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Expression of a *Thermomonospora fusca* Cellulase Gene in *Streptomyces lividans* and *Bacillus subtilis*

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A cellulase gene from *Thermomonospora fusca* coding for endocellulase E_5 was introduced into *Streptomyces lividans* by using shuttle plasmids that can replicate in either *S. lividans* or *Escherichia coli*. Plasmid DNA isolated from *E. coli* was used to transform *S. lividans*, selecting for thiostrepton resistance. The transformants expressed and excreted the endocellulase, but the ability to produce the endocellulase was unstable. This instability was shown to result from deletion of the endocellulase gene from the plasmid. Plasmid DNA prepared from a culture in which plasmid modification had occurred was used to transform *E. coli*, selecting for Amp^+ cells, and all of the transformants were cellulase positive, showing that pBR322 and *T. fusca* DNA were deleted together. When a plasmid was constructed containing only *T. fusca* DNA in plasmid pIJ702, the transformants were more stable, and the level of endocellulase activity produced in the culture supernatant after growth on 0.2% glucose was close to the level produced by *T. fusca* cultures grown on 0.2% cellulose. About 50% of the total protein in the culture supernatant of the *S. lividans* transformant was endocellulase E_5. The enzyme produced by the *S. lividans* transformant was identical to pure *T. fusca* E_5 in its electrophoretic mobility and was completely inhibited by antiserum to E_5. Shuttle plasmids containing the E_5 gene that could replicate in *Bacillus subtilis* and *E. coli* were also constructed and used to transform *B. subtilis*. Again there was extensive deletion of the plasmid DNA during transformation and growth in *B. subtilis*. There was no evidence of E_5 activity, even in those *B. subtilis* transformants that retained the E_5 gene.

Thermomonospora fusca YX is a gram-variable, thermophilic, filamentous soil bacterium that excretes a number of cellulose-degrading enzymes. Biochemical studies on these proteins and cloning experiments indicate that at least five, and probably more, distinct gene products of *T. fusca* are involved in cellulose hydrolysis (4, 6, 9; D. Wilson, Methods Enzymol., in press). Attempts at cloning *T. fusca* DNA in *Escherichia coli* by using plasmids have resulted in the isolation of only two cellulase genes. One of these clones carries the gene for protein E_5 (6), while the cellulase produced by the other clone is not inhibited by antibodies prepared against any of the five purified *T. fusca* cellulases. The inability to isolate clones in *E. coli* which code for the other enzymes may result from the lack of expression of certain *T. fusca* sequences in *E. coli* or the degradation of *T. fusca* DNAs, mRNAs, or proteins, or some of these gene products may be toxic to *E. coli*.

To test other organisms as hosts for cloning the remaining *T. fusca* cellulase genes, the gene coding for enzyme E_5 was introduced into *Streptomyces lividans* and *Bacillus subtilis*, and its expression was measured. *S. lividans* transformants expressed the gene and excreted the enzyme, while no activity was produced in *B. subtilis*. The endoglucanase expressed in *S. lividans* was characterized by its activity, its mobility during gel electrophoresis, and the inhibition of its activity by antibody directed against purified E_5.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Streptomyces* strain, *S. lividans* TK24(Str-6) (5), and plasmid pIJ702 (13) were kindly provided by D. A. Hopwood, John Innes Institute, Norwich, England. Plasmid pIJ702 contains a thiostrepton resistance gene and a tyrosinase gene from *Streptomyces antibioticus*. Recombinant plasmid pIJ702-pBR322 was provided by Stanley Cohen and Charles Omer, Stanford University, Stanford, Calif., and its orientation was determined as shown (Fig. 1).

E. coli HB101(F'*hsdS20* [r_B^- m_B^-] *recA13 ara-14 proA2 lacY galK rpsL strA xyl mtl supE* λ^-) was used as a recipient host for constructing vectors.

B. subtilis CU1065(*trpC2*) and CU2590(*ilvA2 recE4 attSP*β) were kindly provided by S. A. Zahler, Cornell University, Ithaca, N.Y., as were *B. subtilis* plasmids pCJM1 (16) and pSV101 (constructed by Kevin Mangan).

Media and culture conditions. *S. lividans* was grown on R2YE (regeneration yeast extract medium) agar plates at 30°C until sporulation (24). Spore suspensions were prepared from plates with sterile water, filtered, centrifuged, and stored at −20°C in 20% (vol/vol) glycerol. For protoplast preparations, the spores were grown in yeast extract-malt extract containing 0.5% glycine and 5 mM $MgCl_2$ (11). For plasmid preparations, the strains were generally grown in tryptone soya broth. *S. lividans* was also grown in synthetic media for enzyme production (9). Unless otherwise specified, the amount of thiostrepton was 5 μg/ml in liquid media and 50 μg/ml on plates. Thiostrepton was a generous gift from the Squibb Institute, Princeton, N.J.

E. coli was grown on Luria broth (LB) and plated on LB agar plates. The concentration of ampicillin, when used, was 50 μg/ml. Tryptone blood agar base (Difco Laboratories) was used as a plating medium for *B. subtilis*. Penassay broth (Difco) and LB were used as liquid media. GMI and GMII media, used to obtain competent cells for transformation experiments, were prepared essentially as described by Yasbin et al. (26). Liquid cultures were aerated by shaking.

Transformation procedures and selection of transformants. Preparation of *S. lividans* spores and protoplasts and their

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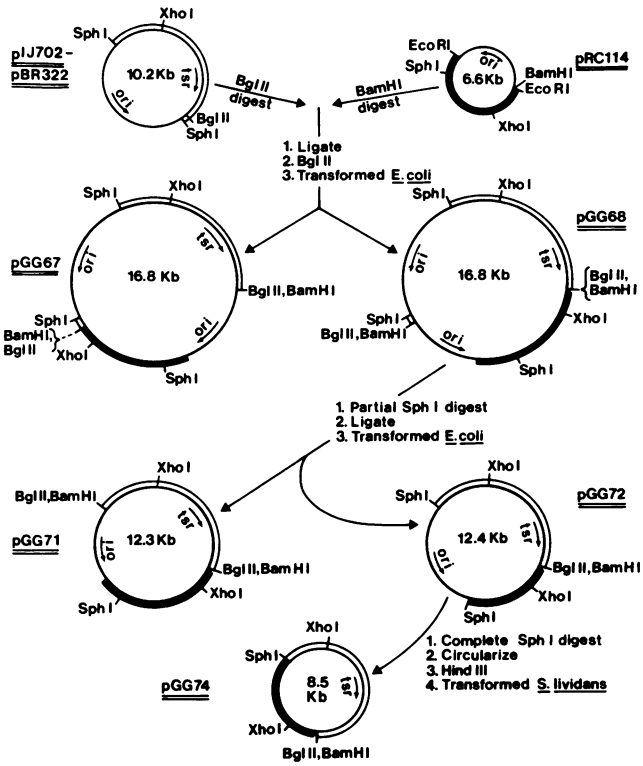

FIG. 1. Cloning strategies for the construction of *S. lividans* plasmids containing a *T. fusca* cellulase gene with and without ColE1-type origin(s) of replication derived from plasmids pBR322 or pUR222 or both. Each ColE1 origin of replication and its direction on the circular maps is represented by an arrow pointing in the direction of replication. ——, pBR322 sequences and pUR222 sequences; ▭, pIJ702 sequences; ▬, *Thermomonospora* sequences carrying the cellulase gene E_5 (6). The pIJ702 origin of replication spans the *Xho*I site and is not shown. Tsr^r, thiostrepton resistance gene which is expressed in *S. lividans*.

polyethylene glycol-assisted transformation were carried out essentially as described by Thompson et al. (25). An overlay containing R2YE medium, 0.4% low-melting-point agarose, and enough thiostrepton to give a final concentration of 50 μg/ml was used. The melanin (Mel$^+$) phenotype of plasmid pIJ702 was scored by adding tyrosine to the overlay agar (300 to 400 μg/ml). Transformation of *E. coli* was carried out by a modification of the Morrison procedure (19). *B. subtilis* cells were made competent by the procedure of Yasbin et al. (26). Competent cells were incubated with DNA for 1 h, diluted, and spread onto selective plates.

DNA preparations. Total DNA was isolated from mycelia by the procedure described elsewhere (5). Large-scale plasmid isolations from *S. lividans* were carried out by the alkaline procedure of Kieser (11, 14). Plasmid isolations from *E. coli* and *B. subtilis* were carried out by either the alkaline procedure or the boiling procedure (10).

Restriction endonuclease digestions and agarose gel electrophoresis. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., New England Biolabs, or International Biotechnologies, Inc. Digestions were carried out under conditions recommended by the suppliers. The sizes of DNA fragments were measured by agarose gel electrophoresis (17), with bacteriophage lambda restriction fragments as standards.

Screening of transformants. *E. coli*, *B. subtilis*, and *S. lividans* transformants were screened for cellulase activity by the carboxymethyl cellulose overlay procedure of Teather and Wood (23).

Enzyme assays. Mycelia and supernatants were separated by centrifugation, and French press extracts were prepared from the washed mycelia in 50 mM KP_i buffer (pH 6.5). The endoglucanase activity was determined by measuring the amount of reducing sugar released from carboxymethyl cellulose by the Miller procedure, essentially as described elsewhere (6, 18), except that the cellulase assays were incubated at 55°C. One international unit of activity is equivalent to 1 μmol of reducing sugar produced per min.

Protein. Protein was estimated by the Coomassie blue dye-binding procedure of Bradford (3), with bovine serum albumin as a standard. The Bradford reagent was prepared as described elsewhere (3) or was purchased from Bio-Rad Laboratories and used according to the instructions of the company. In some cases, proteins were determined by the procedure of Lowry et al. (15).

Gel electrophoresis. Native protein gels were prepared by a modification of the Davis method (7; Wilson, in press). One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the Laemmli procedure as described for the O'Farrell second dimension (20).

Antibody inhibition of cellulase activity. Antisera to five purified *T. fusca* cellulases have been produced in rabbits. Preimmune serum collected before antigen administration was used as control serum. Inhibition of cellulase activity with rabbit antiserum was carried out by mixing antiserum with samples containing 0.002 to 0.008 U of endoglucanase and 50 μl of potassium phosphate (pH 6.5) containing 100 μg of bovine serum albumin per ml. The samples were incubated at room temperature for 1 to 2 h and then at 4°C for 4 to 8 h with occasional mixing. The samples were then assayed directly for endoglucanase activity. The endoglucanase assays were incubated at 55°C for 30 min. Appropriate controls lacking carboxymethyl cellulose, lacking enzyme, or containing preimmune serum were run similarly.

RESULTS

Plasmid constructions. The *Thermomonospora* cellulase E_5 is a 45,000-dalton protein which makes up about 5 to 10% of the total carboxymethyl cellulase activity in *Thermomonospora* supernatants. The gene encoding the E_5 cellulase has been cloned as an *Eco*RI fragment in plasmid pD365 (6). The same *Eco*RI fragment is also present in pRC114, which is a derivative of pUR222 (22; R. Calza, unpublished data).

The E_5 cellulase gene was first transferred to the *E. coli-S. lividans* shuttle vector pIJ702-pBR322, which is an *Sph*I hybrid of pIJ702 (13) and pBR322 (1). The orientation of the pIJ702-pBR322 hybrid plasmid is shown in Fig. 1. There is a single *Bgl*II site in the hybrid plasmid and a single *Bam*HI site in plasmid pRC114. These two plasmids were joined as shown in Fig. 1. Upon transformation of *E. coli*, two plasmids, pGG67 and pGG68, were identified in two different cellulase-positive *E. coli* colonies. These plasmids contain an intact cellulase gene with its original promoter. Plasmids pGG67 and pGG68 each contain the entire pRC114 sequence but in opposite orientations. They also both contain two ColE1 origins of replication. Plasmids pGG71 and pGG72 were constructed from plasmid pGG68 by partial digestion with *Sph*I (Fig. 1). Each plasmid contains only one ColE1 origin of replication, while plasmid pGG74, constructed from

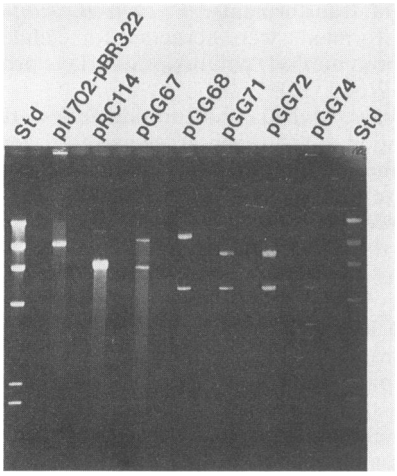

FIG. 2. Electrophoresis of restriction enzyme digests of the plasmids shown in Fig. 1. Std, λ*Hin*dIII digest. Plasmids other than pGG74 were isolated from *E. coli* HB101, and plasmid pGG74 was isolated from *S. lividans* TK24. The purified DNAs were digested with *Xho*I and electrophoresed on an 0.8% agarose gel prepared in TBE running buffer (17). Gels were stained with ethidium bromide and viewed with a UV transilluminator.

plasmid pGG72 by complete digestion with *Sph*I, contains no ColE1 plasmid DNA. The structures of the plasmids were confirmed by restriction analyses (Fig. 2; data not shown).

Transformation experiments. Several different transformations of *S. lividans* were carried out with plasmids pGG68, pGG71, and pGG72 purified from *E. coli* and plasmid pGG74 constructed in vitro (Fig. 1). With the exception of pGG68, which gave only a few Tsr^r colonies, over 500 colonies per μg could be obtained from each of the other plasmids. The colonies obtained from plasmid pGG68 could not be propagated in liquid medium and were lost. Colonies obtained with pGG71 and pGG72 were all cellulase positive (Fig. 3). The cellulase activity of pGG71 transformants on agar plates was consistently higher than that of pGG72 transformants. Interestingly, the same was true of *E. coli* pGG71 and pGG72 transformants (data not shown).

S. lividans transformants containing any of the hybrid plasmids but not pIJ702 gave inefficient and delayed sporulation. There was abundant growth of substrate mycelia on plates and of mycelia in liquid cultures for all transformants

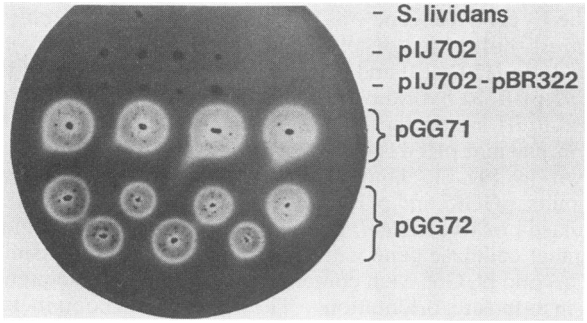

FIG. 3. Detection of cellulase-positive transformants in *S. lividans* by carboxymethyl cellulose overlay method (23). The cells were incubated with the overlay agar for 4 h at 55°C and then stained with Congo red.

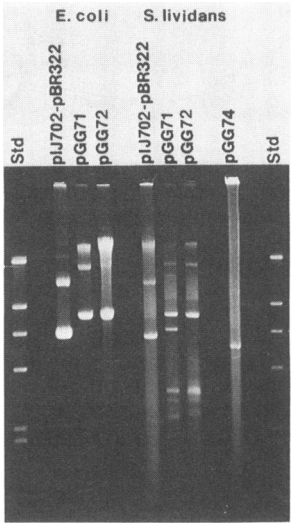

FIG. 4. Plasmid DNAs isolated from primary cultures of *E. coli* and *S. lividans* transformants analyzed on a 0.5% agarose gel. Std, λ*Hin*dIII digest.

except those containing plasmid pGG68. No evidence of mycelium lysis was observed with either untransformed or transformed strains in liquid cultures.

Plasmid DNA stability and maintenance. Plasmid DNA was isolated from various cellulase-positive transformants. The yield of plasmid was 20 to 50 μg/liter of culture. The plasmid DNAs isolated from pGG71 and pGG72 primary clones showed some size variation (Fig. 4). The plasmid DNAs were digested with different restriction enzymes to release fragments containing the cloned inserts. The restriction digests were then fractionated by agarose gel electrophoresis, and the fragment sizes were determined by comparison to marker DNAs. Some of the plasmid fragments were not the correct size and were diffuse, indicating that the plasmid DNA in these clones was unstable.

Unlike the pGG71 and pGG72 primary transformants, the pGG74 transformants did not show changes in plasmid size and produced much higher levels of cellulase activity. Cultures grown with thiostrepton produced about half the cellulase and total protein of cultures grown without the drug.

The cellulase-positive phenotype is particularly unstable during sporulation on solid media. After one round of sporulation on solid media, there was no loss of Tsr^r, but with pGG71 and pGG72 transformants, only about 70% of the germinated spores gave cellulase-positive colonies because of the plasmid instability noted above. Germinated spores from strains containing plasmid pGG74 also gave some cellulase-negative colonies, but they were not studied further.

Enzyme activity of the cellulase-positive clones. The enzyme activity of different clones was determined in cultures prepared in tryptone soya broth and minimal medium. In each experiment, the activity of cellulase-positive transformants was much higher than that of the controls (Table 1). Over 90% of the enzyme activity was excreted into the supernatant. The total amount of activity in primary transformants was as high as 10 U/ml in tryptone soya broth. This amount is similar to the total endoglucanase activity and 5 to 10 times the E_5 activity of *T. fusca* cultures grown on 0.2% cellulose. Subcultures of pGG71 and pGG72 were often low in activity.

TABLE 1. Cellulase activities of *S. lividans* TK24 and *S. lividans* TK24 transformants[a]

Plasmid	Endoglucanase (IU/ml)
None	<0.02
pIJ702	<0.02
pIJ702-pBR322	<0.02
pGG71	3 ± 1
pGG72	4 ± 1
pGG74	8 ± 2

[a] Transformed and control organisms were cultured for 30 to 40 h in tryptone soya broth. The cellulase activity in a synthetic medium (9) containing 0.2% glucose was 10 ± 2 U/ml. The medium for plasmid-containing strains also contained 10 μg of thiostrepton per ml.

This low activity appeared to result from the instability of the cellulase gene in these strains.

The following results show that the cellulase produced by *S. lividans* transformants is the same as the E_5 cellulase purified from *T. fusca*. The cellulase activity produced in *S. lividans* transformants was completely inhibited by antibodies raised against enzyme E_5 purified from *T. fusca* (Fig. 5). In polyacrylamide gels, the major cellulase band was indistinguishable from that of pure E_5 (Fig. 6), while control transformants lacked the cellulase activity band. The results of the determination of the subunit molecular weight and the heat stability of the two enzymes also confirm the identity of the cellulases (Fig. 7 and 8).

Despite the fact that *S. lividans* can grow on cellobiose, the introduction of the *T. fusca* E_5 gene into *S. lividans* did not allow growth on swollen cellulose, Solka-Floc, or Sigmacell in synthetic media. This result indicates that activities other than E_5 activity are necessary to process cellulose to a state that can be utilized by *S. lividans*.

Construction of plasmids and transformation of *B. subtilis*. The bifunctional plasmid vectors pCJM1 and pSV101 were used to clone the E_5 gene into *B. subtilis*. The *E. coli* replicon in plasmid pCJM1 is from plasmid pBR322 (1), and the replicon in pSV101 is from pC194; both pCJM1 and pSV101 contain a *Staphylococcus aureus* replicon that can replicate in *B. subtilis* (8).

Plasmid pGG61 was constructed by inserting the *Eco*RI fragment carrying the E_5 gene from pRC114 into the *Eco*RI site of pCJMI. Plasmid pGG66 was constructed by inserting a gel-purified *Sal*I fragment of pRC114 into pSV101. This *Sal*I fragment also contains the entire sequence of the *T. fusca* endoglucanase gene. The plasmids were isolated by their ability to confer a cellulase-positive phenotype on *E. coli* HB101. The restriction enzyme analyses of these plasmids were consistent with the structures shown (Fig. 9). Both plasmids contain a β-lactamase gene which confers ampicillin resistance in *E. coli*, and plasmid pGG61 carries a kanamycin resistance (Km^r) gene, while pGG66 carries a chloramphenicol resistance (Cm^r) gene, both of which are expressed in *B. subtilis*.

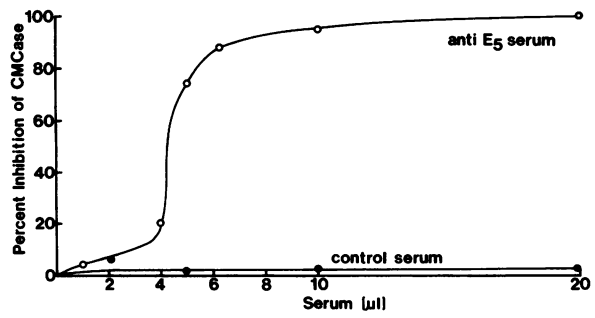

FIG. 5. Antibody inhibition of cellulase activity in culture supernatants of *S. lividans* transformants. CMCase, Carboxymethyl cellulose.

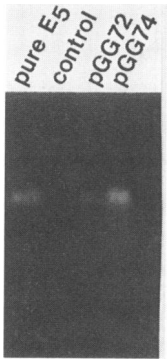

FIG. 6. Migration of enzyme E_5 and the cellulase from an *S. lividans* transformant on an 8.5% native polyacrylamide gel.

Plasmid pGG61 DNA prepared from *E. coli* was introduced into *B. subtilis* CU1065(*trpC2 attSP*β) by transformation by the natural competency of *B. subtilis*, and 10 independent Kan^r colonies were analyzed. Plasmid preparations from these transformants displayed size heterogeneity, as each preparation was different from the rest. These preparations were used to transform *E. coli* cells to Amp^+, and only a few transformants were cellulase positive. Plasmid DNA from one such cellulase-positive colony failed to give Km^r transformants with *B. subtilis* CU1065. These results indicate that the cloned *T. fusca* cellulase DNA sequence

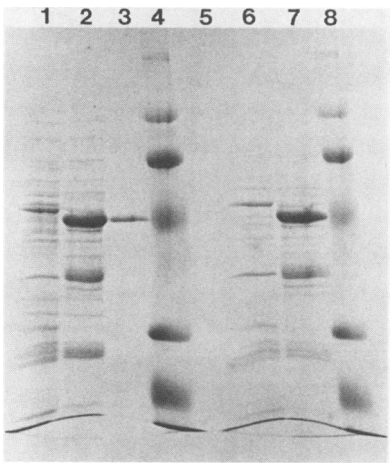

FIG. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the protein excreted by *S. lividans* TK24 transformed by pGG74. The control sample was TK24 transformed by pIJ702-pBR322. Heat treatment involved heating the sample at 65°C for 15 min and centrifugation to remove the denatured proteins. The supernatants were concentrated, and about 20 μg of protein was run in each lane on a 12.5% sodium dodecyl sulfate gel. Lanes: 1, unheated control; 2, unheated pGG74 transformant; 3, purified E_5 from *T. fusca*; 4 and 8, molecular weight standards; 6, heated control; 7, heated pGG74 transformant. The proteins were stained by Coomassie brilliant blue dye.

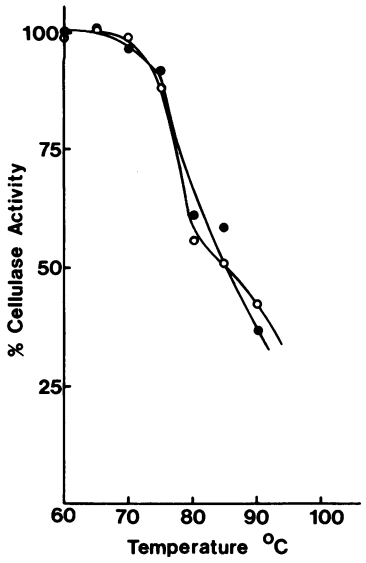

FIG. 8. Heat inactivation at different temperatures of E_5 (●) and the enzyme produced in *S. lividans* (○). The samples were heated for 15 min in a buffer containing 50 mM KP_i and 200 μg of bovine serum albumin per ml and assayed for carboxymethyl cellulase activity as described in the text.

undergoes considerable alteration in the *B. subtilis* background.

We also introduced plasmid pGG66 (Fig. 9) into recombination-deficient strain CU2590(*ilvA2 recE4 attSP*β). Only one of nine randomly analyzed *B. subtilis* transformants contained plasmid DNA that was identical to parental pGG66 DNA. However, this strain produced only the *B. subtilis* cellulase and did not produce any E_5 cellulase activity. These results suggest that the *T. fusca* cellulase gene is not expressed at a significant level in *B. subtilis*.

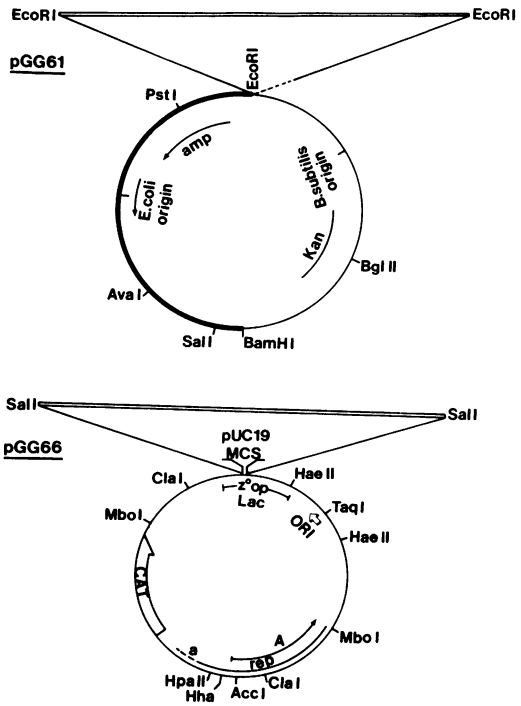

FIG. 9. Structures of *E. coli-B. subtilis* shuttle vectors containing a *T. fusca* E_5 gene.

DISCUSSION

The data presented here show that a *T. fusca* E_5 cellulase gene that is expressed at a low level in *E. coli* is expressed at a high level in *S. lividans*, and the cellulase is excreted. This study is the first report on the expression of a *T. fusca* gene in *S. lividans*.

The reason for the deletion of plasmid DNA in primary transformants of pGG71 and pGG72 is not clear, but the deletion seems to be brought about by the combination of ColE1 replication sequences and *T. fusca* sequences, since plasmids with either sequence alone (i.e., pGG74 and pIJ702-pBR322) are more stable. Evidence has been accumulating that structural instability of *Streptomyces* plasmids and chromosomal genes is widespread (12). These instabilities have been shown to result from rearrangements of DNA sequences, amplifications, or preferential loss of certain segments of DNA.

The ability to introduce and express a *T. fusca* endoglucanase gene efficiently in *S. lividans* points to the possible usefulness of this organism for the identification of other *Thermomonospora* cellulase genes which may be less stable in, or lethal to, *E. coli*. In addition, the high level of expression of the *T. fusca* gene in *S. lividans* makes it easy to purify the cellulases from the *S. lividans* transformants. The E_5 cellulase makes up about 50% of the total protein excreted by *S. lividans* strains containing plasmid pGG74 compared with about 5% of the total protein excreted by *T. fusca*.

We have confirmed the results of a recent investigation suggesting that plasmid pIJ702 transforms and replicates in *T. fusca* (21). The availability of pIJ702 derivatives carrying the *T. fusca* E_5 cellulase gene makes it possible to study the effect of increasing the level of the E_5 enzyme in *T. fusca* on the growth rate and level of other cellulases.

Compared with the *Streptomyces* system, the *B. subtilis* system appears much less suitable for cloning *T. fusca* cellulase genes because of extensive deletions of the plasmid DNA and the lack of expression of the cellulase gene.

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