Characterization of a cloned chromosomal fragment affecting differentiation in Streptomyces azureus ATCC14921

Nishiyama, Takashi
Laboratory of Microbial Genetics Technology, Department of Genetic Resources Technology, Division of Agriculture, Graduate School, Kyushu University

Kamachi, Yasuharu
Laboratory of Microbial Genetics Technology, Department of Genetic Resources Technology, Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University

Yokoyama, Eiji
Laboratory of Microbial Genetics, Division of Applied Genetic Resources, Department of Genetic Resources Technology, Faculty of Agriculture, Graduate School, Kyushu University

Doi, Katsumi
Laboratory of Microbial Genetics, Division of Applied Genetic Resources, Department of Genetic Resources Technology, Faculty of Agriculture, Graduate School, Kyushu University

http://hdl.handle.net/2324/24373
Characterization of a cloned chromosomal fragment affecting differentiation in *Streptomyces azureus* ATCC14921

Takashi Nishiyama*, Yasuharu Kamachi**, Eiji Yokoyama, Katsumi Doi and Seiya Ogata***

Laboratory of Microbial Genetics, Division of Applied Genetic Resources, Department of Genetic Resources Technology, Faculty of Agriculture, Graduate School, Kyushu University, Higashi-ku, Fukuoka 812-8581, Japan

(Received July 31, 2000 and accepted August 18, 2000)

We characterized a transformant BalA1 of thioestrepton–producing strain *Streptomyces azureus* ATCC14921, which carries an approximately 2.5 kb chromosomal DNA fragment in high-copy-number plasmid. While strain BalA1 formed little aerial hyphae, its morphological defect was restored by cultivation with *S. azureus*, *S. laurentii* and *S. coelicolor*. Strain BalA1 strongly inhibited the growth of *B. subtilis* more so than its parent strain, and also inhibited the development of its parent and some *Streptomyces* strains with thioestrepton resistance. Furthermore, it induced *S. coelicolor* A3(2) to produce undecylprodigiosin at an early stage of growth, and to increase in the amount of actinorhodin production. The 2.5 kb fragment contained two *orf1*, *orf1* and truncated *orf2*. The deduced products were somewhat similar to phosphoserine phosphatase–like protein and N-terminal region of MinD–like protein, respectively. The individual function of *orf1* or jointed function of both *orf1* seems to induce particular phenotypes or properties in strain BalA1.

INTRODUCTION

*Streptomyces* undergo a complex cycle of morphological and physiological differentiation resembling that of filamentous fungi. A spore germinates to form substrate mycelium, aerial hyphae rise from the colony surface, and aerial hyphae septate into chains of pigmented spores. Morphological and physiological differentiation of *Streptomyces* are temporally coordinated (Champness and Chater, 1994), suggesting the existence of shared global regulatory elements for differentiation. Genes that promote morphological and physiological differentiation have been identified and characterized, include *bld, whi, aba, abs* and *afs* (Aceti and Champness, 1998; Chater, 1998; Fernandez–Moreno et al., 1992; Umeyama et al., 1999). By cloning of regulatory sequences on high–copy–number plasmids, novel regulators for secondary metabolism and cellular differentiation have been detected and characterized (Scheu et al., 1997; Ueda et al., 1999). These reports encourage the use of high–copy–number plasmid to screen for potential developmental regulatory genes or sequences.

We isolated a 2.5 kb chromosomal DNA fragment from thioestrepton–producing *Streptomyces azureus*, using a high–copy–number plasmid. In this report, we char-
acterized the transformant and determined the sequence of the 2.5 kb fragment.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

*Streptomyces azureus* PK100C, a plasmid–cured derivative of thiostrepton producing *S. azureus* ATCC14921 named as PK0, was earlier isolated in our laboratory (Miyoshi et al., 1986). *Streptomyces coelicolor* A3(2), its pIJ702 (Katz et al., 1983) carrying derivative and another thiostrepton–producing strain *Streptomyces laurentii* ATCC31255 were used as indicators in antimicrobial activity tests. Plasmid pMCP5, that was constructed by insertion of a kanamycin resistance gene from *Streptomyces kanamyceticus* ISP5500 into the BglII site of pIJ702 (Nakano et al., 1984), was kindly provided by H. Ogawara (Meiji Pharmaceutical University, Japan). *Streptomyces* cultures were grown in Bennett’s or Rye media (Ogata et al., 1981). Solid media were prepared by addition of 1.5% agar (Difco). Thiostrepton and kanamycin were added at a concentration of 25 μg ml⁻¹, as required.

*Bacillus subtilis* ATCC6633 was used as an indicator of antimicrobial activity. Solid cultures were prepared by mixing spores into autoclaved LB medium containing 1.5% agar at about 60 °C, then incubated at 37 °C.

*E. coli* strain JM109, host for pUC19 and pUC119, was grown at 37 °C in LB medium supplemented with 50 μg ml⁻¹ ampicillin.

Other bacterial strains used in this work are described in notes and figure legends, and all were grown under suitable conditions and appropriate media.

General recombinant DNA techniques

Transformation, plasmid isolation and DNA manipulation of *Streptomyces* and *E. coli* were done, as described (Hopwood et al., 1985). Restriction endonucleases and other modifying enzymes were purchased from TOYOBO (Japan). DNA sequence was determined using an ALFexpress automated sequencer and Thermo Sequenase fluorescent labelled primer cycle sequencing kits with 7–deaza–dGTP (Amersham Pharmacia Biotech). Computer analysis was made using GENETYX–MAC program (Software Development Co. Ltd., Japan). Similar nucleotide or amino acid sequences were identified using BLAST.

Shotgun cloning

Chromosomal DNA prepared from *S. azureus* PK100C was digested with *KpnI* and ligated into the *KpnI* site of pMCP5. The ligation mixture was used to transform *S. azureus* PK100C, and kanamycin–resistant transformants were selected (Tomura et al., 1993). A colony lacking aerial hyphae was cultured in liquid Bennett’s medium. The recombinant plasmid was extracted and re–transformed strain PK100C to confirm that the aerial hyphae–negative phenotype was the result of cloned DNA fragment.

Physiological activity

Antimicrobial activity was tested using the agar piece method. Pre–cultivated *S. azureus* PK100C and BalA1 in liquid Bennett’s media were spreaded to Rye plates (10^6
colony-forming units per plate). These solid cultures sampled using a cork-borer were transferred onto an indicator lawn of *B. subtilis* ATCC6633. Further physiological activity was observed by streaking BalA1 proximate to indicator strains.

**Southern hybridization**

Chromosomal DNAs of *Streptomyces* strains, prepared as described (Hopwood *et al.*, 1985), were digested with *KpnI*. After agarose gel electrophoresis, the DNA fragments were transferred onto a PROTRAN BA85 nitrocellulose membrane (Schleicher & Schuell, Germany). The 2.5 kb fragment used as a probe was labeled with DIG DNA Labeling and Detection kit (Roche Diagnostics). Hybridization was carried out following the manufacturer’s instructions.

**RESULTS**

**Isolation of transformant BalA1**

One colony impaired in aerial mycelium-formation was isolated out of approximately 30,000 shotgun-cloned transformants (Fig. 1, right). This transformant carried a plasmid pSAB931 which we constructed by insertion of an approximately 2.5 kb chromosomal DNA fragment into the *KpnI* site of pMCP5. The aerial mycelium-negative phenotype was confirmed by re-introduction of pSAB931 into *S. azureus* PK100C, and the transformant was named BalA1. A transformant with only vector pMCP5 did not show the aerial hyphae-negative phenotype and the following properties.

It is well known that medium pH pleiotropically affects morphological development of

![Fig. 1. Growth inhibition toward *B. subtilis* ATCC6633 by *S. azureus* BalA1 and its parent PK100C.](image)

After seven days of growth, agar pieces of strains BalA1 (right) and PK100C (left) were transferred onto a *B. subtilis* seeded plate, and incubated at 37°C for 1 d.
Streptomyces (Süssstrunk et al., 1998). However, the phenotype of strain BalA1 was not suppressed by neutralizing buffer (data not shown).

Physiological activity

As shown in Fig. 1, a piece of solid culture of strain BalA1 not only formed a clear growth-inhibitory zone toward B. subtilis ATCC6633 as large as that of parent strain PK100C, but also formed a turbid area outside of the clear zone (Fig. 1, right). Strain BalA1 inhibited the morphological development of thiostrepton resistant Streptomyces strains, including its parent strain PK100C (Fig. 2) and another thiostrepton producing strain S. laurentii ATCC31255 (Fig. 3, A, B). And strain BalA1 also inhibited morphological development of PK0, wild-type strain of S. azureus, and S. coelicolor A3(2) transformed with pJ702 (Fig. 3, A; Table 1), which carried the thiostrepton resistance gene of S. azureus (Katz et al., 1983). We interpreted these phenomena to mean that strain BalA1 secreted some bioactive substance(s) in addition to thiostrepton.

During growth with S. azureus and S. laurentii ATCC31255, formation of aerial hyphae and spores of strain BalA1 was restored (Fig. 3, A, B). Weaker restoration was also observed with Streptomyces griseus JCM4681 and Streptomyces fradiae JCM4133 (Table 1). These restorations may suggest that strain BalA1 lacks an important substance or regulator for initiating morphological development, or that a developmental inhibitor caused by pSAB931 is inactivated.

Furthermore, strain BalA1 induced S. coelicolor A3(2) early production of red

Fig. 2. Physiological activities of strain BalA1.
Strain BalA1 and its parent PK100C were cultivated proximately for 3 d. Strain BalA1 (A, upper half) inhibited morphological differentiation of PK100C (A, lower half), but not so PK100C carrying the vector pMCP5 (B, upper half).
Fig. 3. Inhibition of morphological development toward *S. coelicolor* and *S. laurentii* by strain BalA1, and restoration of morphological differentiation of strain BalA1 by them.

A: Strain BalA1 (a), *S. coelicolor* A3(2) carrying plJ702 (b) and thioestreptom-producing strain *S. laurentii* ATCC31255 (c) were cultivated proximately for 7 d. Strain BalA1 inhibited morphological development of thioestrepton resistant strains (b, c). Needless to say, no inhibition was observed between strain BalA1s (a). Aerial mycelia and spores of strain BalA1 was restored on the entire lawn.

B: Strain BalA1 (right) and *S. laurentii* ATCC31255 (left) were cultivated proximately for 3 d (Bi). The aerial mycelia of strain BalA1 appeared at the area neighboring lawn of *S. laurentii* (Bi, arrow). After 7 d cultivation, aerial mycelia and spores of strain BalA1 were observed on the entire lawn (Bii).
Table 1. Physiological activities of S. azureus BalA1

<table>
<thead>
<tr>
<th>Thiostrepton resistant strains which were inhibited their morphological differentiation by strain BalA1</th>
<th>Strains which restore the formation of aerial hyphae and spores of strain BalA1 †</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. azureus PK0*</td>
<td>S. azureus PK0*</td>
</tr>
<tr>
<td>S. azureus PK100C</td>
<td>S. azureus PK100C</td>
</tr>
<tr>
<td>S. coelicolor A3(2) with plJ702</td>
<td>S. coelicolor A3(2) with plJ702</td>
</tr>
<tr>
<td>S. laurentii ATCC31255</td>
<td>S. griseus JCM4681</td>
</tr>
<tr>
<td></td>
<td>S. fradiae JCM4133</td>
</tr>
<tr>
<td></td>
<td>S. laurentii ATCC31255</td>
</tr>
</tbody>
</table>

* S. azureus ATCC14921
† Restorations were observed in the same way as Fig. 3.

Fig. 4. Influence of strain BalA1 on pigmentary antibiotics productions of S. coelicolor A3(2). After seven days of growth, agar pieces of strains BalA1 were transferred onto seeded plates of S. coelicolor, and incubated for more than 2 d.
A: Strain BalA1 induced early production of undecylprodgiosin at 2 d or less (lower right).
B: Strain BalA1 had barely induced early production of actinorhodin, but increased in the amount after 2 d (lower right).
Strain PK100C (lower left) and paper disk (8 mm in diameter) containing 10 µg thiostrepton (upper center) did not affect the antibiotics productions.

pigmentary antibiotic undecylprodgiosin (Fig. 4, A). The same effect was obtained with the addition of supernatant of liquid culture of strain BalA1 (data not shown). Strain BalA1 did not induced early production of actinorhodin, but increased in the amount (Fig. 4, B). The parent strain PK100C and its
antibiotic thiostrepton did not affect pigment production of *S. coelicolor* A3(2). These results suggested that strain BalA1 probably produced inducer(s) or regulator(s) for production of secondary metabolites of *Streptomyces*.

**Nucleotide sequence**

The nucleotide sequence of the cloned 2506 bp fragment was determined. FRAME analysis (Bibb *et al.*, 1984) by GENETYX–MAC revealed a complete 834 bp *orf* (*orf1*) and an incomplete 541 bp *orf* (*orf2*) in this region (Fig. 5). The entire nucleotide sequence of the fragment was deposited in DDBJ under accession no. AB004855.

![Restriction map with GC-plot and nucleotide sequence of the cloned fragment.](image)

**Fig. 5.** Restriction map with GC-plot and nucleotide sequence of the cloned fragment. The FRAME analysis was carried out with window size 50 nt.

**Homology search**

The nucleotide sequence and deduced amino acid sequences of *orf1* and *orf2* were compared with sequences in databases. There was no similar gene, protein or sequence relating to the differentiation in *Streptomyces*. Orf1 showed similarity to phosphoserine phosphatase–like protein of *Mycobacterium leprae* (Fig. 6, A) (accession no. U00018, 37% identity and 73% similarity in the overlapped region), and others. Orf2 showed a low level of similarity to MinD, septum site determining protein, of *B. subtilis* (Fig. 6, B) (accession no. AL009126, 14% identity and 58% similarity in the overlapped region) (Varley and Stewart, 1992) and MinD–like proteins of several species. Although MinD protein was seen to have a mononucleotide binding motif and ATPase activity (de Boer *et al.*, 1991), Orf2 lacked the motif replacing the most important lysine residue with alanine (Saraste *et al.*, 1990).
Southern blot analysis

To search for similar sequences, Southern hybridization was done using the 2.5 kb fragment as a probe against total DNAs from many kinds of bacteria. Fig. 7 shows hybridizing bands with *Streptomyces lividans* TK21, *S. lividans* TK24, *S. laurentii* ATCC31255, *S. coelicolor* JCM4357, *S. griseus* JCM4681 and *S. griseus* JCM4319. The same results were obtained with other *Streptomyces* strains, but not from *Gordonia*...
A 2.5 kb chromosomal fragment of *Streptomyces aureus*

![Southern blot of several *Streptomyces* strains.](image)

The probe was the DIG-labelled 2.5 kb fragment. All DNAs were digested with Kpnl. Lanes: 1, the 2.5 kb fragment carried on the plasmid pMCP5; 2, *S. aureus* PK100C; 3, *Streptomyces lividans* TK21; 4, *S. lividans* TK24; 5, *S. laurentii* ATCC31255; 6, *S. coelicolor* JCM4357; 7, *S. griseus* JCM4681; 8, *S. griseus* JCM4319.

**Fig. 7.** Southern blot of several *Streptomyces* strains.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Fragment Size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.1</td>
</tr>
<tr>
<td>2</td>
<td>9.4</td>
</tr>
<tr>
<td>3</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
</tr>
<tr>
<td>5</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*amae* JCM3171, *Nocardia farcinica* JCM3088, *Rhodococcus* sp JCM0376, *B. subtilis* ATCC6633 and *E. coli* JM109 (Table 2). These results indicate that regions similar to the 2.5 kb fragment are widely distributed among *Streptomyces* species. Recently we found a sequence (registered as highly homologous to the 2.5 kb fragment detected in this work) in a database of *Streptomyces coelicolor* A3(2) Genome Project (SCH5.19–20c region of *S. coelicolor* cosmid H5, accession no. AL035636, Nucleotide identity is 81% in overlapped region). These results imply that regulation systems similar to strain BalA1 play important global roles in the early differentiation of *Streptomyces*.

**DISCUSSION**

Strain BalA1, which carried with high-copy-number plasmid including an approximately 2.5 kb fragment, formed little aerial mycelium, yet this morphological defect was restored by growth with *Streptomyces* strains, and this restoration led to the formation of spores. Strain BalA1 produced an unidentified antimicrobial substance in addition to thiostrepton, and induced early production of red pigmentary antibiotic undecylprodigiosin in *S. coelicolor*. These phenomena were reminiscent of regulators such as A-factor, which plays a key role as regulator to activate signal transduction pathways for differentiation (Ohnishi et al., 1999). The repression or de-repression caused by the unidentified regulatory element may be responsible for the particular phenotype of strain
Table 2. Summarized result of Southern hybridization using the 2.5 kb fragment as a probe

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces azureus</em> PK0</td>
<td>+</td>
</tr>
<tr>
<td><em>S. azureus</em> PK100C</td>
<td>+</td>
</tr>
<tr>
<td><em>S. achromogenes</em> JCM4121</td>
<td>+</td>
</tr>
<tr>
<td><em>S. antibioticus</em> JCM4007</td>
<td>+</td>
</tr>
<tr>
<td><em>S. coelicolor</em> JCM4357</td>
<td>+</td>
</tr>
<tr>
<td><em>S. coelicolor</em> A3(2)</td>
<td>+</td>
</tr>
<tr>
<td><em>S. coeruleus</em> JCM4360</td>
<td>+</td>
</tr>
<tr>
<td><em>S. cyaneus</em> JCM4220</td>
<td>+</td>
</tr>
<tr>
<td><em>S. fradiae</em> JCM4133</td>
<td>+</td>
</tr>
<tr>
<td><em>S. glaucescens</em> JCM4377</td>
<td>+</td>
</tr>
<tr>
<td><em>S. griseus</em> JCM2926</td>
<td>+</td>
</tr>
<tr>
<td><em>S. griseus</em> JCM4681</td>
<td>+</td>
</tr>
<tr>
<td><em>S. hawaiiensis</em> JCM4172</td>
<td>+</td>
</tr>
<tr>
<td><em>S. lactamdurae</em> JCM4912</td>
<td>+</td>
</tr>
<tr>
<td><em>S. laurentii</em> ATCC31255</td>
<td>+</td>
</tr>
<tr>
<td><em>S. lavendulae</em> JCM4055</td>
<td>+</td>
</tr>
<tr>
<td><em>S. lividans</em> TK21</td>
<td>+</td>
</tr>
<tr>
<td><em>S. lividans</em> TK24</td>
<td>+</td>
</tr>
<tr>
<td><em>S. siogaeensis</em> JCM4418</td>
<td>+</td>
</tr>
<tr>
<td><em>Gordonia amarae</em> JCM3171</td>
<td>-</td>
</tr>
<tr>
<td><em>Nocardia farcinica</em> JCM3088</td>
<td>-</td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp JCM3376</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC6633</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM109</td>
<td>-</td>
</tr>
</tbody>
</table>

+, Hybridized with the 2.5 kb fragment; -, No hybridization
Total DNA of these strains were digested with KpnI and hybridized with the 2.5 kb fragment.

BalA1, however, the A–factor did not affect the properties of strain BalA1 (data not shown).

The 2.5 kb fragment is expected to influence the regulation systems of morphological differentiation and secondary metabolite, and/or to contain some regulatory element(s) involved in differentiation. It has been reported that putative regulators inserted in high–copy–number plasmids influenced a carbon–source–dependent morphological defect in *S. griseus* and secondary metabolite production of *S. lividans* (Scheu et al., 1997; Ueda et al., 1999). The 2.5 kb fragment showed no similarity to previously characterized genes or to sequences for differentiation in *Streptomyces*, judging from the sequence of the 2.5 kb fragment and two orfs. However, these orfs showed little similarity to phosphoserine phosphatase–like proteins and MinD (and MinD–like) proteins. Phosphoserine phosphatase and MinD are involved in serine biosynthesis and inhibition of septum formation at potential division sites, respectively (Hu and Lutkenhaus, 1999; Neuwald and Stauffer, 1985). Thus, the orfs may influence serine concentration or cell division in mycelium. These quantitative or qualitative changes are possibly key elements for differentiation in *Streptomyces*, or the orfs themselves may be novel regulatory
elements. Thus, functional analysis of orf1 and orf2 may contribute greatly to understanding of mechanisms of differentiation in *Streptomyces*. Detailed characteristics of orf1 and orf2 will be reported in ongoing studies.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research (C, no. 06660115) from the Ministry of Education, Science, Sports and Culture of Japan and by the Suzuki Fellowship Foundation (Tokyo). We are grateful to M. Ohara for helpful comments and advice on the manuscript.

REFERENCES


Hu, Z. and J. Lutkenhaus 1999 Topological regulation of cell division in *Escherichia coli* involves rapid pole to pole oscillation of the division inhibitor MinC under the control of MinD and MinE. *Mol. Microbiol.*, **34**: 82–90.


