GENE 03365

Cloning and characterization of the gene encoding a blasticidin S acetyltransferase from *Streptoverticillum* sp.

(Recombinant DNA; *Streptomyces*; antibiotic resistance; plasmid vector; nucleotide sequence; promoter activity)

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SUMMARY

A gene (*bls*) encoding a blasticidin S-acetyltransferase from *Streptoverticillum* sp. JCM4673 was cloned in the *Streptomyces* plasmid pIJ702 and expressed in *S. lividans* 1326. Four of the recombinant plasmids contained a common 1129-bp *Bcl*I-*Pst*I DNA fragment, which by subcloning experiments was shown to carry the *bls* gene. Sequencing of the 1129-bp *Bcl*I-*Pst*I fragment indicated that it contains two open reading frames of 408 and 366 nucleotides (nt), both starting with ATG and terminating at TGA triplets. Deletion analysis showed that the correct *bls* sequence is that of 408 nt, whose deduced amino acid sequence conforms to a polypeptide of M_r 15073. A 175-bp DNA fragment from the 5' region of the *bls* gene had promoter activity in the promoter-probe plasmid pIJ486.

INTRODUCTION

In antibiotic-producing organisms, the presence of a self-resistance mechanism is required whenever the drug pro-

duct is potentially harmful to the producer. Resistance may be achieved by several ways including antibiotic inactivation by acetylation and/or phosphorylation. Enzymic modification by acetyltransferases has been found in a variety of Actinomycetes that are producers of either aminoglycoside (reviewed by Cundliffe, 1984), nucleoside or other antibiotics (Pérez-González et al., 1983; Sugiyama et al., 1986; Horinouchi et al., 1987). The acetyltransferase activities from *Streptomyces alboniger*, *Streptomyces lavendulae* and *Streptoverticillum* sp., the producers of puromycin, streptothricin and Bs, respectively, have been biochemically characterized (Vara et al., 1985b; Sugiyama et al., 1985; 1988; Horinouchi et al., 1987).

The genes *pac* and *staI*, encoding puromycin- and streptothricin-*N*-acetyltransferase, respectively, have been cloned in *E. coli* and *S. lividans* and their nt sequences determined (Vara et al., 1985a; Lacalle et al., 1989; Horinouchi et al., 1987). In addition, the *pac* gene has been expressed in mammalian cells (Vara et al., 1986). Plasmid constructs carrying *pac*, which use puromycin as the selective dominant marker, can be used as cloning vectors for several

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Abbreviations: A, absorbance; aa, amino acid(s); *aac7*, gene encoding an aminoglycoside acetyltransferase; Ap, ampicillin; APH II, aminoglycoside 3'-phosphotransferase II; BAC, Bs acetyltransferase; *bls*, gene encoding BAC; bp, base pair(s); Bs, blasticidin S; IPTG, isopropyl- β -D-thiogalactopyranoside; kb, 1000 bp; Km, kanamycin; *lacZ'*, gene encoding β -galactosidase (3' truncated); MM, minimal medium; mu, milliunit; nt, nucleotide(s); ORF, open reading frame; PAC, puromycin *N*-acetyltransferase; *pac*, gene encoding PAC; PAGE, polyacrylamide-gel electrophoresis; ^R, resistance; R5, regeneration medium for *Streptomyces* (Hopwood et al., 1985); RBS, ribosome-binding site; S., *Streptomyces*; SDS, sodium dodecyl sulfate; *staI*, gene encoding streptothricin *N*-acetyltransferase; TGE, 10 mM Tris · HCl pH 8.0/10% glycerol/1 mM EDTA; Ts, thiostrepton; *tsr*, gene encoding Ts resistance; XGal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; YEME, rich liquid medium (Hopwood et al., 1985); [], denotes plasmid-carrier state.

mammalian cells (de la Luna et al., 1988). In this respect, Bs (produced by Kaken Pharmaceutical Co. Ltd., Tokyo, Japan), which is mainly used as an antifungal agent against *Piricularia oryzae* infection of rice fields, is an interesting antibiotic because it strongly inhibits growth of the yeast *Saccharomyces cerevisiae* and other eukaryotic cells by specifically blocking protein synthesis (reviewed by Vázquez, 1979, and Korzybski et al., 1978). Therefore, the availability of a gene determining a dominant resistance to Bs could be useful to construct cloning vectors for yeast, mycelial fungi and other eukaryotic cells. The present work reports the cloning and characterization of the gene (*bls*) for Bs acetyltransferase from *Streptovercillum* sp. JCM4673, the Bs producer.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the *bls* gene in *Streptomyces lividans*

A total of 16 Bs-resistant *S. lividans* transformants were obtained from the *Streptovercillum* genomic library as

described in Table I, footnote a. Although all of these transformants contained pIJ702 with inserted DNA, only four (named BS1, BS4, BS6 and BS14) contained BAC activity (Table I). In addition, plasmid DNA isolated from these transformants (named pBS1, pBS4, pBS6 and pBS14, respectively), but not that isolated from the other twelve transformants, was able to retransform *S. lividans* to produce both Bs-resistant and BAC-containing clones. Therefore, plasmids pBS1, 4, 6 and 14, whose restriction maps are presented in Fig. 1, should contain a *bls* gene. The mechanism(s) determining the Bs-resistance phenotype of the other twelve transformants is unknown; it could be due to chromosomal mutation(s) inducing a permeability barrier. For instance, we noted that some Bs-resistant colonies appeared when MM plates containing 100 µg Bs/ml were seeded with a high amount of *S. lividans* spores.

The restriction maps of the DNA inserts of plasmids pBS1, pBS4, pBS6 and pBS14 show that they contain a common 2.2-kb *PvuII*-*PstI* fragment. This sequence should, most likely, contain the *bls* gene. However, different restriction site patterns were present in the DNA regions 5'

TABLE I

BAC activity, plasmids and Bs-resistance of different bacterial strains

Strain ^a	Plasmid	kb ^b	BAC activity (µg/mg protein) ^c	Bs resistance (µg Bs/ml) ^d
<i>S. lividans</i> 1326	—	—	0.0	80
<i>S. lividans</i> 1326	pIJ702	5.8	0.0	80
<i>Streptovercillum</i> sp. JCM4673	—	—	0.5	> 1000
<i>S. lividans</i> BS1	pBS1	11.5	10.2	> 1000
<i>S. lividans</i> BS4	pBS4	12.8	8.6	> 1000
<i>S. lividans</i> BS6	pBS6	11.8	9.3	> 1000
<i>S. lividans</i> BS14	pBS14	13.3	7.9	> 1000
<i>S. lividans</i> BS1.3	pBS1.3	6.5	22.9	> 1000

^a *Streptovercillum* sp. JCM4673, the producer of Bs, was provided by Dr. A. Seino (Institute of Physical and Chemical Research, Tokyo, Japan). *S. lividans* 1326, the host for transformation, and plasmid pIJ702 (Hopwood et al., 1985) were provided by Dr. D.A. Hopwood (John Innes Institute, Norwich, U.K.). Actinomycetes were grown in liquid medium YEME (Hopwood et al., 1985) supplemented with 34% sucrose and 5 mM MgCl₂. Solid medium R5, overlay agar and solid MM were described previously (Hopwood et al., 1985). *S. lividans* 1326 was found to be highly resistant (> 500 µg Bs/ml) to Bs on R5 plates. However, it does not grow on MM plates supplemented with 100 µg Bs/ml. Total DNA from *Streptovercillum* and plasmid DNA from *Streptomyces* were prepared as described by Hopwood et al. (1985). DNA restriction fragments were isolated as reported by Vara et al. (1985b). Transformation of *S. lividans* was performed as described by Hopwood et al. (1985). A genomic library was prepared by inserting *SauIII*A-partials (2–15 kb average size) from *Streptovercillum* DNA in the *BglII* site of pIJ702. *S. lividans* 1326 spheroplasts were transformed with this library and transformants were selected on R5 plates containing 25 µg Ts/ml. Transformants (approx. 15 500) were then replicated on MM plates containing 100 µg Bs/ml. A total of 16 Bs-resistant clones were obtained by this procedure.

^b DNA size of plasmid pIJ702 was taken from published data (Hopwood et al., 1985). Other plasmid (Fig. 1) sizes were deduced by gel electrophoresis of restricted DNA preparations using appropriate standards.

^c Mycelia from late log-phase *Streptovercillum* liquid cultures (100 ml) were collected by centrifugation and washed twice with TGE buffer. Cells (2 g/g wet weight) were broken with alumina at 4°C, resuspended in 1 vol. TGE buffer and centrifuged at 17 000 × g for 10 min. The resulting supernatant was centrifuged at 100 000 × g for 2 h. The supernatant (S100 fraction) was used as a source of BAC. BAC activity was determined by a spectrophotometric method in a reaction mixture (1 ml) containing 100 mM Tris · HCl pH 8.0/50 µM Bs/200 µM acetyl-CoA/40 µg 5,5'-dithiobis(2-nitrobenzoic acid)/20 µl S100 fraction per ml. Background was automatically subtracted from a parallel reaction lacking Bs. Reactions were incubated at 30°C and the increase in *A*_{412nm} served to estimate the amount of acetyl groups transferred to Bs by using an extinction coefficient of 13.6/µM (Shaw, 1975). One unit of BAC activity corresponded to 1 µmol of acetyl groups transferred to Bs/mg protein/min.

^d Determined by the ability of the cells to grow on MM agar plates supplemented with 1–1000 µg Bs/ml.

Fig. 1. Restriction maps of several plasmids and of the 1129-bp fragment containing *bls*. (A) Plasmids pBS1, pBS4, pBS6 and pBS14 were prepared as described in section a. Plasmid pBS1.3 was prepared by inserting the 1.1-kb *BclI-PstI* DNA fragment from pBS1 (indicated by the vertical dashed line) in the *BglII-PstI* replication fragment from pIJ702. The 1-kb mark refers to the size of the DNA inserts but not to the vector size. Arrows indicate the direction of transcription. B, *BamHI*; Bc, *BclI*; Bg, *BglII*; Bs, *BstEII*; M, *MboI*; N, *NcoI*; P, *PstI*; Pv, *PvuII*; S, *SalI*; Ss, *SstII*. (B) Sequencing strategy. The 1.1-kb *BclI-PstI* from pBS1.3 was inserted in the polylinker region of pUC19. From this construct, specific DNA fragments were subcloned in pUC19 (Yanisch-Perron et al., 1985) for sequencing by the dideoxynucleotide chain-termination method (Sanger et al., 1977) as modified by Hattori and Sakaki (1986). The arrows indicate the direction and length of sequence determined. Arrows starting by a closed circle indicate that the 'dideoxy' sequencing method was followed by the chemical destruction of the C residues by the methoxyamine plus Na · bisulfite method. This treatment was required to prevent compressions of the C residues (Ambartsumyan and Mazo, 1980). *E. coli* JM83 (Vieira and Messing, 1982), used for cloning experiments, was grown on liquid or solid LB medium supplemented with 40 µg XGal/ml and 100 µg Ap/ml (Miller, 1972) as required. *E. coli* was transformed as described by Hopwood et al. (1985).

and 3' to the common 2.2-kb *PvuII-PstI* fragment in pBS1, pBS4, pBS6 and pBS14 (Fig. 1A). This could be due to ligation of noncontiguous *MboI* *Streptovorticillum* chromosomal fragments into pIJ702 or to the presence of multiple *bls* genes in *Streptovorticillum*. Southern analysis showed that *bls* is present as a single copy in the *Streptovorticillum* genome (results not shown).

By successive subcloning experiments in pIJ702, the *bls* gene was located in a 1.1-kb *BclI-PstI* DNA fragment from pBS1 (plasmid pBS1.3; Fig. 1A and Table I).

(b) The *bls* gene sequence

The restriction map and sequencing strategy for a 1129-bp *BclI-PstI* DNA fragment containing *bls* are presented in Fig. 1B. The nt sequence is outlined in Fig. 2. A computer-aided search for ORFs, showed that only two ORFs, each starting by ATG (nt 148 and 226) and terminating by TGA (nt 556), could correspond to *bls*. The deduced aa sequences indicated polypeptides of 15073 (ORF1) and 12101 Da (ORF2). The molecular size of

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1  BamHI/BclI 60
  GGATCAGCGCGGCCCCCACCAGGACTGTGCATCAGCGTACGGCCGGGTACGACAACGG

61  AAGCGGATTGGCAAAACTGCCTGGCCCCGGTGTATGTTAGCTTTATGTTTCAGTATTG 120

121  AGCGGTGAACGACCCGGAACGACGCGAATGTTGCTTGGCAGGTTGCAGACCGTCAAC 180
      RBS      ThaI      HincII
      MetLeuSerLeuProArgLeuGlnThrValAsn

181  GACGAACGTTCCCGCCCTGCGGGCGTTGCGGCGCACGCCGGTGTAGGAGCGCGGCGG 240
      AspGluArgSerProAlaLeuArgAlaLeuArgArgThrProValMetGluAlaArgPro

241  CTGGAGGTGTACGCCACGTACGCTGCGGCGAGCGCGGGAGCTGGCGGGCGGGCTCGTC 300
      LeuGluValTyrAlaThrTyrAlaCysGlyGluArgGlyGluLeuAlaGlyGlyLeuVal

301  GGTATGTGCAGTGGCAATGGCTGCACGTGGACCTGCTGTGGGTGGACGCGGGGCCCGC 360
      GlyHisValGlnTrpGlnTrpLeuHisValAspLeuLeuTrpValAlaGlyAlaArg

361  GGGCGGGGCTGGGCTCGCGTTGATCGCGCGGGCGGAGGCCCGCGCCCGGAGGAGTTC 420
      GlyAlaGlyLeuGlySerArgLeuIleAlaArgAlaGluAlaArgAlaArgGluGluPhe

421  GGCTGCATCGGCAGCCAGGTGGAGACCTGGGACTTCCAGGCGCGGGGTTCTACGACGG 480
      GlyCysIleGlySerGlnValGluThrTrpAspPheGlnAlaProGlyPheTyrGlnArg

481  GTGGGGTATCGCTCGCGGCGAGCATCCGGACTATCCGCCCGGATCAGAGCCACCTG 540
      ValGlyTyrArgLeuAlaAlaSerIleProAspTyrProProGlyIleThrSerHisLeu

541  CTGGTGAAGGAGCTTTGAGGCGCCCGCTCAGGGCGCGGGGCCCTTACTCCGGGGCTCGG 600
      LeuValLysGluLeuEnd

601  CCCCAGACCCCGGCGGCGCGCTGACTGCGGGCCGGTGGGGCTTGTCGCGCAGTTCC 660
      SmaI      HincII

661  CGCGCCCTTACGGGGCGCTGGTCGCGCCACGCGCGGAGCGGCATCGAGCACAGC 720

721  CCGCGCCCTTACGGGGCGCTGCTCTAGGCCACCGCGCGTGGCCCTCCCGCGCGCGG 780

781  TGCCGAACAGTCGTGCGGTCCCAAGTGCCTCGGTGACACCTTGCTACCTTTCTCTCAT 840
      BstEII      BstEII

841  CTGCCCATCCACCAAGCGGATTGCCGAGCGCGCAAGCCGCGCCGTCATCCGGGCCC 900

901  CCAGGGCCCCCGCTTACCGCGCGTCTCCACCACCACGTCGAATTCGGCACAGGACACCC 960

961  GGAAGTCGTCGCCAGCGAGGCGTGCCCTCCGTCAGCAAGTGGCCACAGCCCTCGCAT 1020

1021  CCCCCGCGGCCAGCAGGGCCGCGACCCGCTCCACCCGTCGTTCTCGGTCACCACGTGAC 1080

1081  GGACCAAGCGCGCTCCGCGGACGGCAACTCACCAGTGGCCGCTGCAAG 1129
      PstI

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Fig. 2. The nt sequence of the 1129-bp *bls* fragment (Fig. 1B). The ATG potential start codons are underlined. A potential RBS is overlined. The small g at position 1 indicates that it is not from the *Streptovorticillum* DNA but from the *Bam*HI site of the pUC vector. The indicated *Tha*I site is not unique in the sequenced DNA; it is intended to mark a fragment used to prepare plasmid pBLS.TI (see section b). Facing arrows indicate inverted repeat sequences.

BAC was estimated to be approx. 15 kDa by SDS-PAGE and 13 kDa by gel filtration (Sugiyama et al., 1988). These findings suggest that ORF1 is the correct one. Nevertheless, to discriminate between these possibilities, two different gene constructs were prepared; the 478-bp *Tha*I(nt 145)-*Hinc*II(nt 622) fragment containing ORF1 and the 446-bp *Hinc*II(nt 177-622) fragment containing ORF2 (Fig. 2) were inserted in pUC19 downstream from the *lacZ'* promoter, resulting in plasmids pBLS.TI and pHs, respectively (results not shown). *E. coli* transformants containing either pBLS.TI (carrying ORF1) or pHs (carrying ORF2) were examined for Bs resistance. Whereas *E. coli*[pBLS.TI] grew on agar plates at higher than 100 µg Bs/ml, *E. coli*[pHs] did not grow at 25 µg Bs/ml. In addition, expression of BAC activity in *E. coli* JM103[pBLS.TI] was

stimulated by IPTG, indicating that transcription of the coding region of *bls* took place from the *lacZ'* promoter of pUC19 (not shown). These results demonstrate that ORF1 is the correct coding sequence for *bls*. This conclusion is in agreement with the presence of a putative RBS (GGAA) which is optimally located 8 bp 5' to the ATG start codon of ORF1. The %G + C in the first, second and third positions of ORF1 codons is 66.9, 48.1 and 84.9%, respectively, which is in accordance with the findings of Bibb et al. (1984) for *Streptomyces* genes. The codon usage for *bls* (not shown) is very similar to that found for other *Streptomyces* genes (for a review, see Seno and Baltz, 1989). The aa sequence deduced for BAC has no significant homology with the sequence of other antibiotic-modifying acetyltransferases (not shown). Several inverted repeats are

located 3' to the *bls* coding sequence. Of these, only the two strongest ones, nt 559–624 (–55 kcal/mol) and nt 660–743 (–65 kcal/mol), are presented (Fig. 2). These motifs have been implicated in Rho-independent transcription termination events (Seno and Baltz, 1989).

(c) Isolation of the *bls* promoter

A 185-bp *HincII* DNA fragment containing 175 bp of the upstream region of *bls* (Fig. 2) was isolated from a pUC19-derived plasmid carrying the 1.1-kb *BclI*-*PstI* DNA fragment and then inserted in the polylinker region of the promoter-probe plasmid pIJ486 (Ward et al., 1986). Plasmids pBLP1 and pBLP2 (not shown), differing in the orientation of the inserted DNA, were obtained from several *S. lividans* transformants able to grow in R5 agar plates containing 25 µg Ts/ml. These transformants had both APHII activity and Km resistance (Table II), which indicated that the 185-bp *HincII* fragment had promoter activity in both orientations. However, the promoter activity of pBLP1, in which transcription from the *bls* promoter coincided with transcription of the gene encoding APHII, was much higher than that of pBLP2. A similar result was previously found with the *aacC7* promoter inserted, in both orientations, in pIJ486 (López-Cabrera et al., 1989).

(d) Conclusions

(1) The gene *bls*, encoding a BAC from *Streptoverticillum* sp., contains a coding sequence of 408 nt that starts with an ATG codon and encodes a polypeptide of M_r 15073.

(2) The *bls* promoter has been isolated in a 175-bp fragment of the 5' region of the *bls* gene.

(3) Bs is known to be a strong inhibitor of plant, animal, yeast and mycelial fungi. Therefore, the *bls* gene deter-

mining Bs-resistance could be used to prepare useful cloning vectors for some or all of these organisms.

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TABLE II

Expression of the *aph* gene encoded in the recombinant plasmids

Cells ^a	Plasmid ^b	Km resistance (µg Km/ml) ^c	APHII activity (mu) ^d
<i>S. lividans</i> 1326	—	<2	0
<i>S. lividans</i> 486	pIJ486	<5	30
<i>S. lividans</i> BL1	pBLP1	>100	562
<i>S. lividans</i> BL2	pBLP2	15	53

^a See Table I, footnote a.

^b See section c.

^c Determined by the ability of the cells to grow on MM agar plates supplemented with 1–100 µg Km/ml.

^d Determined as described by Haas and Dowding (1975). One mu is equivalent to 1 pmol of phosphorylated Km/mg of protein/min. Protein estimations were performed as described by Bradford (1976).

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