

Streptomyces lividans Blasticidin S Deaminase and Its Application in Engineering a Blasticidin S-Producing Strain for Ease of Genetic Manipulation

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Blasticidin S is a peptidyl nucleoside antibiotic produced by *Streptomyces griseochromogenes* that exhibits strong fungicidal activity. To circumvent an effective DNA uptake barrier system in the native producer and investigate its biosynthesis *in vivo*, the blasticidin S biosynthetic gene cluster (*bls*) was engrafted to the chromosome of *Streptomyces lividans*. However, the resulting mutant, LL2, produced the inactive deaminohydroxyblasticidin S instead of blasticidin S. Subsequently, a blasticidin S deaminase (SLBSD, for *S. lividans* blasticidin S deaminase) was identified in *S. lividans* and shown to govern this *in vivo* conversion. Purified SLBSD was found to be capable of transforming blasticidin S to deaminohydroxyblasticidin S *in vitro*. It also catalyzed deamination of the cytosine moiety of cytosylglucuronic acid, an intermediate in blasticidin S biosynthesis. Disruption of the SLBSD gene in *S. lividans* LL2 led to successful production of active blasticidin S in the resultant mutant, *S. lividans* WJ2. To demonstrate the easy manipulation of the blasticidin S biosynthetic gene cluster, *blsE*, *blsF*, and *blsL*, encoding a predicted radical S-adenosylmethionine (SAM) protein, an unknown protein, and a guanidino methyltransferase, were individually inactivated to access their role in blasticidin S biosynthesis.

Blasticidin S (Fig. 1) belongs to the peptidyl nucleoside family of antibiotics. This compound, which is produced by *Streptomyces griseochromogenes*, was first identified in 1958 (1) and was the first antibiotic used to control rice blast in eastern Asia (2). The cytidine core of blasticidin S can bind tightly to the corresponding guanine base at the P site of the 50S subunit of the ribosome through a Watson-Crick base pair (3), which makes it a potent inhibitor of protein synthesis in both prokaryotic and eukaryotic cells (4, 5). There have been three kinds of blasticidin S resistance genes reported to date: *BSD* from *Aspergillus terreus* (6), *bsr* from *Bacillus cereus* (7), and a blasticidin S acetyltransferase gene from *Streptovorticillium* sp. (8). Both *BSD* and *bsr* encode blasticidin S deaminase (BSD), which can convert blasticidin S to the nontoxic deaminohydroxyblasticidin S, the base moiety of which is converted to uridine, thereby losing the capability of binding to the ribosome. On the other hand, the blasticidin S acetyltransferase gene encodes an acetyltransferase which, along with acetyl-coenzyme A, can block the inhibition of protein synthesis by blasticidin S in a cell-free system (8). Because of these traits, blasticidin S is widely used to select transformed cells that have been engineered to carry a resistance gene in biological research (9).

Puromycin (10), polyoxin (11), nikkomycin (12), and pacidamycin (13) are representative examples of the peptidyl nucleoside family, the biosynthetic pathways of which have been well studied (11, 14). Unlike the above-mentioned peptidyl nucleosides that typically have five-membered rings, the structure of blasticidin S and mildiomycin (Fig. 1) features a glucuronic acid-derived hexose that is coupled to cytosine or hydroxymethyl cytosine. The biosynthetic gene cluster of blasticidin S was first reported in 1998 and heterologously expressed in *Streptomyces lividans* (15). However, except for some putative intermediates, blasticidin S could not be identified from *S. lividans* heterologous production. Although the biosynthesis gene cluster for blasticidin S was se-

quenced and analyzed (16), there were no *in vivo* studies of the biosynthetic pathway due either to the difficult genetic manipulation system of *S. griseochromogenes* or to the failure of production of blasticidin S in *S. lividans*. Hence, the biosynthetic pathway of blasticidin S was proposed largely on the basis of labeled precursor incorporation, biochemical studies, and bioinformatic analysis (17–20), and several steps in the pathway still remain cryptic.

In this study, we identified a blasticidin S deaminase in *S. lividans* (SLBSD) that is able to transform the cytotoxic blasticidin S to its inactive form, deaminohydroxyblasticidin S. The activity of SLBSD was measured with several nucleosides *in vitro*. The blasticidin S biosynthetic gene cluster was then engrafted into a *S. lividans* strain with the SLBSD gene disrupted to generate the mutant WJ2 that produced blasticidin S. Furthermore, to specifically investigate the functions of *blsE*, *blsF*, and *blsL* in blasticidin S biosynthesis *in vivo*, these genes were individually disrupted, and the fermentation broths of the resultant mutants were analyzed by liquid chromatograph-mass spectrometry (LC-MS).

MATERIALS AND METHODS

Strains and culture conditions. Key strains and plasmids are listed in Table S1 in the supplemental material. *S. lividans* 66 and its derivatives

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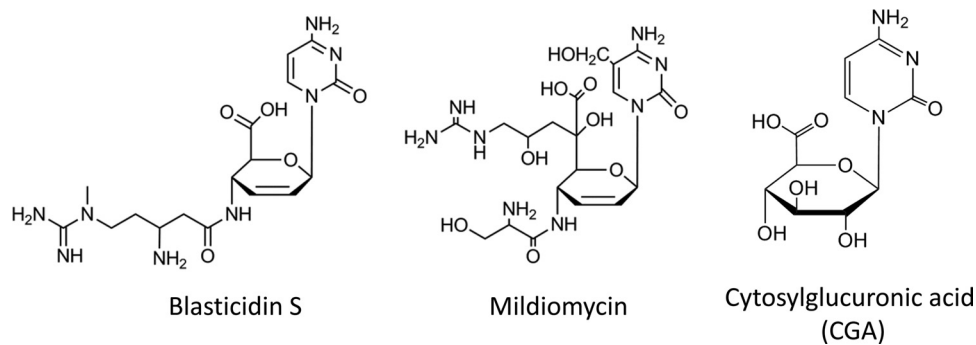


FIG 1 Chemical structures of blasticidin S, mildiomycin, and cytosylglucuronic acid.

were grown at 30°C on soy flour-mannitol (SFM) agar plates for sporulation or in 10.3% TSBY (tryptic soy broth [TSB] supplemented with 10.3% sucrose [wt/vol] and 1% yeast extract [wt/vol]) medium for growth of mycelia. Exconjugants were grown on SFM agar plates containing the appropriate antibiotic (150 µg/ml spectinomycin or 15 µg/ml thiostrepton) or in TSBY (10.3%) medium with the appropriate antibiotic (50 µg/ml spectinomycin or 5 µg/ml thiostrepton). For secondary metabolite analysis, seed cultures were prepared by inoculating a loop of stock culture into a 250-ml baffled flask containing 25 ml of seed medium [per liter, 20 g of glucose, 30 g of soybean flour, 5.0 g of yeast extract, 1.0 g of (NH₄)₂SO₄, 0.5 g of MgSO₄ · 7H₂O, 3.0 g of CaCO₃] and incubated in a rotary shaker for 40 h at 30°C and 200 rpm. Then, 5 ml of this seed culture was transferred into a 500-ml baffled flask containing 50 ml of fermentation medium [per liter, 80 g of glucose, 40 g of soybean flour, 1.0 g of (NH₄)₂SO₄, 0.5 g of MgSO₄ · 7H₂O, 10.0 g of CaCO₃, 0.05 g of FeSO₄, 0.4 g of K₂HPO₄] and incubated another 6 days under the same conditions. For LC-MS analysis of the production of blasticidin S and its intermediates, strains were cultured in TSBY (10.3%) medium in baffled flasks at 30°C and at 220 rpm for 6 days.

General molecular biology methods. Isolation of total DNA, protoplast preparation, and transformations were performed according to Kieser et al. (21). Manipulations of *Escherichia coli* strains were conducted according to Sambrook et al. (22). DNA fragments and PCR products were purified from agarose gels (0.8%) using a DNA Gel Extraction Kit (V-Gene Biotechnology Ltd.). The PCR product for SLBSD expression was inserted into pMD18-T vector (TaKaRa, Dalian, China) and verified by sequencing. For the Southern hybridization experiments, all the DNA samples were cleaved with BamHI, separated on an agarose gel (0.8%), and transferred onto Hybond-N⁺ nylon membranes (Amersham Biosciences). BamHI-digested fosmid 7D11 was labeled with radioactive [α -³²P]dCTP (Beijing Furui Bio-Engineering Co., Ltd.) using a Random Priming Kit (Roche) as the probe. The Southern blot hybridization was carried out overnight at 65°C, followed by a high-stringency wash (0.1 × SSC [0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] SDS) at the same temperature, and detected with a phosphorimager (Fujifilm). Restriction enzymes, T4 DNA ligase, *Taq* polymerase, and alkaline phosphatase were purchased from MBI Fermentas. Blasticidin S was purchased from Sigma-Aldrich. Cytosylglucuronic acid was purchased from Shanghai Violetpharm Chemical Technology Co., Ltd. CMP was purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Bioassay method. To measure the resistance level of *S. lividans* and its *bsd* knockout mutant to blasticidin S, a serially diluted blasticidin S standard was added to an Oxford cup placed on SFM medium plates that were preinoculated with spores of the *S. lividans* strains. For bioassays, the *Streptomyces* strains were grown at 30°C for 5 days on SFM plates for the production of secondary metabolite; agar patches were transferred to potato dextrose agar (PDA; 200 g of diced potatoes, 20 g of glucose, and 15 g of agar in 1 liter of tap water) that contained *Rhodotorula rubra* AS2.166 (see Table S1 in the supplemental material), a yeast that is sensitive to blasticidin S.

LC-MS analysis. The fermentation broth (25 ml) was harvested and adjusted to pH 5.0 and centrifuged at 12,000 × *g* for 5 min. The supernatant was applied to Supelclean LC-SCX solid-phase extraction (SPE) columns (bed weight, 500 mg; volume, 3 ml) (Supelco) and washed with 2 ml of water and then with 2 ml of 0.5% NH₄OH. The fraction eluting with 3% NH₄OH was filtered through a 2-µm-pore-size membrane before injection. The LC-MS analysis was done using an Agilent 1100 LC/MSD (mass-selective detector) with a C₁₈ column (TC-C₁₈; 250 mm by 4.6 mm) (Agilent). The isocratic mobile phase was 10 mM trichloroacetic acid-MeCN, 82:18 (vol/vol), and the flow rate was 0.3 ml/min at room temperature. Elution was monitored with a photodiode array detector at 272 nm, and electrospray ionization (ESI) MS analysis was carried out in the positive mode.

Q-TOF MS analysis. Quadrupole time of flight (Q-TOF) MS analysis was done using an Agilent 6530 Q-TOF LC-MS with a C₁₈ column (Zorbax StableBond SB-C18; 3.5-µm particle size; 2.1 mm by 150 mm) (Agilent). The isocratic mobile phase was MeCN-water with a gradient from 5% to 100%, and the flow rate was 0.2 ml/min at room temperature. The ion source was ESI, and the analysis was carried out in the positive mode.

Sequencing and bioinformatic analysis. The genomic library of *S. griseochromogenes* was constructed in the pCC1FOS vector according to the provided protocol (Epicentre Biotechnologies). DNA sequencing was done at Life Technologies Corporation, Shanghai, China. Open reading frames were predicted using FramePlot, version 3.0 beta (<http://watson.nih.gov/jp/~jun/cgi-bin/frameplot-3.0b.pl>), and multiple-sequence alignment was performed with BioEdit, version 7.0. Similarity comparisons of nucleotide or amino acid sequences against public databases were done using the BLAST program on the NCBI website (<http://ncbi.nlm.nih.gov/blast>) (23).

Construction of the recombinant strains. To integrate the entire blasticidin S gene cluster into the genome of *S. lividans*, fosmid 7D11, containing the published blasticidin S gene cluster as well as its downstream 8,780-bp sequence, was selected from the genomic library of the blasticidin S native producer *S. griseochromogenes*. Fosmid 7D11 was then cut by XbaI and SpeI and precipitated with 70% isopropanol and 10% 3 M sodium acetate. The purified digestion products were ligated at the XbaI-SpeI site of pJTU1289 (24) to construct pJTU1780 by rapid screening of the transformants, which contained the complete blasticidin S biosynthesis gene cluster. pJTU1780 was then digested with StuI and ligated with an *aadA* cassette amplified by the *aadA*-T primer set, with pIJ779 as the template to replace most of the insertion containing the blasticidin S gene cluster, generating construct pJTU1785. A 4.85-kb dispensable fragment covering the insertion site for the genomic island SLG (accession number EF210454) (34) in the *S. lividans* HXY16 chromosome was amplified with HXY16 forward and reverse primers and inserted at the EcoRV site of pOJ260 to construct pJTU1528. A 0.25-kb internal region of the 4.85-kb fragment was cut off by StuI, and its flanking 1.6-kb and 3.0-kb fragments were employed as two homologous arms for introduction of foreign DNA sequences. The insertion part of pJTU1785 was excised with EcoRV and ligated with the StuI-cut pJTU1528 to obtain pJTU1786.

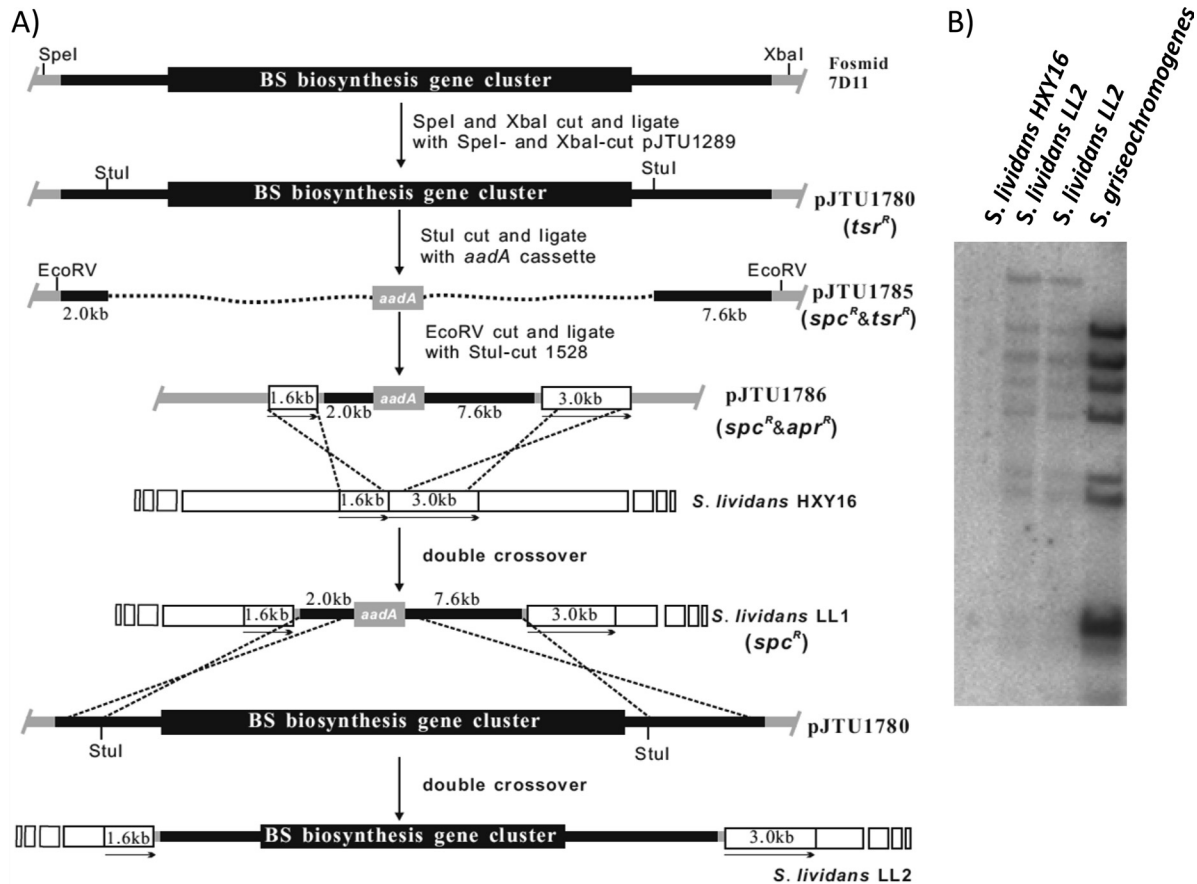


FIG 2 Construction of the *S. lividans*-derived strain LL2 that was engrafted with the blasticidin S biosynthetic gene cluster. (A) Schematic representation of the construction of *S. lividans* LL2. (B) Confirmation of the engraftment of the *bls* gene cluster to *S. lividans* HXY16 by Southern blotting. Genomic DNA preparations from *S. lividans* HXY16 (lane 1), two individual strains of *S. lividans* LL2 (lanes 2 and 3), and *S. griseochromogenes* (lane 4) were digested with BamHI, separated by agarose gel electrophoresis, and probed with BamHI-cut pJTU1780. *spc*, spectinomycin; *tsr*, thiostrepton; *apr*, apramycin.

To construct the *blsE*, *blsF*, and *blsL* knockout mutants, a 13-kb fragment covering *blsE*-*blsN* was excised from pJTU1780 using BglII and ligated with pJTU412 (25) to construct pWJ5. pWJ5 was then modified by module exchanges using ReDirect technology essentially as described previously with the primers Tar-*blsE*, Tar-*blsF*, and Tar-*blsL* (see Table S2 in the supplemental material) (26). The genes *blsE*, *blsF*, and *blsL* were deleted individually by recombinational exchange with an *aadA* cassette. The unmethylated recombinant plasmids, prepared in *E. coli* ET12567, were then separately introduced to *S. lividans* WJ2 by protoplast transformation. The resulting putative double crossover strains were confirmed by PCR with the primers Con-*blsE*, Con-*blsF*, and Con-*blsL* listed in Table S2 in the supplemental material.

Expression and purification of SLBSD. The SLBSD gene was amplified from the genomic DNA of *S. lividans* HXY16 by PCR with SLBSD forward and reverse primers (see Table S2 in the supplemental material; NdeI and EcoRI sites are underlined). PCR was carried out with high-fidelity DNA polymerase (KOD-Plus; Toyobo), and products were gel purified, digested with the appropriate enzymes, and cloned into the corresponding restriction sites of the pET28a(+) vector (Novagen). The resulting plasmid, named pWJ4, was used to transform *E. coli* DH10B for sequencing. For expression studies, the plasmid was introduced to *E. coli* BL21(DE3)/pLysE (Novagen) and grown overnight at 37°C in liquid LB medium containing chloramphenicol and kanamycin (34 µg/ml and 50 µg/ml, respectively). The seed cultures (10 ml) were used to inoculate 1-liter production cultures of LB medium with the corresponding antibiotics. The cells were grown at

37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced with isopropyl- β -D-thiogalactopyranoside (1 mM). The culture was then grown for an additional 5 h at 30°C. After centrifugation, the cells were resuspended in 40 ml of binding buffer (20 mM sodium phosphate, 20 mM imidazole, and 0.5 M NaCl, pH 7.4) and lysed by sonication in an ice bath (10 times for 60 s at 15 W with 60-s pauses). After another round of centrifugation (at 10,000 $\times g$ for 1 h), the supernatant was applied to a HisTrap HP column (GE Healthcare) and purified using AKTA fast protein liquid chromatography (FPLC) (GE Healthcare), by eluting with elution buffer (20 mM sodium phosphate, 500 mM imidazole, and 0.5 M NaCl, pH 7.4) in a linear gradient. The purified His-tagged SLBSD was desalted using a HiTrap desalting column (GE Healthcare) and stored in 10 mM Tris-HCl buffer (pH 8.0) with 20% glycerol at -80°C. The purified His-tagged SLBSD was analyzed by 15% SDS-PAGE, and protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad).

In vitro assay of the purified SLBSD. The assays of recombinant SLBSD were carried out at 30°C overnight in a total volume of 100 µl containing Tris-HCl buffer (10 mM, pH 8.0), blasticidin S-mildiomycin-cytosylglucuronic acid (at 0.5 mM, 0.5 mM, and 0.5 mM, respectively), and the corresponding His-tagged SLBSD (10 µM). The reactions were quenched by the addition of chloroform. The products and substrates were analyzed by LC-MS as described above.

Nucleotide sequence accession number. By chromosomal walking (details not shown), an 8,780-bp DNA fragment immediately down-

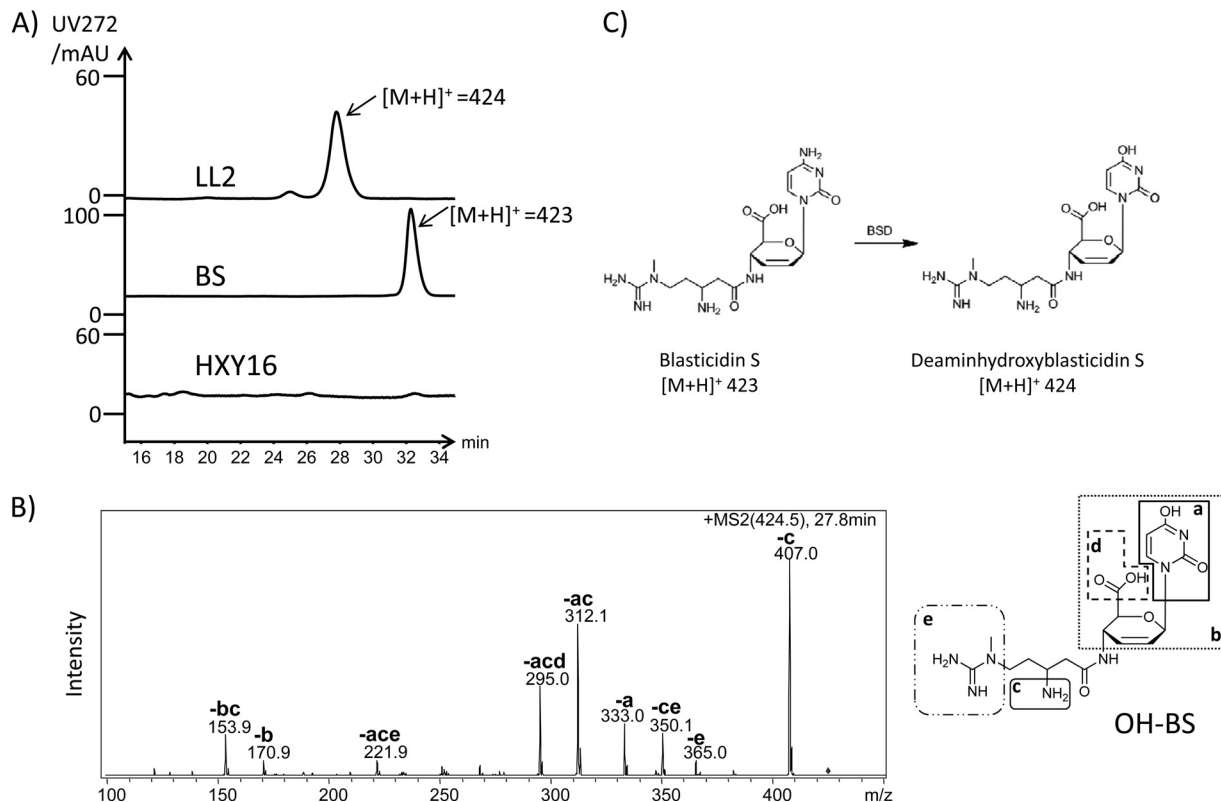


FIG 3 LC-MS analysis of the metabolite production of *S. lividans* LL2. (A) High-performance liquid chromatography comparison of the blasticidin S standard to the purified fermentation broth of *S. lividans* LL2 and *S. lividans* HXY16. LC-MS analysis revealed the peak presented in the fermentation broth of LL2, having a dominant peak at m/z 424. (B) MS/MS analysis of the m/z 424 parent ion. Each daughter peak (corresponding structures are shown in the inset) labeled could be derived from deaminohydroxyblasticidin S. (C) Blasticidin S was transformed to deaminohydroxyblasticidin S by BSD. BS, blasticidin S; AU, arbitrary units.

stream of *blsN* was cloned, sequenced, and deposited under accession number [JX244070](https://www.ncbi.nlm.nih.gov/nuclseq/JX244070).

RESULTS

Engraftment of blasticidin S biosynthetic gene cluster to *S. lividans*. The blasticidin S producer has a very strong barrier system against introduction of foreign DNA, which made prior genetic engineering of the blasticidin S gene cluster unsuccessful. To bypass this tough genetic manipulation system, attachment of the biosynthetic gene cluster onto the genome of *S. lividans* has been accomplished to generate a genetically stable heterologous host.

As depicted in [Fig. 2A](#), the complete blasticidin S gene cluster and flanking sequence were excised from 7D11 (see [Fig. S1A](#) in the supplemental material) and inserted into pJTU1289 to generate pJTU1780 (see [Table S1](#) and [Fig. S1A](#) and [B](#)). All internal *StuI* fragments of the insertion were removed and replaced with a blunt-ended *aadA* cassette to form pJTU1785 (see [Fig. S1C](#)), leaving 2.0-kb and 7.6-kb DNA fragments at the 5' end and 3' end of the insertion, respectively. A 4.85-kb dispensable DNA fragment from *S. lividans* HXY16 on pJTU1528 (see [Fig. S1D](#)) was separated into 1.6 kb and 3.0 kb by replacing its internal 0.25-kb *StuI* fragment with the 2.0 kb-*aadA*-7.6 kb(*EcoRV*) fragment from pJTU1785, generating pJTU1786 (see [Fig. S1E](#)). The 1.6-kb and 3.0-kb arms were then employed as the target sites for integration of the sandwiched 2.0 kb-*aadA*-7.6 kb fragment into the *S. lividans* HXY16 chromosome via double crossover to give mutant *S. lividans* LL1 (*aadA*). Finally, pJTU1780 was transferred to *S. lividans*

LL1 to replace the 2.0 kb-*aadA*-7.6 kb fragment with the blasticidin S biosynthetic gene cluster and the flanking sequences. Thus, *S. lividans* LL2, a mutant strain containing the blasticidin S gene cluster, was constructed and confirmed by Southern blotting ([Fig. 2B](#)).

When assayed for antifungal activity, *S. lividans* LL2 showed a very slight inhibitory effect versus the indicator strain *Rhodotorula rubra* (see [Fig. S2](#) in the supplemental material). To more accurately measure the blasticidin S production in *S. lividans* LL2, the fermentation broth was analyzed by LC-MS, but no peak corresponding to a blasticidin S standard was observed in the LC profile. However, a new compound was detected in the fermentation broth with a molecular size one mass unit greater than that of blasticidin S ($[M+H]^+$ of 424) ([Fig. 3A](#)). The tandem MS (MS/MS) analysis of this compound identified it as the nontoxic deaminohydroxyblasticidin S ([Fig. 3B](#)), which is the known product of blasticidin S deaminase (BSD) ([Fig. 3C](#)) (27). To confirm this result, this compound was further purified and analyzed by Q-TOF MS (see [Fig. S3](#) in the supplemental material).

Identification of a BSD gene in *S. lividans* HXY16. The existence of deaminohydroxyblasticidin S in the fermentation broth of LL2 prompted us to search for a homolog of the *Aspergillus terreus* BSD in the genome of *S. lividans*. Hence, we identified a cytidine deaminase (accession number [EFD66414](https://www.ncbi.nlm.nih.gov/nuclseq/EFD66414)) in *S. lividans* TK24 that showed 51% identity and 67% similarity to the *A. terreus* BSD. The *S. lividans* HXY16 strain was also found to contain

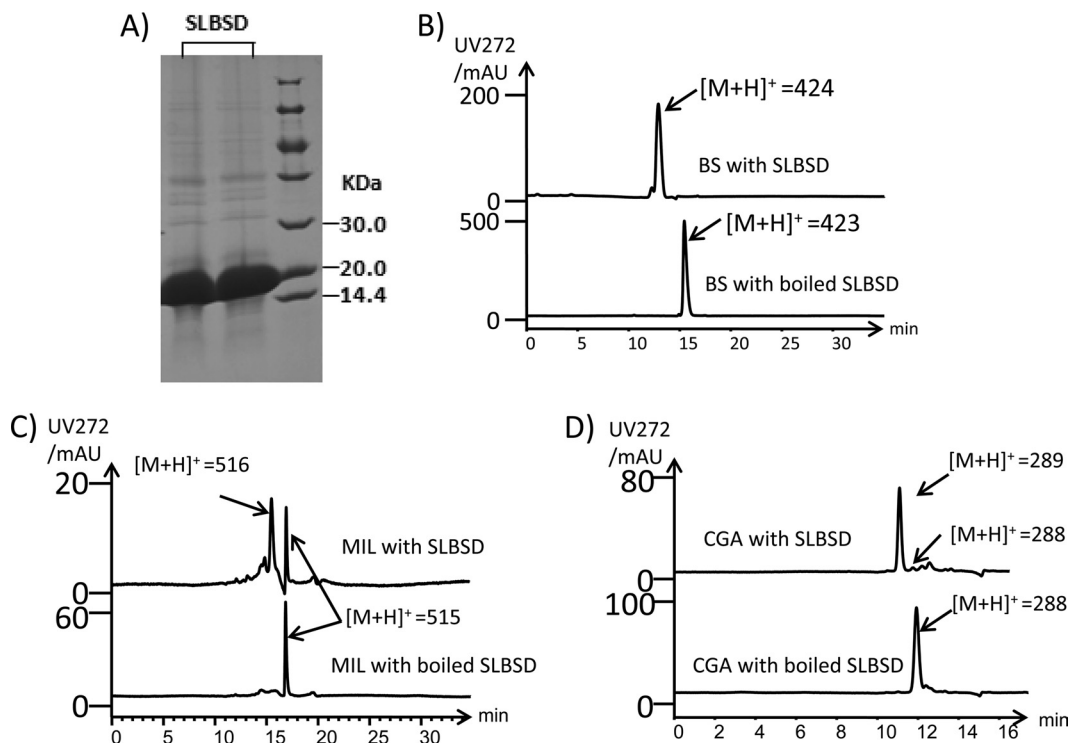


FIG 4 *In vitro* and *in vivo* analysis of SLBSD. (A) SDS-PAGE analysis of purified SLBSD. First two lanes, purified SLBSD; right lane, protein marker. The calculated molecular mass of His-tagged SLBSD was 16.9 kDa. (B) LC-MS analysis of products after overnight incubation with SLBSD and boiled SLBSD, with blastidicin S as the substrate. (C) LC-MS analysis of products after overnight incubation with SLBSD and boiled SLBSD, with mildiomycin as the substrate. (D) LC-MS analysis of products after overnight incubation with SLBSD and boiled SLBSD, with cytosylglucuronic acid as the substrate.

a BSD homolog, named SLBSD, that is almost identical to the TK24 enzyme except for a single mutation (E112D) (see Fig. S4 in the supplemental material).

To provide a means to measure the ability of SLBSD to convert blastidicin S to deaminohydroxyblastidicin S, the SLBSD gene was heterologously expressed in *E. coli*. A purified soluble protein 16.9 kDa in size, consistent with the calculated molecular size of the His-tagged SLBSD, was obtained (Fig. 4A). LC-MS analysis of an overnight reaction incubating SLBSD with blastidicin S showed that blastidicin S ($[M+H]^+$ of 423) was completely transformed to deaminohydroxyblastidicin S ($[M+H]^+$ of 424) (Fig. 4B). Moreover, the substrate specificity of SLBSD was surveyed by examining its activity on mildiomycin, a nucleoside with similar structure to blastidicin S, cytosylglucuronic acid, an intermediate in blastidicin S biosynthesis, and CMP. The deaminase activity was observed with mildiomycin and cytosylglucuronic acid as substrates, but conversion was comparatively low (Fig. 4C and D). However, SLBSD did not catalyze the deamination of CMP (data not shown), suggesting that the hexose moiety may be an essential component for recognition by SLBSD.

Inactivation of the SLBSD gene in *S. lividans* LL2 leads to the production of blastidicin S. To stop the conversion of blastidicin S to deaminohydroxyblastidicin S by the SLBSD gene in the blastidicin S-producing *S. lividans* LL2, the SLBSD gene was disrupted via double crossover. The resulting strain was termed *S. lividans* WJ2, and the correct genotype was confirmed by PCR (Fig. 5A). LC-MS analysis of the purified fermentation broth of mutant WJ2 grown in production medium revealed a peak with same retention

time as the blastidicin S standard. MS analysis of this peak further confirmed the production of blastidicin S in the mutant (Fig. 5B).

To compare the effect of the disruption of the SLBSD gene in *S. lividans* strains with different genotypes, the SLBSD gene was also inactivated in *S. lividans* HXY16 via double crossover to generate mutant *S. lividans* WJ1 (see Fig. S5A in the supplemental material). WJ1, *S. lividans* HXY16, and WJ2 were then assayed for their resistance levels against blastidicin S at different concentrations (20 μg to 200 $\mu\text{g}/\text{ml}$). The growth of HXY16 was not greatly inhibited by blastidicin S at a concentration of 200 $\mu\text{g}/\text{ml}$, while the SLBSD mutant WJ1 was completely inhibited by blastidicin S at a concentration of 100 $\mu\text{g}/\text{ml}$, as indicated by a clear zone on an agar test plate. Moreover, WJ2, containing the blastidicin S self-resistance gene *blsJ*, was also not affected by blastidicin S (see Fig. S5B). This observation suggested that the heterologous production of blastidicin S in WJ2 should not significantly affect growth of the host strain.

We previously found that the mildiomycin producer, *Streptomyces rimofaciens* ZJU5119, could produce three additional mildiomycin derivatives in the seed/fermentation broth (28). To check the secondary metabolite production by *S. lividans* WJ2, the strain was grown in the standard seed/fermentation medium, and analysis revealed a new peak exhibiting a dominant ion at $[M+H]^+$ of 409 in the LC profile of the fermentation broth (Fig. 6A). This product was identified as demethylblastidicin S by MS/MS analysis (Fig. 6B) and Q-TOF MS (see Fig. S6 in the supplemental material). However, only blastidicin S was detected

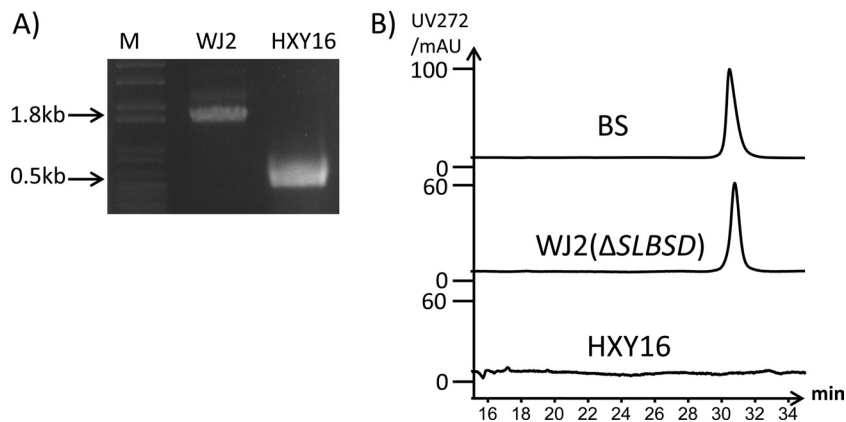


FIG 5 High-performance liquid chromatography analysis of the metabolite production of *S. lividans* WJ2. (A) Confirmation of the genotype of the SLBSD mutant WJ2 by PCR with primer Con-BKO (see Table S2 in the supplemental material). M, DNA marker; HXY16, PCR product targeting the SLBSD gene from *S. lividans* HXY16; WJ2, PCR product targeting the SLBSD gene from *S. lividans* WJ2. (B) High-performance liquid chromatography comparison of the blasticidin S standard to the purified fermentation broth of *S. lividans* WJ2 and *S. lividans* HXY16.

when *S. griseochromogenes* was grown in the same seed/fermentation medium (data not shown).

Generation of *blsE*, *blsF*, and *blsL* disruption mutants. To demonstrate the ability to manipulate the blasticidin S gene clus-

ter in a heterologous host, three genes, *blsE*, *blsF*, and *blsL*, were disrupted individually, and the fermentation broths from the resulting mutant strains were analyzed by LC-MS.

Because demethylblasticidin S accumulated in the fermentation

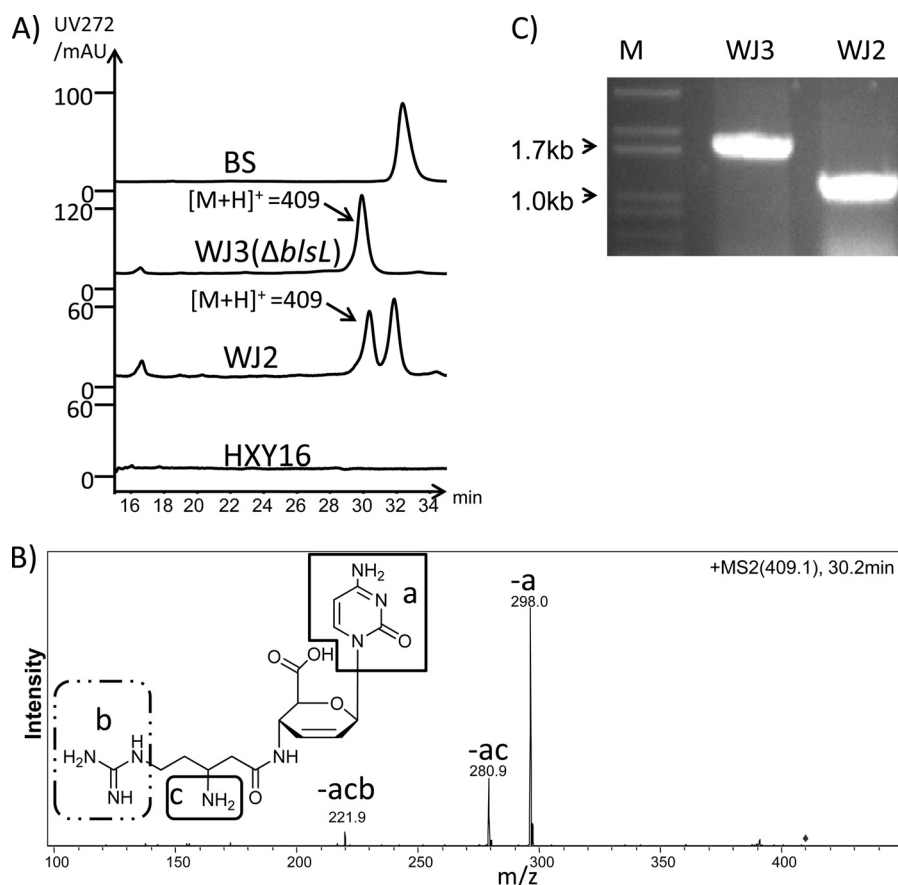


FIG 6 Assay of the blasticidin S production of the $\Delta blsL$ mutant strain *S. lividans* WJ3. (A) High-performance liquid chromatography comparison of the blasticidin S standard to the purified fermentation broth of WJ3, WJ2, and HXY16. LC-MS analysis revealed the peak presented in the fermentation broth of WJ2 and WJ3, with a dominant peak at m/z 409. (B) MS/MS analysis of the m/z 409 parent ion. Each labeled daughter peak (corresponding structures are shown in the inset) could be derived from demethylblasticidin S. (C) PCR confirmation of the genotype of the $\Delta blsL$ mutant WJ3 with primer Con-blsl (see Table S2 in the supplemental material). M, DNA marker; WJ2, PCR product targeting *blsL* from *S. lividans* WJ2; WJ3, PCR product targeting *blsL* from *S. lividans* WJ3.

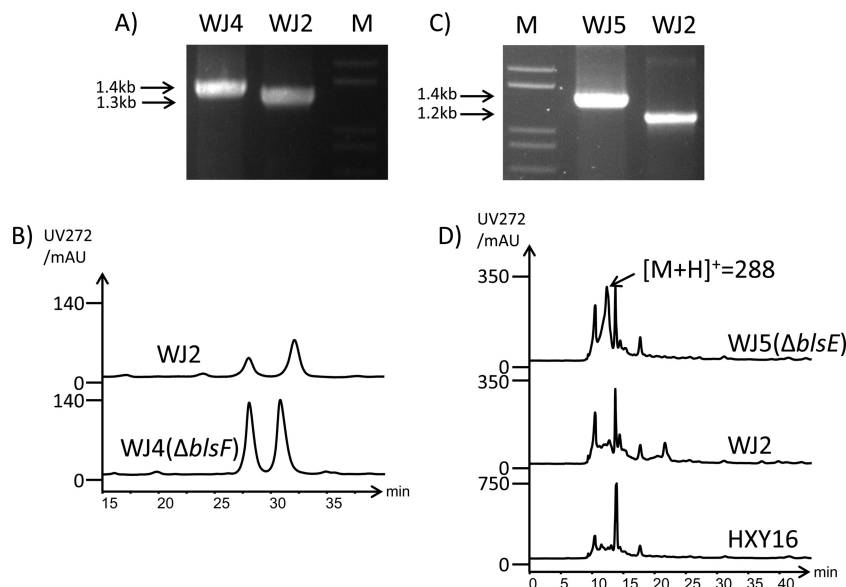


FIG 7 Assay of the blasticidin S production of the $\Delta blsF$ and $\Delta blsE$ mutant strains, *S. lividans* WJ4 and *S. lividans* WJ5, respectively. (A) Confirmation of the $\Delta blsF$ mutant WJ4 by PCR with primer Con-*blsF* (see Table S2 in the supplemental material). M, DNA marker; WJ2, PCR product targeting *blsF* from *S. lividans* WJ2; WJ4, PCR product targeting *blsF* from *S. lividans* WJ4. (B) High-performance liquid chromatography comparison of the purified fermentation broth of WJ4 to that of WJ2. (C) Confirmation of the $\Delta blsE$ mutant WJ5 by PCR with primer Con-*blsE* (see Table S2). M, DNA marker; WJ2, PCR product targeting *blsE* from *S. lividans* WJ2; WJ5, PCR product targeting *blsE* from *S. lividans* WJ5. (D) High-performance liquid chromatography comparison of the untreated fermentation broth of WJ5 to that of WJ2 and HXY16.

broth of WJ2 and was considered an intermediate in the blasticidin S biosynthetic pathway (15), the formation of demethylblasticidin S in WJ2 was investigated. An *N*-methyltransferase is the predicted product of *blsL*; thus, this gene was selectively inactivated, and the genotype of the resulting mutant WJ3 was confirmed by PCR (Fig. 6C).

There remain several steps in the biosynthetic pathway to blasticidin S that are unclear. To narrow down the genes involved in blasticidin S assembly, *blsF* a gene homologous to *milL* in the mildiomycin gene cluster, which is predicted to encode a protein of unknown function (29), was disrupted. Disruption of *blsF* (Fig. 7A) did not abolish the production of blasticidin S. On the contrary, the production of blasticidin S was increased in the mutant, *S. lividans* WJ4 (Fig. 7B). Moreover, in the crude extract of the fermentation broth of WJ4, a known intermediate of the blasticidin S biosynthetic pathway, cytosylglucuronic acid, accumulated (see Fig. S7 in the supplemental material).

The accumulation of cytosylglucuronic acid in the WJ4 mutant implied that the enzyme acting on this intermediate was very important in increasing the production of blasticidin S. BLAST analysis showed that *BlS*E belonged to the radical *S*-adenosylmethionine (SAM) superfamily, members of which are capable of catalyzing multiple reaction types, such as decarboxylation, methylation, and dehydration (30). In our earlier work, the disruption of *milG*, a homologue to *blsE* in the mildiomycin biosynthetic pathway, led to the accumulation of hydroxymethyl cytosylglucuronic acid (29). To analyze the function of *blsE* in blasticidin S formation, the gene was inactivated, and the resulting mutant, *S. lividans* WJ5, was confirmed by PCR (Fig. 7C). As expected, production of blasticidin S was abolished in WJ5 (see Fig. S8 in the supplemental material), and the accumulation of cytosylglucuronic acid was observed in the fermentation broth of this mutant (Fig. 7D).

DISCUSSION

As introduction of alien DNA into the chromosome of the blasticidin S native producer *S. griseochromogenes* was never successful, *in vivo* functional studies of the role of each gene involved in blasticidin S biosynthesis was hindered. To bypass the strong DNA uptake barrier system of *S. griseochromogenes*, we set forth to engineer an *S. lividans*-derived blasticidin S-producing host that was genetically stable and manipulable. However, the resulting *S. lividans* LL2 mutant that carried the intact blasticidin S gene cluster was found to produce only a blasticidin S derivative, deaminohydroxyblasticidin S; this observation led to the discovery of a blasticidin S deaminase in *S. lividans*.

BSD was first discovered in *Aspergillus terreus* in 1975 (31). It was shown to be a zinc-dependent enzyme (27), and the blasticidin S-bound crystal structure of BSD revealed that the position of the zinc ion was very close to C-2 of the cytosine base of blasticidin S (32). This is the site occupied by a hydroxymethyl group in mildiomycin and may thus hinder its coordination to the active zinc ion center. This assumption may in part explain the low *in vitro* transformation efficiency (Fig. 4C) of mildiomycin to deaminohydroxymildiomycin by SLBSD. Consistent with this assumption, mildiomycin was successfully produced in *S. lividans* (29), whereas all of the blasticidin S was converted into deaminohydroxyblasticidin S. As for cytosylglucuronic acid, another substrate for SLBSD, this compound was nearly completely transformed into deaminohydroxycytosylglucuronic acid. The detection of cytosylglucuronic acid in the fermentation broth of the earlier heterologous expression experiments (15) might be the result of the high yield of this compound. Finally, the finding that CMP was not recognized by SLBSD might be due to the lack of a carboxyl group on its sugar ring or the negative charge carried by

the phosphate group, both of which were essential for the recognition of blasticidin S to BSD as proposed by Kumasaka et al. (32).

The disruption of the SLBSD gene led to the successful heterologous expression of blasticidin S for the first time. This engineered blasticidin S-producing strain provided the necessary means to investigate the blasticidin S biosynthesis pathway *in vivo*. The disruption of *blsF*, a homologue of *milL* that had been confirmed as a gene unnecessary for mildiomycin biosynthesis (29), raised the yields of blasticidin S and other intermediates instead of abolishing their production. This result suggested that *blsF* might have a negative effect on blasticidin S biosynthesis. Moreover, accumulation of cytosylglucuronic acid and demethylblasticidin S in WJ4 suggested that the production of the cytotoxic blasticidin S repressed or slowed down the metabolic flow toward the final product. However, in the native producer, the coevolution of blasticidin S and a self-resistant mechanism can efficiently counter the cytotoxic activity. A good case for this is that there exist two chromosomal fragments containing putative resistance genes to blasticidin S (15). One fragment contains a transporter-encoding gene, *blsJ* (16), while the other resistant element is located outside the blasticidin S biosynthetic gene cluster.

The accumulation of cytosylglucuronic acid in the WJ4 mutant may also result from the inefficient expression, or the deficient performance, of some gene(s) or gene products in the heterologous host. Identification of the proteins responsible for directly processing cytosylglucuronic acid to advanced intermediates is therefore very important for fully utilizing the substrates and increasing the yield of blasticidin S in a heterologous expression host. In mildiomycin biosynthesis, disruption of *milG*, a *blsE* homologue, led to the accumulation of hydroxymethyl cytosylglucuronic acid (29). This result provided a hint that *blsE* was a key gene for the accumulation of cytosylglucuronic acid. As expected, disruption of *blsE* abolished the production of blasticidin S while the accumulation of cytosylglucuronic acid remained.

The wild-type *S. griseochromogenes* can produce demethylblasticidin S when inoculated in yeast extract-malt extract (YEME) medium (15), and demethylblasticidin S can be transformed to blasticidin S by a cell extract of this strain (33). It is very likely that BlsL alone is in charge of this transformation. In a previous report, the nontoxic leucyl blasticidin S was detected in the heterologous expression strain and a route involving leucyl demethylblasticidin S and leucyl blasticidin S as precursors to the blasticidin S pathway was proposed (16). However, in present study, leucyl blasticidin S was not regularly detected in the fermentation broth. A blasticidin S acetyltransferase was discovered in *Streptovercillium* sp. in 1990 (8), and a homolog exists in many *Streptomyces* strains including *S. lividans* (accession number ZP_06530511). The formation of leucyl blasticidin S in the original study thus may have resulted from a host resistance or detoxification mechanism rather than from an enzyme in the blasticidin S biosynthetic pathway. Furthermore, the inactivation of *blsL* led to the production solely of demethylblasticidin S, which suggests that the direct conversion of demethylblasticidin S to blasticidin S is more likely.

In conclusion, blasticidin S is a very important nucleoside antibiotic both in agriculture and in laboratory research. The successful heterologous expression of this antibiotic in *S. lividans* HXY16 bypassed the difficult genetic manipulation of its native producer and led to the discovery of a blasticidin S deaminase from the *S. lividans* HXY16. Moreover, the easy genetic manipulation of *S. lividans* unveiled the function of

several genes in the biosynthetic gene cluster. This is very important for the demonstration of the whole biosynthetic pathway of blasticidin S and for increasing its production in both native and heterologous hosts.

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