INTRODUCTION

Cellulose, a linear polysaccharide consisting of a large number of β-1,4-linked D-glucose units, is one of the most abundant organic compounds present as a main component of the plants on the earth. Cellulosic biomass, such as rice husks and straw, wood waste, and forest scraps, contains a large quantity of cellulose but remains unused as a biomass resource all over the world.

Streptomycetes are filamentous bacteria inhabiting soils, ponds, and plant bodies and there exist streptomycetes which degrade cellulose into glucose by the function of three types of cellulases; endoglucanase (EG), exoglucanase (ExG), and β-glucosidase (BGL) [2–5]. These streptomycetes initially secrete EG and ExG outside the cells: EG randomly cleaves the amorphous region inside the cellulose chain and ExG excises to release cellobiose from the end of the sugar chain. Cellobiose is taken into the cell via an ABC transporter to be degraded into glucose by BGL in the cell [6].

Streptomyces thermocarboxydus strain C42 was isolated from a soil sample and selected on the basis of its high cellulase secretion. It can grow on cellulose compounds including microcrystalline cellulose, was isolated from soil for genetic breeding of streptomycetes that produce antibiotics from cellulosic biomass. Draft genome sequencing revealed putative genes encoding nine cellulases and one xyloglucanase dispersed on the chromosome. All these genes were isolated and rearranged on a chromosome-integration vector for streptomycetes pTYM19 to construct cellulase-expression plasmids pBOM51 and pBOM66 for streptomycete host strains. The cellulase gene cluster on pBOM66 was further introduced into pTYM18, another Streptomyces integration vector, to yield pBOM67. To investigate cellulase secretion and antibiotic production, the resulting plasmids were introduced into Streptomyces avermitilis K139, the producer of avermectin and oligomycin. Remarkable cellulose-degrading activity for filter paper was observed by pBOM66-carrying transformants. Oligomycin alone was produced by the transformed strain with pBOM67 only when cultivated in a medium containing glucose: not in a medium containing microcrystalline cellulose as a carbon source. Using antibiotic-producing streptomycetes for antibiotic production from cellulosic biomass therefore appears to be infeasible, even when transformed with cellulase genes of Streptomyces origin.

Keywords: cellulase, streptomycetes, cellulosic biomass
streptomycese cannot assimilate cellulose, cellulosic compounds have not been used as fermentation ingredients for the antibiotic production.

The goal of our study is to foster antibiotic-producing streptomyeze strains that can utilize cellulotic biomass as ingredients in the fermentation. Specifically, in order to turn antibiotic-producing S. avermitilis into a cellulose-assimilable strain, 10 cellulase genes found in the genome of strain C42 were ligated into a cluster to construct cellulose expression plasmids and S. avermitilis transformed with the plasmids were evaluated for expression of cellulase activity and OLM production.

2 MATERIALS AND METHODS

Bacterial strains and cultivation

Streptomyces thermocarboxybus strain C42 was cultivated as described in the previous report [7], and Streptomyces avermitilis K139 [10] was also cultured in the same manner as above. Escherichia coli JM109 and SCS110 were cultured as described in the previous paper [9]. For cultures of the transformants, appropriate antibiotics were added to the medium at the following concentrations: ampicillin sodium salt, 50 μg/mL; kanamycin sulfate 50 μg/mL; thiopentin, 20 μg/mL.

DNA manipulation

In this study, pTYM18 and pTYM19 [11] were used as plasmid vectors for gene introduction into streptomycese. Primers for PCR were designed using Genetyx-Mac version 16.07 (Genetyx Co.) and were synthesized commercially (Hokkaido System Science Co. Ltd.). DNA fragments for cloning were amplified by PCR using KOD-Plus- ver. 2 (TOYOBO) and the PCR primers. For DNA ligation, DNA Ligation Kit Mighty Mix (Takara Bio Inc.) and In-Fusion HD Cloning Kit (Takara Bio Inc.) were used. DNA manipulation using E. coli other than the above was performed in the same manner as the previous report [9].

Evaluation of cellulase activity

The degradation activity of filter paper cellulose (FPase) was measured as follows by modifying the method of Adney and Baker [12]. Strain of S. avermitilis was cultivated in 50 mL GPY and CBPY liquid medium containing 0.5% (w/v) glucose and cellulose at 30°C for 7 days. The supernatant was harvested from the culture broth by centrifugation. One piece of Whatman No. 1 qualitative filter paper (1 cm x 1 cm) was added to a test tube containing 400 μL of 50 mM sodium citrate buffer (pH 4.8) and 100 μL of the supernatant, and was incubated at 50°C for 18 hr. The reducing sugar released in the reaction mixture was determined by reacting with 3,5-dinitrosalicylic acid. The experiment was conducted in triplicate. One unit of FPase activity was defined as the amount of enzyme releasing 1 μmole of glucose equivalent per minute.

Antibiotic production by S. avermitilis strains

For seed culture, a strain of S. avermitilis was inoculated into 2 mL vegetative medium [13] in a test tube and cultured at 30°C for 3 days on a reciprocal shaker at 120 strokes/min. For antibiotic production, 2% of the seed culture was inoculated into 10 mL of synthetic medium [14] in a 100 mL Erlenmeyer flask and cultivated at 28°C for 7 days on a rotary shaker at 180 rpm. The culture was vigorously mixed with equal volume of methanol, which was shaken for 30 min. After centrifugation, the supernatant was analyzed by high-performance liquid chromatography (HPLC) with SenshuPak PEGASIL ODS (100 x 4.6 mm i.d.; Senshu Scientific Co. Ltd.) at 246 nm and 40°C with acetonitrile – methanol – water (54 : 18 : 28) as the mobile phase at a flow rate of 1.0 mL/min.

3 RESULTS AND DISCUSSION

Construction of cellulase expression plasmids, pBOM51 and pBOM66

A cellulase expression plasmid, pBOM51 was constructed by cloning 10 putative cellulose- and xylulose-degrading genes dispersed on the genome of strain C42 (Figure 1). Each of the genes was amplified separately with their intrinsic promoter regions by PCR, except the clustered group of genes, cel5A, cel48A, and xeg74A. The amplified DNA fragments were connected one after another as depicted in Figure 1 to form a 17.5-kb gene cassette for insertion into pTYM19 [11], a chromosome integration vector, resulting in pBOM51. The cel genes located downstream of CebR box on the plasmid were designed to be induced through derepression by cellobiose. Most of the genes in the cassette encoded cellulases with a signal peptide sequence [15], and were therefore predicted to express the individual enzyme activity secreted.

The facts that CebR box was absent upstream of cel5A gene and that the heterologous expression in Streptomyces lividans elicited high CMC-degrading activities [7] strongly suggested that the cel5A gene is expressed constitutively for the initial degradation of cellulose. Therefore, another gene cassette was constructed in which cel5A was under the control of P<sub>rpsJ</sub>, the constitutively-expressed strong promoter of rpsJ gene encoding ribosomal protein S10 from Streptomyces avermitilis [16], in the same manner as described above and inserted into pTYM19 to give pBOM66.

Expression of cellulases from the expression plasmids in Streptomyces avermitilis

Plasmids pBOM51 and pBOM66 were introduced into S. avermitilis K139, a producer of AVM and OLM, and culture supernatants of the transformants cultivated in GPY and CBPY media were examined for FPase activity (Figure 2). As a result, GPY cultures of strain K139 transformed with pBOM66 exhibited significantly higher activities than that of control strain carrying pBOM51 and pBOM51 showed 1.8 and 4.3 times higher FPase activities than...
that of the control pTYM19-transformant.

These results indicate that the constitutive cel5A expression and the presence of 0.5% cellobiose in the medium enhanced the cellulase activities elicited by the transformants with the cellulase gene cassette. It is most likely that the addition of cellobiose in the medium induced cellulose-responsive cel genes, i.e. cel6A, cel9A, cel12A, and cel48A and xeg74A in the cassette, all of which are located downstream of CebR box, to increase FPase activity. The pBOM66 transformant exhibited the highest activity, suggesting the synergistic effect on the total enzyme activity by cel5A and the other cel and xeg genes expressed. Strain C42, on the other hand, failed to induce the cellulase activity even in the presence of cellobiose.

The reducing sugar formation from a square filter paper (1.0 cm²) using the supernatants of CBPY cultures of the transformants was examined by incubation for 10 days at 30°C (Figure 3). Strain K139 with pBOM51 produced a significantly larger amount of reducing sugars than the control strain with pTYM19 and reached the same level of production as strain C42. Furthermore, strain K139 carrying pBOM66 exhibited the highest production, thus indicating that the cellulase gene clusters on these two plasmids, pBOM66 in particular, brought about the acquisition of cellulase highly secreting phenotype on S. avermitilis.

Antibiotic production by the transformants of S. avermitilis with cellulase expression plasmid

In order to avoid detrimental effects on gene expression as observed by the thiostrepton resistance gene on pTYM19 [17], another cellulase expression plasmid, pBOM67 (Figure 1), was constructed by insertion of the whole cellulase gene cassette of pBOM66 into pTYM18. The transformant of K139 with pBOM67 did not grow on the synthetic medium supplemented with Avicel as the sole source of carbon, and it, however, secreted FPase activity in the same level as that of the transformant with pBOM66 in an experiment similar to that shown in Figure 2 (data not shown).

A wild type strain of S. avermitilis K139 and the transformants with pTYM18 and pBOM67 were cultivated in the synthetic medium containing low concentrations of glucose, i.e. 0.5, 1.0, and 2.0% with and without 1.0% Avicel at 28°C for 7 days to examine OLM production and FPase activity in the supernatants (Figure 4). Under these cultivation conditions, HPLC analysis of the culture extracts revealed OLM on the basis of the UV spectra at retention time of 14.5 min, but not AVM (data not shown).

Under 2.0% glucose condition, addition of Avicel gave rise to no significant increase in FPase activities of the three strains (Figure 4B). Meanwhile, under a lower concentration of glucose (0.5%), the strain K139 with pBOM67 exhibited higher FPase activities than that with pTYM18 irrespective of Avicel addition (Figure 4F), which implied that the cellulase genes introduced were expressed in the transformant. In addition, the presence of Avicel appeared to induce the intrinsic cellulase activity in strain K139. In a concentration of 1.0% glucose, the addition of Avicel tended to increase FPase activities of all the strains (Figure 4D). These results strongly suggest that the introduction of pBOM67 together with addition of Avicel induced expression of cellulase activity in S. avermitilis at lower concentrations of glucose.

Glucose at 2% concentration in the medium were able to support the substantial production of OLM (Figure 4A), while the concentrations at 1% and 0.5% generally yielded lower levels of the production (Figure 4C and 4E); the amount of OLM produced appeared to be related to the amount of glucose added to the medium. Furthermore, the addition of Avicel to the cultures did not bring about the significant increase in OLM production except for those of strain K139 at 2% glucose. The increase would not be caused by the utilization of Avicel, because no significant increase in FPase activity was observed in the culture (Figure 4B). It may be due to the apparent fragmentation of mycelium in the culture, probably caused by cellulose addition. These results suggested that the glucose added exclusively would be the major carbon source for OLM production, to which Avicel would not contribute.

In addition to malonyl CoA and methylmalonyl CoA, a large amount NADPH is required for OLM biosynthesis, and the metabolism of glucose via pentose phosphate cycle is essential for the formation of NADPH. The transformants constructed in this study were able to express a certain level of cellulase but not to utilize cellulose directly. Actually, the transformant with pBOM67
antibiotic productivity was decreased in the culture of low glucose concentrations, where cellulase activity was fully detected. Therefore, at this stage, antibiotic production from cellulolytic biomass as fermentation ingredients appears to be infeasible by antibiotic-producing streptomycetes transformed with cellulase and xyloglucanase genes of *Streptomyces* origin.

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