Cloning and Heterologous Expression of Blasticidin S Biosynthetic Genes from *Streptomyces griseochromogenes*

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Two small chromosomal DNA fragments (2.6 and 4.8 kb) from the blasticidin S producer *Streptomyces griseochromogenes* were cloned in the high copy number vector pIJ702 and shown to confer increased resistance to blasticidin S upon *S. lividans* TK24. These fragments were used to screen a library of *S. griseochromogenes* DNA prepared in the cosmid shuttle vector pOJ446. Cosmids containing DNA inserts of at least 23 kb were identified which hybridized to one or the other resistance fragment, but not to both. Transformation of *S. lividans* TK24 with several cosmids hybridizing with the 4.8 kb resistance fragment resulted in clones that produced cytosylglucuronic acid, the first intermediate of the blasticidin S biosynthetic pathway, and other blasticidin-related metabolites. A strain of *S. lividans* TK24 harboring both the 4.8 kb-hybridizing cosmid and the 2.6 kb resistance fragment cloned in pIJ702 produced 12.5 times as much demethylblasticidin S as the transformant harboring the cosmid alone.

Blasticidin S (BS, **1**) was first isolated from *Streptomyces griseochromogenes*[1] as part of an effort to replace mercury-based fungicides for prevention of *Piricularia oryzae* infection of rice plants in Japan[2]. Blasticidin S is a peptidyl nucleoside derived from the primary precursors cytosine, glucose, arginine and methionine[3]. The involvement of β-arginine[4], cytosylglucuronic acid (CGA, **2**)[5], and cytosinine[6] in the pathway has also been demonstrated (Scheme 1). More recently, cytosylglucuronic acid synthase, the enzyme catalyzing the first step in blasticidin biosynthesis and the first prokaryotic UDP-glucuronosyl transferase identified, has been purified and characterized[7].

Our interest in further studying CGA synthase, as well as our interest in identifying the genes for the entire biosynthetic pathway, led us to undertake the cloning of this pathway and expression in a heterologous host, *S. lividans*. Peptidyl nucleosides[8] constitute a subclass of a large family of antibiotics, the nucleosides,[9] of which only the biosynthetic genes for puromycin[10] and nikkomycin[11] have been cloned. Our strategy for cloning the biosynthetic pathway for blasticidin S was first to identify the resistance genes. Because resistance genes in antibiotic-producing prokaryotic organisms are generally located with the biosynthetic genes[12], these markers can often be used as probes for the DNA of the biosynthetic cluster. We report here the identification of two small fragments of DNA from *S. griseochromogenes* which confer increased resistance to blasticidin S upon *S. lividans*. These resistance fragments were further used to clone larger DNA fragments which, when expressed in *S. lividans*, result in the production of CGA (**2**) and other blasticidin S-related metabolites.

Materials and Methods

Bacterial Strains and Plasmids

The blasticidin S producer *Streptomyces griseochromogenes* was obtained from Dr. Y. Miyazaki (Kaken Chemical Co., Ltd., Chiba, Japan) and maintained as previously described[7]. *Streptomyces lividans* TK24 and plasmid pIJ702 were obtained from Prof. D. A.

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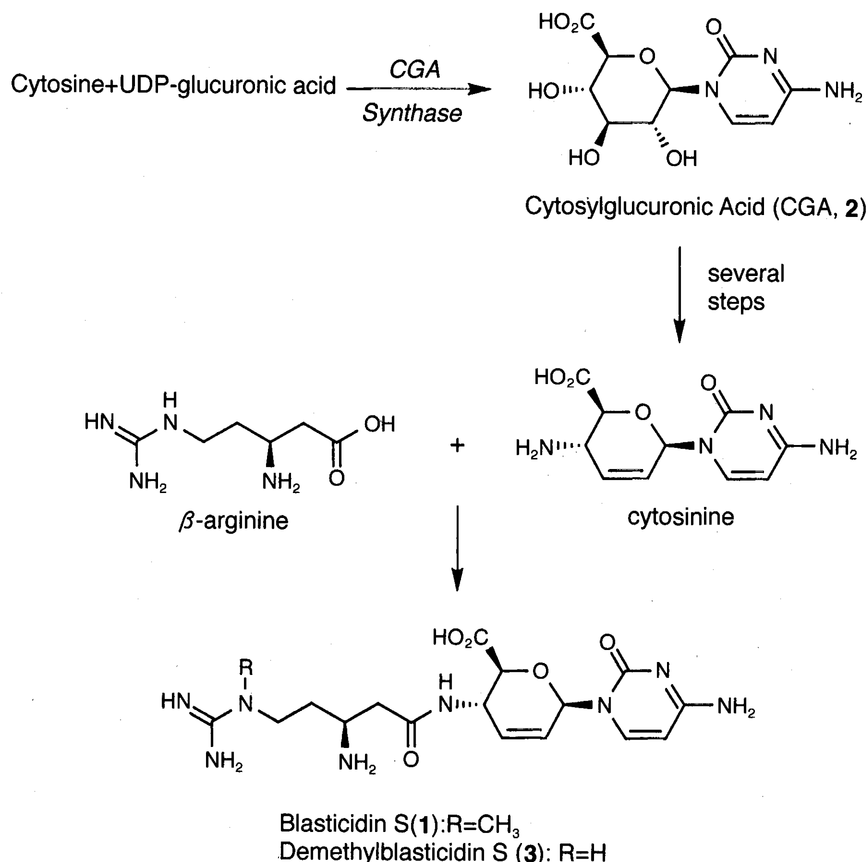
Scheme 1.



HOPWOOD (John Innes Inst., Norwich). *Streptomyces* cultures were grown in either YEME medium[13] or GPS medium[14] and manipulated by standard procedures[13]. *E. coli-Streptomyces* shuttle cosmid pOJ446, obtained from Lilly Research Laboratories, was used for constructing a *S. griseochromogenes* chromosomal DNA library[15]. The *E. coli* cloning vector pGEM3Z(+) and *E. coli* strain JM109 were purchased from Promega Corp. (Madison, WI) and used in all subcloning experiments. The *E. coli* vector pBluescript II KS(+) was obtained from Stratagene (La Jolla, CA). All *E. coli* manipulations were performed according to standard protocols[16].

Cloning of BS Resistance Genes

S. griseochromogenes chromosomal DNA was isolated, digested to completion with *Bam*HI, and ligated into the *Bgl*II site of pIJ702. *S. lividans* TK24, previously determined to be sensitive to BS at a concentration of 200 μg/ml (data not shown), was transformed with the ligation mixture, and transformants were allowed to sporulate. Approximately 5000 transformants were replica-plated to R2YE agar containing 200~600 μg/ml BS. All colonies capable of growth at 200 μg/ml BS were also capable of growth at 600 μg/ml. Plasmid DNA was isolated from resistant colonies and analyzed by restriction digestion.

Construction of a Cosmid Library

S. griseochromogenes chromosomal DNA was partially digested with *Sau*3A to give DNA fragments 25~35 kb in size. The chromosomal DNA was treated with alkaline phosphatase and ligated into pOJ446, previously digested with *Hpa*I and *Bam*HI. Ligated DNA was packaged using a Gigapack II XL packaging kit from Stratagene Corp. (La Jolla, CA) according to the manufacturer's specifications. A 4.8 kb BS resistance fragment was amplified by PCR using primers specific to flanking pIJ702 DNA sequence[17] and purified by agarose gel electrophoresis. The DNA fragment was recovered from the agarose using the Gene Clean Kit (Bio101, Vista, CA) and labelled with ^{32}P-dATP using the Random Primed Labelling Kit (Boehringer Mannheim, Indianapolis, IN). The cosmid library was screened with the labelled DNA fragment by colony blot hybridization. Positive colonies were reprobed and grown up for plasmid isolation, and the presence of a 4.8 kb *Bam*HI fragment was confirmed by restriction digestion and hybridization.

HPLC Analysis of Metabolites

Mycelia were removed from liquid cultures (5 ml) by centrifugation, and the supernatant adjusted to pH 3.0 with 0.1 N HCl. The samples were kept at room temperature for approximately 10 minutes, and then centrifuged for 10 minutes at 12,000 × *g*. The resulting supernatant was filtered (0.45 μm) and an aliquot (10 μl) of the solution was analyzed by ion exchange HPLC (Waters model 600 system) using a polysulfoethylaspartamide column (4.6 × 200 mm, NEST Group, Southboro, MA) with gradient elution (30 minutes linear gradient of 0 ~ 0.25 M KCl in 5 mM potassium phosphate, pH 3.0, containing 25% MeCN, at a flow rate of 1.0 ml/minute). The UV region from 200 ~ 300 nm was scanned with a photodiode array detector (Waters model 996 or 990+) and these spectra were compared with authentic materials. Chromatograms were printed out for absorption at 275 nm, corresponding to the cytosine chromophore.

LC-MS Identification of Metabolites

A Finnigan-MAT LCQ ion trap mass spectrometer coupled to a Hewlett-Packard HP1100 binary LC system with photodiode array detection was used for all analyses. Metabolites were eluted from a Whatman SCX column (4.6 × 250 mm) with acetonitrile/water (1 : 1) containing 0.1% TFA at 1.0 ml/minute at 40 °C. A 10 : 1 split was used for introduction to the LC-MS interface.

Results

Identification of Resistance Genes

Chromosomal DNA prepared from *S. griseochromogenes* and completely digested with *Bam*HI was cloned into the *Bgl*II site of the high copy *Streptomyces* vector pIJ702 and used to transform *S. lividans* TK24. Transformants were screened for blasticidin S (BS) resistance by replica plating with BS concentrations of 200, 400 and 600 μg/ml. Forty colonies selected from the two higher BS concentrations were screened in liquid culture and seven recombinant plasmids were identified which conferred resistance to BS at 600 μg/ml. Southern analysis of *Bam*HI digests of *S. griseochromogenes* chromosomal DNA, using three of the recombinant plasmids as probes, highlighted fragments of approximately 2.6 and 4.8 kb. In order to remove the inserts from pIJ702 for further study, PCR was performed on the recombinant plasmids using 28-mer primers incorporating sequence on either side of the *Bgl*II site in the *S. antibioticus* tyrosinase (*mel*) gene[18] along with new restriction sites: Res702E (*Eco*RI site is underlined), 5′-CATGCGAATTCCCGC CTTCGACGACATC-3′; Res702H (*Hin*dIII site is underlined), 5′-CGTACAAGCTTGTATCCGGCGGCC CTTG-3′. PCR of the seven resistance plasmids yielded only two products, either 2.6 kb or 4.8 kb. The PCR products were cloned into the *E. coli* vector pGEM-3Z(+) for use in preparing radiolabelled probes. It should be noted that this strategy would not detect a resistance gene having an internal *Bam*HI site. Thus, the existence of other resistance determinants cannot be ruled out.

Preparation and Screening of *S. griseochromogenes* Chromosomal Library

Partial digestion of *S. griseochromogenes* chromosomal DNA with *Sau*3A yielded fragments greater than 25 ~ 35 kb which were ligated into the cosmid shuttle vector pOJ446 and packaged into phage for transfection into *E. coli*. Approximately 3000 recombinant *E. coli* colonies were probed with each of the two resistance fragments. Four cosmid clones hybridized with the 2.6 kb resistance fragment and eleven cosmid clones hybridized with the 4.8 kb resistance fragment. None of the clones were found to hybridize with both probes. The 2.6 kb and 4.8 kb *Bam*HI resistance fragments were identified in the cosmids, purified, and subcloned in pGEM-3Z(+) for sequencing.

Heterologous Expression of Blasticidin Biosynthesis

Four cosmids from each of the two resistance fragment-hybridizing groups were used to transform *S. lividans* TK24 protoplasts, and a large inoculum for production was taken directly from the primary transformation plate without further subculture. The transformants were inoculated into three media for production from the primary liquid culture. The cultures were grown for seven days and then screened for metabolite production using ion exchange HPLC coupled with photodiode array detection. Controls for the experiment included *S. lividans* TK24 alone and transformed with the cosmid vector without insert. One cosmid clone of the 4.8 kb-hybridizing group (cos9) was found to produce cytosylglucuronic acid (CGA) in GPS medium. The identity of the metabolite was suggested by comparison of the retention time and UV/vis spectrum with those of authentic CGA (Fig. 1). Confirmation that the compound was CGA was found in coinjection studies and independently by LC-MS analysis (*m/z* 288, $[M+H]^+$). The remaining seven 4.8 kb hybridizing cosmids were also

Fig. 1. HPLC chromatograms of transformant metabolites.

A: cos9 transformant (hybridizing with the 4.8 kb fragment); B: *S. griseochromogenes* grown in GPS medium; C: cos4 transformant (hybridizing with the 2.6 kb fragment); and D: pOJ446 (vector).

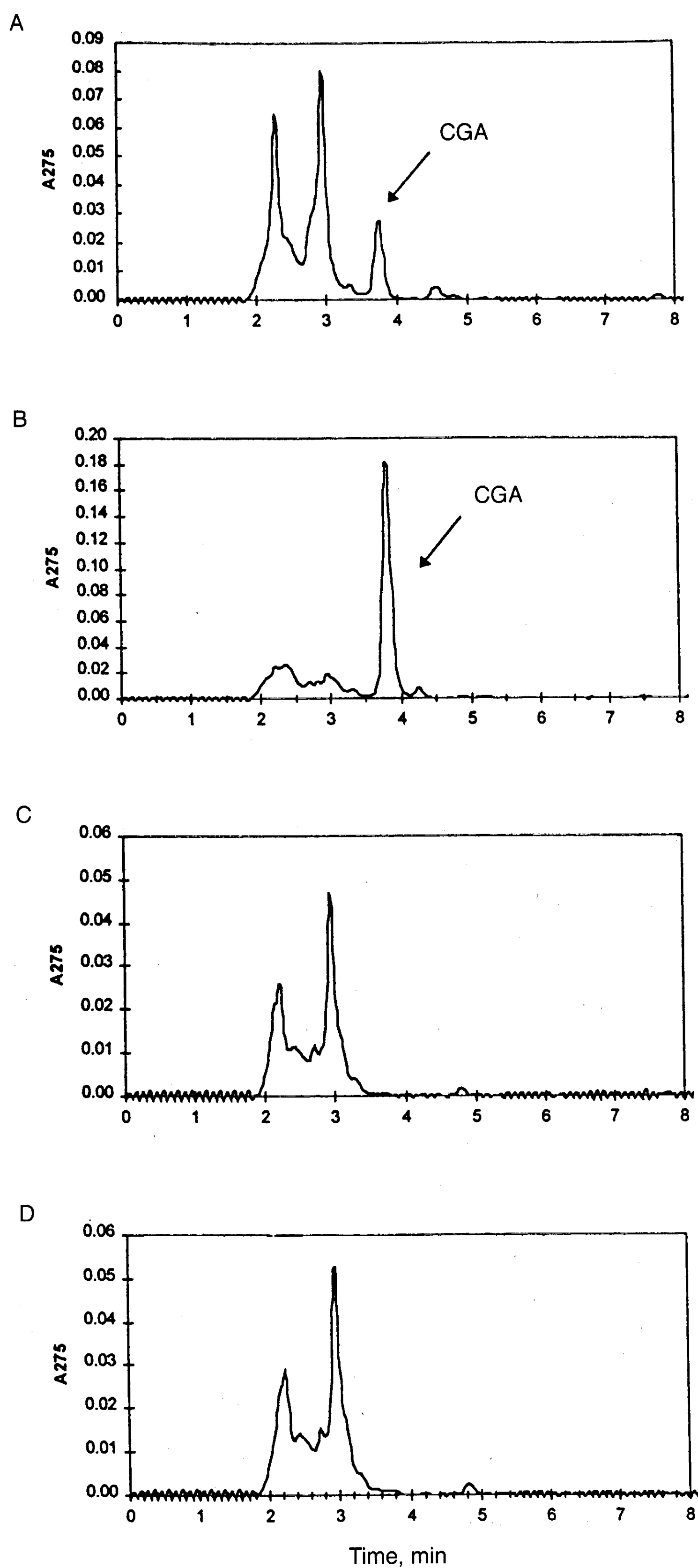
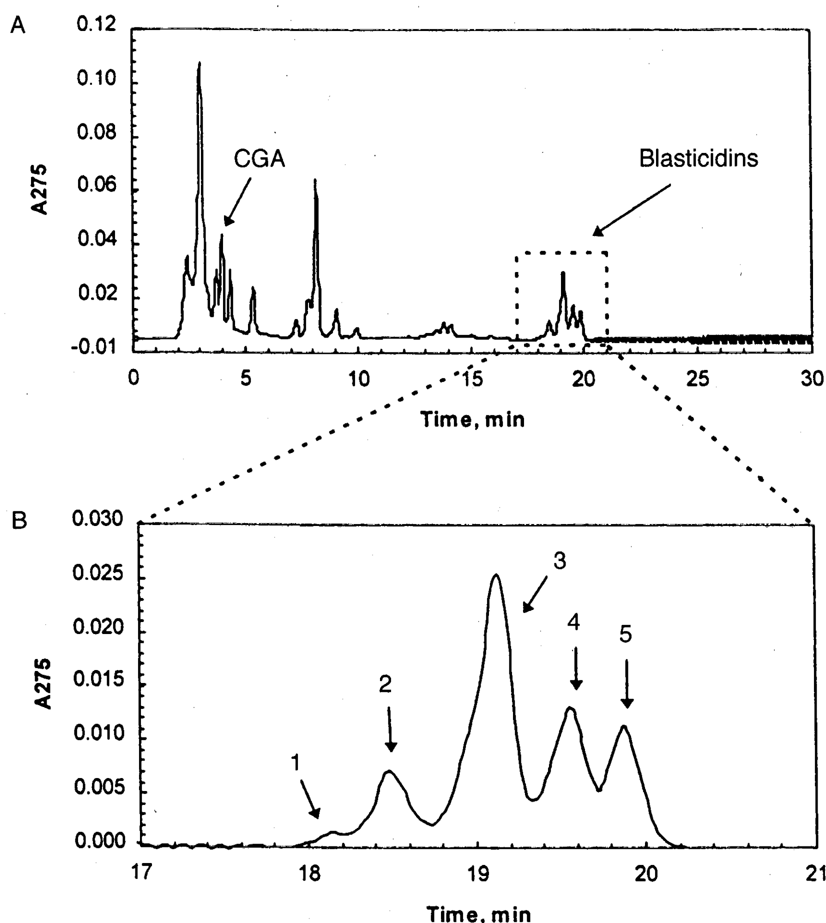


Fig. 2. HPLC chromatogram of a cos9 transformant grown in YEME medium.



Expansion of chromatogram **A** between 17 and 21 minutes.

used to transform *S. lividans* TK24 and the cultures were screened for expression of the BS biosynthetic genes. Three more cosmid clones (cos12, cos14 and cos16) were identified which produced CGA. None of the clones hybridizing with the 2.6 kb resistance fragment produced CGA.

Further testing of the clones positive for CGA production led to the detection of additional blasticidin S-related metabolites. The production of these metabolites was lower but more reproducible when the clones were grown in YEME medium, a standard medium for the culture of *S. lividans*. In YEME medium, *S. griseochromogenes* produced a large amount of two late-eluting metabolites (blasticidin S and demethylblasticidin S), whereas the transformants produced CGA and a complex of five late-eluting metabolites (Fig. 2A and 2B). These closely-eluting metabolites contain a chromophore closely matching that of blasticidin S (peaks 1~4) and demethylblasticidin S (peak 5) (data not shown). Careful coinjection experiments indicated that metabolite 5 is most probably demethylblasticidin S, and metabolite 4 may be blasticidin S. Definitive identification of these metabolites at this scale was more complicated than it was for CGA because the compounds were not separated using low-salt buffers and were thus unsuitable for LC-MS analysis. Furthermore, scale-up and conventional isolation and structure elucidation of the transformant metabolites was hampered by the instability of metabolite expression.

Double transformation of *S. lividans* TK24 with both BS Resistance Fragments

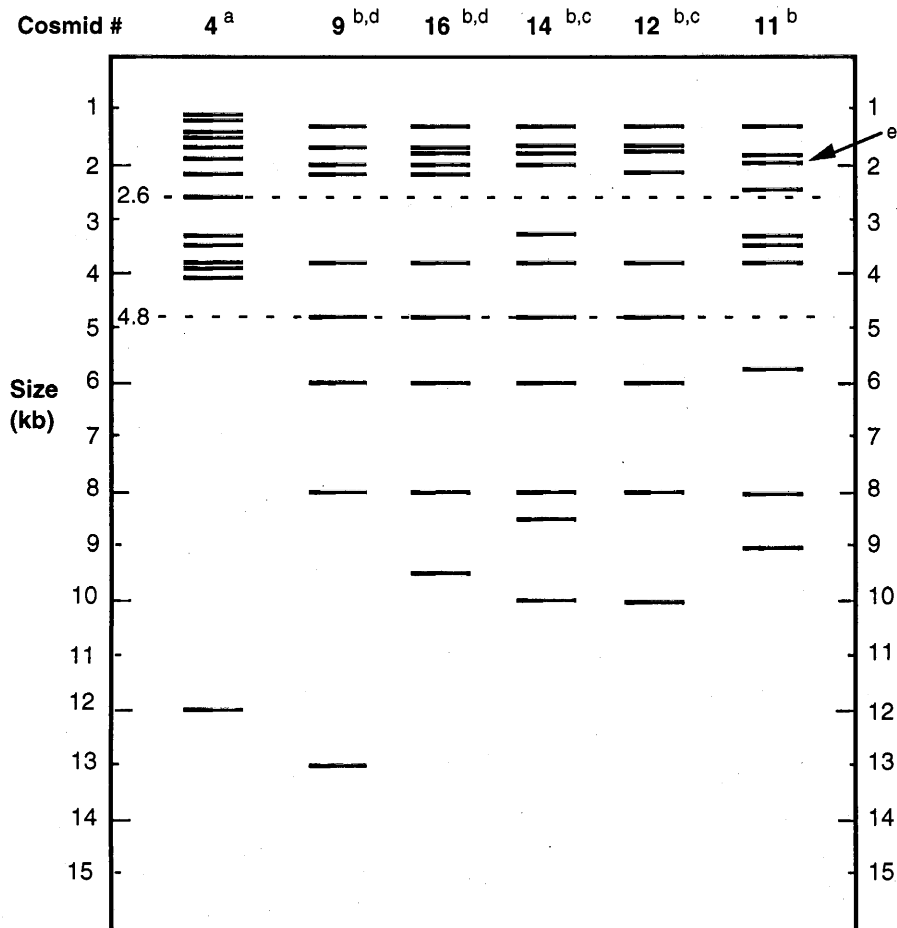
In an attempt to improve the level and stability of BS metabolite production in *S. lividans* TK24, we sought to simultaneously express both resistance genes. A double transformation experiment was performed with cos9 and the 2.6 kb resistance fragment inserted in pIJ702. The transformation frequency with both constructs was one tenth that of either alone. Controls for the experiment included transformation using both vectors with no insert. Four colonies were identified that were resistant to both apramycin (marker for cos9) and thiostrepton

Table 1. Amounts of blasticidin complex produced by double transformant DT1 (cos9 + 2.6 kb fragment in pIJ702) and single transformant (cos9 alone).

Metabolite			Amount[a] produced by:		Ratio
Peak	t_R, minutes	Match[b]	cos9	DT1	DT1/cos9
1	18.14	BS 968	3.4	3.2	0.92
2	18.47	BS 976	22.0	20.1	0.91
3	19.10	BS 986	68.6	71.9	1.1
4	19.55	BS 988	13.3	34.6	2.6
5	19.88	deMeBS 994	2.1	25.6	12.5

[a] Area under peak in units of $A_{275} \times \min \times 10^{-4}$.
[b] UV-vis spectrum match number according to Waters 990 detector (1000 = perfect match).

Fig. 3. *Bam*HI restriction patterns of recombinant cosmids hybridizing with 2.6 kb or 4.8 kb resistance fragments.



Fragment size was determined by reference to a 1 kb DNA ladder (GIBCO BRL). Fragments <1 kb are not shown.

[a] Hybridizes with 2.6 kb resistance fragment.
[b] Hybridizes with 4.8 kb resistance fragment.
[c] *S. lividans* transformant produces CGA (**2**).
[d] *S. lividans* transformant produces demethylblasticidin S (**3**).
[e] *Bam*HI fragment (2 kb) that hybridizes with 4.8 kb resistance fragment.

(marker for pIJ702). Of these four, only one (DT1) produced blasticidin-related metabolites. This culture initially grew very slowly but on continued subculture grew more rapidly in the presence of both antibiotics. Levels of production of the five metabolites of the blasticidin complex by the double transformant and the cos9 clone are compared in Table 1.

The amounts of metabolites 1 ~ 3 were insignificantly changed in the double transformant. However, levels of metabolite 4 (tentatively assigned as blasticidin S) and metabolite 5 (demethylblasticidin S) were threefold and 12.5-fold higher, respectively, in the double transformant.

Characteristics of Cosmid DNA

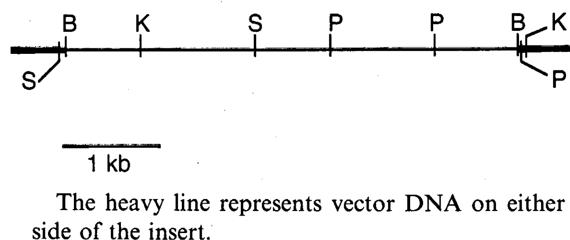
The reason for lack of metabolite production by three of the four initial isolates in the double transformation experiments was not explored further. However, the cosmids in these strains may have undergone some rearrangements or deletions. We have detected this type of rearrangement in some of the cosmids which initially hybridized with the 4.8 kb probe. These cosmids at first showed *Bam*HI restriction patterns similar to the 4.8 kb hybridizing cosmids depicted in Figure 3 but on subculture the restriction patterns were drastically altered and their transformants produced no blasticidin-related metabolites (data not shown).

Figure 3 compares the *Bam*HI restriction pattern of a cosmid hybridizing with the 2.6 kb probe (#4) with cosmids hybridizing with the 4.8 kb resistance fragment and producing CGA (#'s 12 and 14); those hybridizing with the 4.8 kb resistance fragment and producing blastidicidins (#'s 9 and 16); and those hybridizing with the 4.8 kb resistance fragment but producing no metabolites (#11). In the case of cosmid #11, the band which hybridized with the 4.8 kb probe is only 2.0 kb in size. Similarities are clearly evident in the digests of those cosmids able to transform *S. lividans* TK24 into strains synthesizing blasticidin metabolites. The original Southern analysis suggested the 2.6 kb and 4.8 kb resistance fragments did not hybridize to any of the same cosmids. However, the *Bam*HI maps in Figure 3 show that fragments of 1.7 and 3.8 kb are found in cos4 and most of the cosmids carrying the BS biosynthetic genes. These fragments are candidates for overlap of the 2.6 kb and 4.8 kb hybridizing cosmids and may be useful in future studies to investigate the proximity of both resistance genes to the biosynthetic cluster and to one another.

We are also interested in the mechanism of BS self-resistance in *S. griseochromogenes*. Additional restriction

Fig. 4. Restriction map of 4.8 kb resistance fragment.

B, *Bam*HI; K, *Kpn* I; P, *Pst*I; S, *Sst*I.



The heavy line represents vector DNA on either side of the insert.

mapping performed on the 4.8 kb resistance fragment is presented in Figure 4, and the subcloning and sequencing of this DNA fragment are underway. Sequence analysis of the 2.6 kb resistance fragment indicates this piece of DNA carries a gene encoding a protein with two ATP-binding domains that may be involved in antibiotic transport (PETRICH and GOULD, unpublished data).

Discussion

We have reported here the cloning of blasticidin S biosynthesis genes *from S. griseochromogenes* and have demonstrated their expression in a heterologous host. Expression of the pathway has been achieved at least up to the penultimate metabolite, demethylblasticidin S[19]. Several transformants harboring the biosynthetic genes accumulate a complex of blasticidin-related metabolites, in contrast to the producing organism in which only blasticidin S (**1**) and demethylblasticidin S (**3**) are found at significant levels. This group of metabolites may be normal intermediates in the biosynthesis and excretion of the antibiotic. The difference in the spectrum of accumulated products between producer and transformants may result from the inefficiency of the pathway components in the transformants, or the lack of one or more elements necessary for optimal expression of the pathway. Blasticidin S inhibits protein chain elongation in bacterial and mammalian cells, in the same manner as the well-studied nucleoside antibiotic puromycin[10]. The ribosomes of the puromycin producing organism, *S. alboniger*, are sensitive to its inhibitory action and the mechanism of self-resistance is proposed to involve formation of an inactive *N*-acetyl derivative. This derivative is then transported from the cell and hydrolyzed by an extracellular *N*-acetyl hydrolase[20]. Small amounts of *N*-acetylpuromycin are found in the culture medium of both transformants and the producing or-

ganism[20]. A gene encoding a Blasticidin S *N*-acetyltransferase activity has been cloned and characterized from *Streptoverticillium* sp. JCM 4673, another blasticidin producer[21]. This raises the possibility that one or more of the metabolites in Fig. 2B could be *N*-acetylated blasticidins. Preliminary results in our laboratory indicate that while blasticidin S *N*-acetyl transferase activity is present in *S. griseochromogenes* the activity is not detectable in *S. lividans* carrying either cosmid 9 or the 4.8 kb resistance fragment in pIJ702 (ZHANG and ZABRISKIE, unpublished results). Another modified blasticidin produced by *S. griseochromogenes* is leucylblasticidin, which can accumulate under certain physiological conditions and can be converted to blasticidin S[22]. Efforts to firmly identify all the BS-related compounds produced by the transformants are continuing and should provide valuable insight into the biosynthesis of BS and its self-resistance mechanism.

The blasticidin S biosynthetic gene cluster is currently being mapped and sequenced, and further studies will determine if cosmids containing the 2.6 kb resistance fragment overlap with those containing the 4.8 kb resistance fragment. The major focus is to identify the product of the resistance gene on the 4.8 kb DNA fragment and determine its function, as well as to express and characterize other individual genes and their products in the blasticidin S biosynthetic cluster.

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