in both orientations in pZV2 (Fig. 1, left).

Plasmids introduced into *Bacillus* or *Streptomyces* protoplasts were controlled by restriction analysis to verify the structural identities with their counterparts in *E. coli*. In the case of pUFus1/2, all ligation sites were tested by sequencing for putative frameshifts.

Sequencing of ORF1. Genes encoding proteins which are required for certain steps of a metabolic pathway are often clustered or organized as a transcriptional unit (7, 14, 23, 28). In order to identify genes encoding additional enzymes for the breakdown of cellulose or possible regulatory proteins, both strands of the upstream region of *cel-1* were sequenced and analyzed. The resulting DNA sequence of 677 bp and the deduced amino acids of one complete reading frame (ORF1) are shown in Fig. 2. The coding sequence has an overall G+C content of 73.2%, with 76.4% in the first, 49% in the second, and 94.3% in the third position. Eight base pairs upstream of the ATG start codon, a typical Shine-Dalgarno sequence is located. In order to detect putative transmembrane-spanning regions, a hydropathy profile was deduced from the protein, according to the method of Kyte and Doolittle (18). No hydrophobic region could be found; therefore, an intracellular location of the gene product is proposed. By using the EMBL and Swissprot databases, no significant similarity to other known proteins was found.

At 11 bp downstream of the translation stop codon, a 28-bp inverted repeat with a loop of 8 bp could be a transcriptional terminator signal. The free energy of this hairpin structure was calculated to be −28 kcal/mol (ca. −120 kJ).

Distribution of ORF1 and *cel-1*-like genes within genomes of different *Streptomyces* strains. In order to study the distribution of *cel-1*-like genes, Southern blots of total DNA (digested with *Sma*I) from 10 different *Streptomyces* strains were hybridized with a 1,285-bp *Sty*I-*Pst*I fragment encoding a large portion of the catalytic domain of the Avicelase. All strains used were able to produce enzymes which degrade soluble forms of cel-

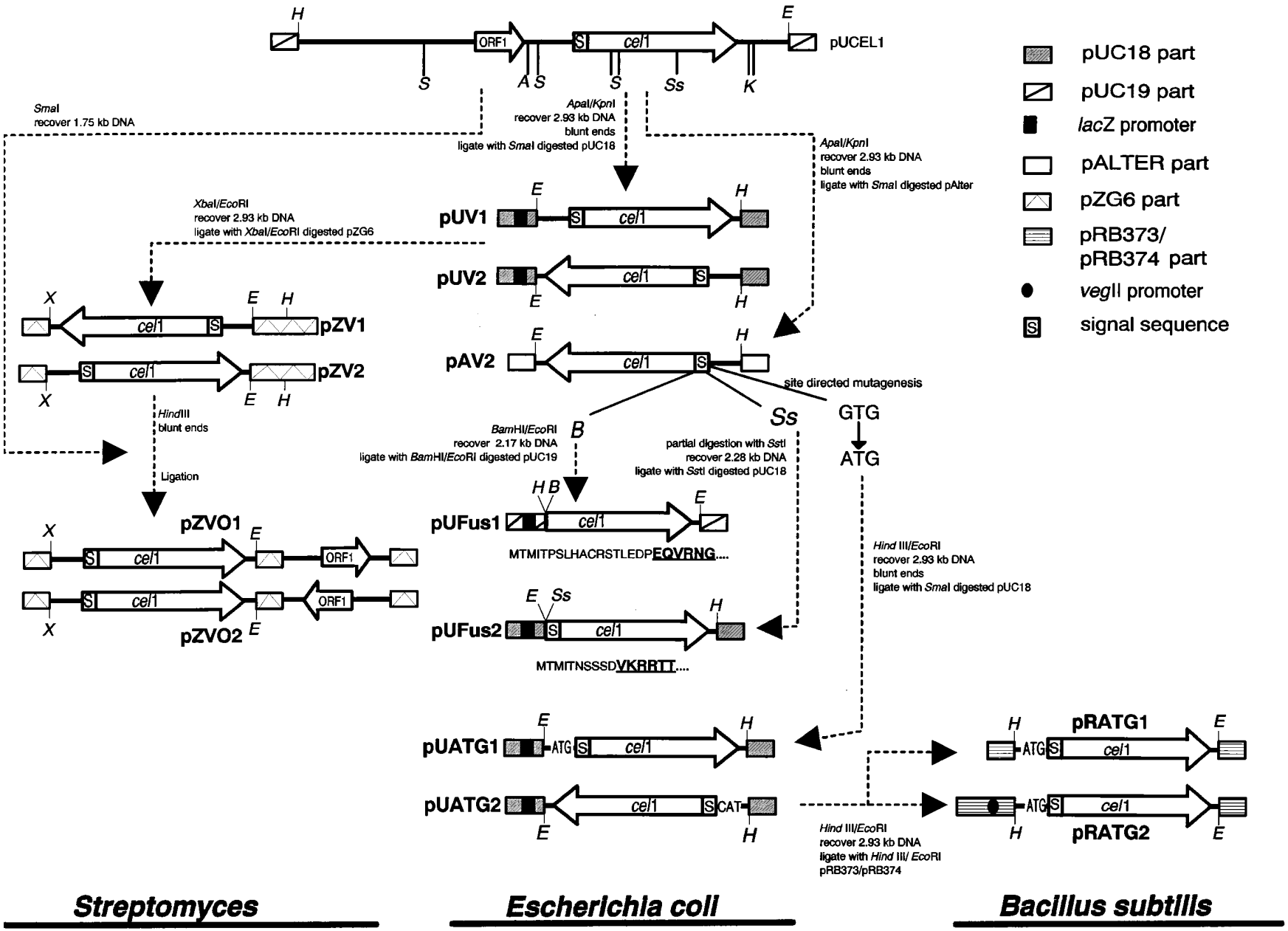

FIG. 1. Construction of plasmids. Only restriction sites relevant to the cloning strategy in this study are shown and named as following: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sma*I; Ss, *Sst*I; and X, *Xba*I. The NH_2-terminal amino acids of the fusion proteins are given in the single-letter code under the corresponding plasmids. The Avicelase parts are indicated by boldface and underlined letters.

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        10                  30                  50                  70                  90                 110
GTCGCGAGGA GGATCAGCAC CGAGGAACTG CGCGCATGCT GCGGCTGCGG CAGGCCGAGC TGGAGGCCGG CATGGCCGCC GCGGCCGGAC GGCTGGTCCA GGTCGAGGCG AGGCTCCGGT
                            M   L   R   L   R   Q   A   E   L   E   A   G   M   A   A   A   A   G   R   L   V   Q   V   E   A   R   L   R   S

        130                 150                 170                 190                 210                 230
CGATCGAGAG CGAGGGGCAC ATGCCCACGA ACGACGTCGT CGTCAAGAAC GTCCCGGCGA TCCGGGTGGC GGAACTCACC GCGACCGCCG CGAGCTTCGC GCCCGAGCAC ATCGGCCCGG
  I   E   S   E   G   H   M   P   T   N   D   V   V   V   K   N   V   P   A   I   R   V   A   E   L   T   A   T   A   A   S   F   A   P   E   H   I   G   P   V

        250                 270                 290                 310                 330                 350
TCTACGGACC GCTCTACGAC GAGCTGTTCC GGCGCCTGGA CGCGGCGGGC GTCACGCCCA CGGGCCCCGG TGTCGCCTTC TACGAGGACG CGCCAGAGGG CGGCGGCAGG ATCGTCGTCC
  Y   G   P   L   Y   D   E   L   F   R   R   L   D   A   A   G   V   T   P   T   G   P   G   V   A   F   Y   E   D   A   P   E   G   G   G   R   I   V   V   H

        370                 390                 410                 430                 450                 470
ACGCCGCCGT CCAGGTCGCG GCGCCCCTTC AGGACGGCGC GTTCCGGGTG CTCGACCTGC CACCCGTCGA CCAGGCGGCG ACGATCGTGC ACCGCGGCTC GATGGACGCG GTCCTGCCCA
  A   A   V   Q   V   A   A   P   L   Q   D   G   A   F   R   V   L   D   L   P   P   V   D   Q   A   A   T   I   V   H   R   G   S   M   D   A   V   L   P   T

        490                 510                 530                 550                 570                 590
CGGCGCAGGC CCTGGCCCGC TGGATCGACG GCAACGGCTA CCGGTCGGCC GGATACCCGC GGGACATCAA CCTGGAGTGC CCCGAGAACC GCGACGACTG GGTCACCGAA CTCCAGGCAC
  A   Q   A   L   A   R   W   I   D   G   N   G   Y   R   S   A   G   Y   P   R   D   I   N   L   E   C   P   E   N   R   D   D   W   V   T   E   L   Q   A   P

        610                 630                 650                 670
CGGTGGCCAA GCCCTGAGCC GCGGCGGTCC TGGCCGGTTC GGTGACCTCC CCGAGCCGGT CAGGTGCCGA GGGGCCC
  V   A   K   P   *
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FIG. 2. Nucleotide sequence of ORF1 and the deduced amino acid sequence. The putative Shine-Dalgarno sequence is indicated by a double underline, and the transcription terminator is indicated by a thin underline.

lulose such as HEC or carboxymethylcellulose (42). So far, only *S. reticuli* (34) and *S. flavogriseus* (21) were shown to additionally synthesize an Avicelase at a high and a low level, respectively. Neither *S. flavogriseus* nor any of the other strains harbored a gene with significant homologies to the *cel-1* gene of *S. reticuli* (Fig. 3A). In addition, an internal fragment of ORF1 (*Sph*I-*Sal*I fragment) was hybridized with the chromosomal DNA of the same strains. Since only *S. reticuli* contained this ORF1 (Fig. 3B), it was speculated that the gene product assists the Avicelase or regulates its expression.

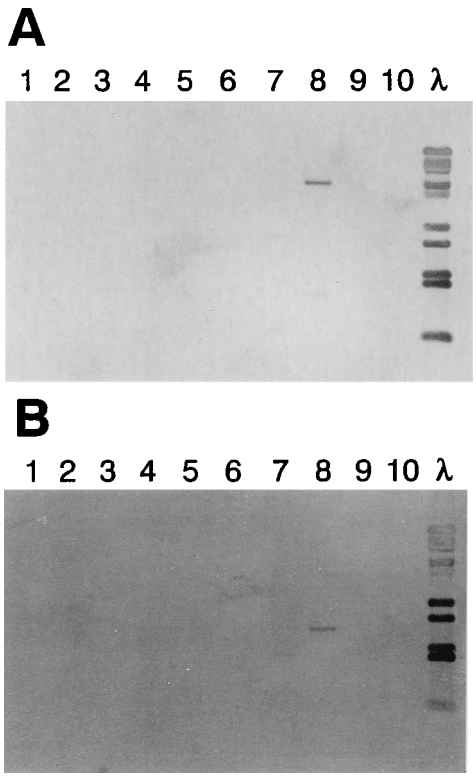

FIG. 3. Southern hybridization with *Sma*I fragments of total DNA, isolated from several *Streptomyces* strains with DNA probes corresponding to a fragment encoding a part of the catalytic domain of the Avicelase (A) and an internal fragment of ORF1 (B). Lanes: 1, *S. lividans* 66; 2, *S. coelicolor* A3(2); 3, *S. antibioticus*; 4, *S. parvulus*; 5, *S. vinaceus*; 6, *S. olivaceoviridis*; 7, *S. ambofaciens*; 8, *S. reticuli*; 9, *S. flavogriseus*; 10, *S. venezuelae*; λ, λ DNA, *Bst*EII digested.

Expression studies using different *Streptomyces* strains. As examples for strains lacking the *cel-1*-like genes, *S. lividans* 66, *S. coelicolor* A3(2), and *S. vinaceus* were chosen to study the production of the Avicelase. Transformants of these strains carrying the plasmids listed in Table 1 were grown as shaken cultures in minimal media with different C sources. Samples were taken daily, and the Avicelase production was analyzed by determination of the pNPCase activity or with the help of HEC-containing PAA gels (Fig. 4A). For this purpose, filtrates were prepared from Avicel-grown cultures of *S. reticuli*, as well as of transformants of *S. lividans* and *S. vinaceus* containing pZG6 (as control) and the *cel-1*-containing plasmids pZV1 and pZV2.

In addition to the inherent HECase activities of the individual control strains, several cellulolytic proteins were found to be produced in strains in which the *cel-1* gene had been intro-

TABLE 1. Growth, pNPCase activity, and repression

Strain	Plasmid	Growth with Avicel[a]	pNPCase activity[b] (mU/ml) with:		Repression by glucose[c]
			Avicel	Glucose	
S. lividans	pZG6	−	0	0	ND
	pZV1	+	0.45	0	+
	pZV2	+	0.43	0	+
	pZVO2	+	0.49	0	+
	pZVO1	+	0.45	0	+
S. vinaceus	pZG6	−	0	0	ND
	pZV1	+	0.44	0	+
	pZV2	+	0.43	0	+
S. coelicolor	pZG6	−	0	0	ND
	pZV1	+	0.68	0	+
	pZV2	+	0.71	0	+
S. reticuli		++	1.2	0	+

[a] Growth of the strains was determined by microscopical control (−, none; +, good; ++, very good).

[b] pNPCase activity in the culture filtrates was measured after 5 days of cultivation.

[c] The repression of the Avicelase synthesis by glucose was measured with pNPC as substrate. After 2 days of cultivation in minimal medium with Avicel, glucose was added. After another day, the enzyme activity was determined and compared with that of culture containing only Avicel as C source. +, decrease of pNPCase activity by more than 90%; ND, not determined.

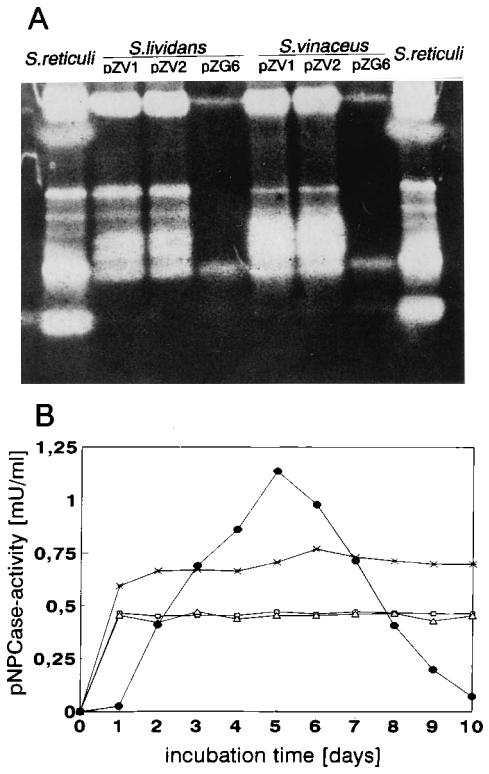

FIG. 4. Analysis of cellulolytic activities in *Streptomyces* strains. All strains were grown in minimal medium with 1% Avicel as sole carbon source. (A) Samples of the culture filtrates were loaded onto an HEC-containing PAA gel. After electrophoresis and protein renaturation, the gel was stained with Congo red. (B) The pNPCase activities in the culture filtrates from *S. coelicolor* (pZV1) (⊠), *S. lividans* (pZV1) (□), *S. vinaceus* (pZV1) (△), and *S. reticuli* (●) were measured once per day.

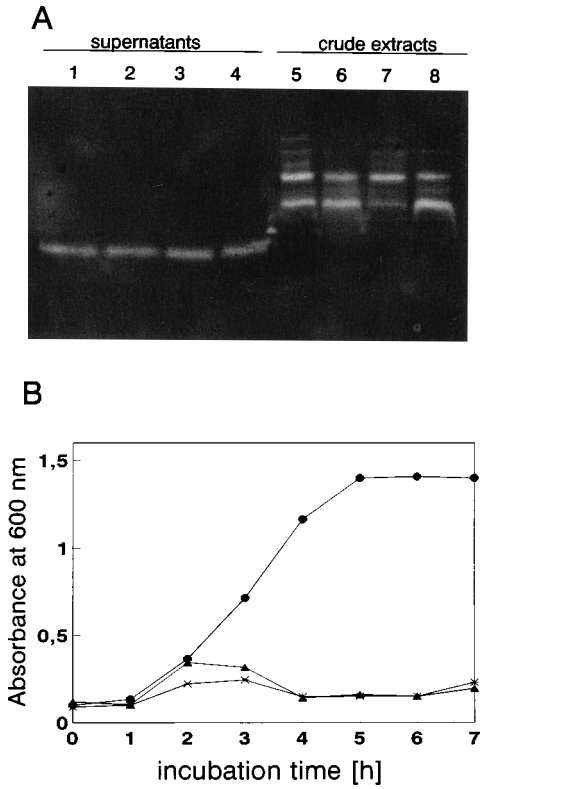

FIG. 5. Test for cellulolytic activities in *E. coli* JM109. (A) *E. coli* JM109 containing different plasmids was grown in the presence of IPTG. When A_{600} reached 1.0, the supernatants and the cells were separated by centrifugation. A total of 40 μl of each supernatant and 30 μg of proteins of the whole-cell extract were loaded on an HEC-containing PAA gel. After electrophoresis, cellulolytic activities were detected by staining the gel with Congo red. Lanes 1 and 5, *E. coli* JM109; lanes 2 and 6, *E. coli* JM109 (pUC18); lanes 3 and 7, *E. coli* JM109 (pUFus1); lanes 4 and 8, *E. coli* JM109 (pUFus2). (B) The growth of *E. coli* JM109 containing pUC18 (●), pUFus1 (▲), or pUFus2 (⊠) was determined by measuring the A_{600} after the induction with IPTG (time zero).

duced (Fig. 4A). The series of protein bands with cellulolytic activity can be attributed to the following: (i) The Avicelase tends to aggregate (34); (ii) under nondenaturing electrophoresis conditions, the mobility of the enzyme is altered (35); and (iii) the Avicelase is processed proteolytically, as in the *S. reticuli* wild type.

The fact that the Avicelase is processed in its natural host and other *Streptomyces* strains indicates a specific feature of the Avicelase which allows a release of the catalytically active part from the crystalline substrate by proteases.

The pNPCase activities in the culture filtrates were measured during 10 days of cultivation of *S. lividans* (pZV1), *S. vinaceus* (pZV1), *S. coelicolor* (pZV1), and *S. reticuli* wild type (Fig. 4B). After 2 days of cultivation, the three *Streptomyces* species containing the *cel-1* gene on the multicopy vector had already reached their highest level of pNPCase activity, which was, however, two to three times lower than that of *S. reticuli* synthesized after 5 days (Fig. 4B).

As the repression of the *cel-1* gene via glucose was found to be similar in *S. reticuli* and in the *Streptomyces* strains containing the *cel-1* gene (Table 1), the low expression level is probably due to the lack of positive regulatory elements present in *S. reticuli*. ORF1, which could encode such a putative activating protein, was cloned in pZGV2, 2.7 kb downstream of the *cel-1* gene in both the same (pZVO2) and the opposite (pZVO1) direction (Fig. 1, left). These constructs were transformed into *S. lividans* 66, and the Avicelase production was analyzed as described above. In Table 1, all results are summarized. (i) Having obtained the *cel-1* gene, Avicelase-negative strains are able to hydrolyze crystalline cellulose. (ii) Within transformants carrying the *cel-1* gene on a multicopy vector, the level of enzyme synthesis is two to three times lower than that of *S. reticuli*. (iii) ORF1 had an effect neither on the induction nor on the repression of the *cel-1* gene. (iv) The transcriptional orientation of the *cel-1* gene had no influence on the expression rate, suggesting that the promoter of the *cel-1* gene was used in all strains.

Expression studies using *E. coli* JM109. For the expression studies in *E. coli*, the transcriptional and translational start sites of the *cel-1* gene from *S. reticuli* were replaced by those of *E. coli*. Thus, the *cel-1* gene was cloned with (pUFus2) and without (pUFus1) the signal sequence in phase with the *lacZ* gene (Fig. 1). The resulting transformants were grown for 4 h after the induction with IPTG (see Materials and Methods).

To detect any additional cellulolytic activities, the supernatant and the whole-cell extract were analyzed with the help of HEC-containing PAA gels (Fig. 5A). No additional cellulase activity could be determined by using *E. coli* with or without pUC18 as control and *E. coli* containing pUFus1 or pUFus2. However, we discovered that during the cultivation in LB medium *E. coli* JM109 produced so-far-undescribed enzymes degrading HEC. Two such activities were localized in the periplasm or cytoplasm, and one was found extracellularly.

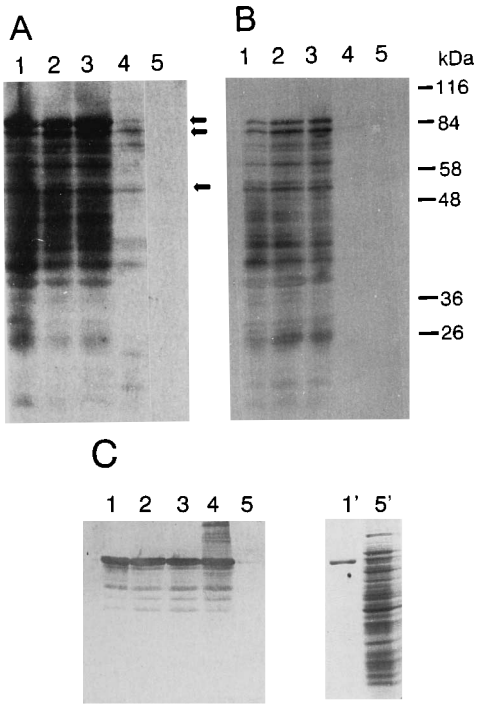

FIG. 6. Analysis with antibodies. A total of 30 μg of proteins of the whole-cell extract or 50 μl of the culture filtrates was loaded on an SDS-containing PAA gel, electrophoresed, blotted, and subsequently incubated with antibodies raised against the Avicelase. (A and B) *E. coli* JM109 containing pUFus1 (A) or pUFus2 (B) was grown in LB. When A_{600} reached 0.3, IPTG was added and whole-cell extract was prepared from samples taken after 0 h (lanes 5), 1 h (lanes 4), 2 h (lanes 3), 4 h (lanes 2), and 6 h (lanes 1). Proteins whose NH_2-terminal ends were sequenced are indicated by arrows. The size of the largest of these proteins corresponds to that of the fusion protein whose NH_2-terminal amino acids are as expected (Fig. 1). (C) The partially purified Avicelase from *S. reticuli* was incubated at 37°C for 1 h with whole-cell extract prepared from *E. coli* JM109 (pUC18). After electrophoresis, the Avicelase was immunodetected (left panel), and the proteins were stained with Coomassie blue (right panel). Lanes 1 and 1′, Avicelase; lane 2, Avicelase and cell extract; lane 3, Avicelase and heat-denatured cell extract; lane 4, heat-denatured Avicelase and cell extract; lanes 5 and 5′, cell extract.

Furthermore, the expression of the *cel-1* gene in *E. coli* caused a significant lysis of the cells (Fig. 5B). Therefore, a separation of the different cell compartments to study the secretion was impossible. In order to analyze whether *E. coli* (pUFus1) or *E. coli* (pUFus2) could synthesize the Avicelase intracellularly, whole-cell extracts were prepared from cells which had been grown with induction with IPTG for different times. Though no functional Avicelase could be detected, antibody studies revealed (Fig. 6A and B) that large quantities of truncated forms of the enzyme had been produced. NH_2-terminal protein sequencing (data not shown) indicated that these truncated forms (marked in Fig. 6A and B) were generated by a proteolytic cleavage of the *cel-1* gene product and not by a degradation of the corresponding RNA. When the Avicelase had been partially purified from *S. reticuli*, neither its native nor its heat-denatured form was processed by proteases present in the whole-cell extract of *E. coli* (pUC18) (Fig. 6C).

The plasmids pUV1/2 and pUATG1/2 were introduced into *E. coli* JM109 by transformation in order to test the functionality of the *S. reticuli* promoter upstream of the *cel-1* gene in *E. coli*. Cell extracts prepared from induced and uninduced cultures were analyzed for the production of the *cel-1* gene product with antibodies raised against the Avicelase. Only *E. coli* (pUATG2) synthesized a small amount of the gene product (data not shown). These results allowed the following conclusions. (i) The sequence upstream of *cel-1* does not contain a promoter which functions within *E. coli*, and (ii) the translation start site of *cel-1* is only efficiently used if the GUG start codon is changed to an AUG.

Expression studies using *B. subtilis*. Both plasmids containing the *cel-1* gene under its own promoter (pRATG1) and under the control of a strong *B. subtilis veg*II promoter (pRATG2) were transformed into *B. subtilis*. The resulting clones were grown under induced conditions, and samples were taken after different times (ranging between 2 and 24 h). Neither in the culture filtrate nor in the whole-cell extract could Avicelase activities or nonenzymatic truncated forms of the cellulase (tested with the help of antibodies) be discovered.

DISCUSSION

During the growth on microcrystalline cellulose (Avicel) as carbon source, *S. reticuli* wild type secretes an 82-kDa Avicelase (Cel-1) in large quantities, which hydrolyzes this macromolecule to cellobiose (34). Three Avicelase-negative strains [i.e., *S. coelicolor* A3(2), *S. lividans* 66, and *S. vinaceus*] acquire the ability to utilize Avicel after transformation with a multicopy plasmid containing the *cel-1* gene. However, the induction of the expression was found to be about two to three times lower than in the *S. reticuli* wild type. This difference may suggest either the requirement of specific factors for the induction or the inability of the regulatory proteins to recognize the target sequence upstream of the *cel-1* gene.

The expression level could not be increased by a cotransformation of *cel-1* with ORF1, which (i) is naturally located directly upstream of the *S. reticuli cel-1* gene, (ii) codes for an intracellular protein of 20.5 kDa, sharing no homology with other known proteins, and (iii) has up to now been found only in conjunction with the *cel-1* gene in *S. reticuli* and not in any other tested *Streptomyces* strains. On the basis of these data, we conclude that ORF1 encodes a protein which is not involved in the regulation of the Avicelase synthesis or that the specific activation mechanisms (i.e., phosphorylation, production of inducers) are missing in the *Streptomyces* hosts. However, the repression of the Avicelase synthesis within all studied *Streptomyces* strains corresponds to that of the *S. reticuli* wild type.

In contrast to our data, several genes from different streptomycetes and actinomycetes had been especially overexpressed in *S. lividans* 66: α-amylase from *Streptomyces griseus* (40), chitinase from *S. olivaceoviridis* (3), protein protease inhibitor from *Streptomyces lividans* and *Streptomyces longisporus* (37), and β-lactamase from *Streptomyces albus* (8) (for a review, see reference 1). Cellulases were also successfully overproduced in streptomycetes, i.e., cellobiohydrolase of *Microbispora bispora* (16); E1, E4, and E5 from *Thermomonospora fusca* (11, 17); and an exoglucanase of *C. fimi* (22). Therefore, streptomycetes are generally useful for overproducing heterologous polypeptides if no specific regulatory system is required for their synthesis. Thus, an esterase-encoding gene from *Streptomyces scabies* (13) which requires zinc ions is induced at a level four to five times lower within *S. lividans*. Our recent data (43) have revealed that specific regulators are necessary for the synthesis of the Avicelase within *S. reticuli*, since the *cel-1* gene is induced only by crystalline cellulose via an unknown signal transduction cascade.

The expression studies using *E. coli* JM109 as host revealed (i) the absence of a promoter which can be recognized by the *E. coli* RNA polymerases, (ii) lysis of the cells if the cellulase (Avicelase) with or without the signal peptide is overexpressed as fusion protein, (iii) the absence of any enzymatically active

protein, and (iv) an extensive proteolytic degradation of the gene product. Cell lysis appears to be likely due to a toxic effect of the heterologous protein. Thus, only its massive proteolysis could ensure survival of *E. coli.*

If isolated from *S. reticuli*, the Avicelase is protected against *E. coli* proteases, and therefore, a posttranslational modification of the enzyme by *S. reticuli* is proposed. The nature of this modification is up to now unknown, but recently, it has been shown that *S. lividans* is able to glycosylate an exoglucanase from *C. fimi* (22). However, nuclear magnetic resonance studies, performed with the Avicelase from *S. reticuli*, showed no protein-associated sugars (24).

It is possible that other heterologous proteins are also not modified in an appropriate manner in *E. coli*. In this case, the real characteristics of such proteins in this new host cannot be studied.

Surprisingly, it could be shown that *E. coli* JM109 produces activities degrading HEC; two are located in the cytoplasm or periplasm, and one was found extracellularly. The occurrence and the location of these enzymes are very unusual for *E. coli* (for a review, see reference 31) and have not yet been reported. Therefore, further investigations should be done to study the enzymes biochemically in more detail.

In addition to the above-cited problems with *E. coli* as host, the newly identified inherent cellulolytic activities exclude this strain as host for the expression of cellulase genes from any organism.

Earlier, only one unsuccessful attempt to express a *T. fusca* (11) cellulase gene in *B. subtilis* was reported. In contrast to this experiment, we fused the strong *Bacillus* promoter *veg*II upstream of the *cel-1* gene to guarantee the transcription. Despite these precautions, neither active, inactive, nor proteolytically cleaved Avicelase could be detected.

As neither *E. coli* nor *B. subtilis* proved to be a suitable host, further studies concerning the *cel-1* gene, its regulation, and its gene product will be performed with *S. reticuli* and other *Streptomyces* strains.

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