

# A Branch Point of *Streptomyces* Sulfur Amino Acid Metabolism Controls the Production of Albomycin

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**Albomycin (ABM), also known as grisein, is a sulfur-containing metabolite produced by *Streptomyces griseus* ATCC 700974. Genes predicted to be involved in the biosynthesis of ABM and ABM-like molecules are found in the genomes of other actinomycetes. ABM has potent antibacterial activity, and as a result, many attempts have been made to develop ABM into a drug since the last century. Although the productivity of *S. griseus* can be increased with random mutagenesis methods, understanding of *Streptomyces* sulfur amino acid (SAA) metabolism, which supplies a precursor for ABM biosynthesis, could lead to improved and stable production. We previously characterized the gene cluster (*abm*) in the genome-sequenced *S. griseus* strain and proposed that the sulfur atom of ABM is derived from either cysteine (Cys) or homocysteine (Hcy). The gene product, AbmD, appears to be an important link between primary and secondary sulfur metabolic pathways. Here, we show that propargylglycine or iron supplementation in growth media increased ABM production by significantly changing the relative concentrations of intracellular Cys and Hcy. An SAA metabolic network of *S. griseus* was constructed. Pathways toward increasing Hcy were shown to positively impact ABM production. The *abmD* gene and five genes that increased the Hcy/Cys ratio were assembled downstream of *hrdBp* promoter sequences and integrated into the chromosome for overexpression. The ABM titer of one engineered strain, SCAK3, in a chemically defined medium was consistently improved to levels ~400% of the wild type. Finally, we analyzed the production and growth of SCAK3 in shake flasks for further process development.**

The *Streptomyces* genus was established at the beginning of the golden age of antibiotic discovery (1). *Streptomyces griseus*, a representative organism of the genus (2), was shown during this time to produce streptomycin (3), which has been clinically used to treat bacterial infections and other human diseases. It is well known that *Streptomyces* organisms in general have a large biosynthetic potential, being capable of producing many bioactive secondary metabolites—the *S. griseus* strains producing albomycin (ABM) are no exception. ABM was named by former Soviet Union scientists and underwent clinical investigations well before the information was released to the English-speaking scientific world (4). The biological activity and chemical constitution of ABM were later reported to be nearly identical to those of grisein, which was isolated from a distinct subtype of *S. griseus* by U.S. scientists in the 1940s (5–7). Despite its remarkable properties, continued studies of ABM were not pursued, partly because of the poor yields (8) and unpublicized research on the microorganism (9, 10). At present, the wealth of *Streptomyces* genomic information that is publically available provides the possibility of using a systems biology approach to uncover new aspects of *Streptomyces* metabolism and potentially overcome the production bottleneck.

ABM and ABM-like secondary metabolites are structurally categorized as peptidyl nucleoside antibiotics (Fig. 1). The N terminus of the peptide is a ferrichrome siderophore moiety in ABM; thus, many human-pathogenic bacteria (e.g., pneumococci and enterics) actively take up ABM through an iron transport system as a mechanism to acquire iron from the environment (11). Once inside the pathogen, a serine-containing nucleosyl dipeptide, termed SB-217452 (12), at the C terminus of ABM is enzymatically released through hydrolysis. SB-217452 has been proposed to mimic seryl-AMP and therefore function as an antibiotic by inhibiting seryl-tRNA synthetase (SerRS), an essential enzyme involved in protein synthesis. The nucleoside component of

SB-217452 consists of a highly unusual cyclic thioether group (—C—S—C—) that, along with several other features, including the C-3' stereochemical configuration and additional base modifications, differentiates it from the canonical nucleosides. It was reported that the sulfur atom of ABM cannot be replaced with an oxygen; a chemically synthesized compound with the oxygen substitution completely lost biological activity (13). Studies aimed at defining ABM biosynthesis, such as the assembly of the empowered siderophore and the intriguing mechanism for the formation of this rare thiosugar-containing amino acid, including the identity of the precursor metabolite(s) in the ABM-producing *S. griseus*, could help alleviate the bottlenecks to bringing an ABM-inspired antibiotic to the clinic.

Bacterial sulfur amino acid (SAA) metabolism is highly adaptive and diversified (14). Biosynthesis of cysteine (Cys) and methionine (Met) via homocysteine (Hcy) are linked through bidirectional transsulfuration pathways. Each transsulfuration direction involves two sequential reactions that are catalyzed by different enzymes that often have relaxed specificities (Fig. 1). Interconversion between Hcy and Met occurs in the activated

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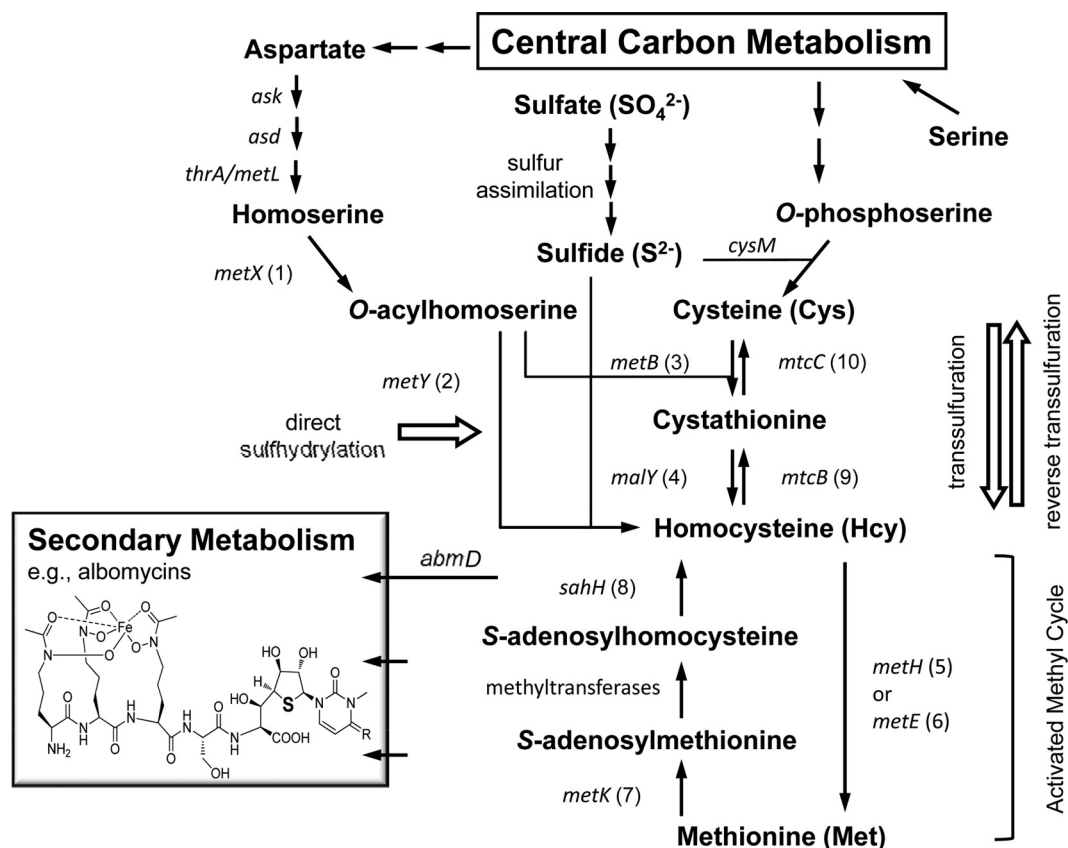
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**FIG 1** Constructed sulfur amino acid metabolism and the linked metabolic network in *Streptomyces griseus*. Corresponding to the conventional name shown, the identified *S. griseus* ORF and its product are as follows: (1) *sgr6646*, homoserine *O*-acetyltransferase; (2) *sgr6647*, *O*-acetylhomoserine sulfhydrylase; (3) *sgr2579*, cystathionine- $\gamma$ -synthase (CGS); (4) *sgr3417*, type II cystathionine- $\beta$ -lyase (CBL); (5) *sgr5847*,  $\text{B}_{12}$ -dependent methionine synthase; (6) *sgr1212*,  $\text{B}_{12}$ -independent methionine synthase; (7) *sgr6058*, *S*-adenosylmethionine (SAM) synthetase; (8) *sgr4513*, *S*-adenosylhomocysteine (SAH) hydrolase; (9) *sgr4452*, cystathionine- $\beta$ -synthase (CBS); and (10) *sgr3660*, cystathionine- $\gamma$ -lyase (CGL). The *abmD* gene product is proposed to be a critical link between sulfur amino acid metabolism and ABM biosynthesis.

methyl cycle (AMC)—the so-called Met salvage pathway—which involves ATP and additional cofactors, such as cobalamin (vitamin  $\text{B}_{12}$ ) or 5-methyl-tetrahydrofolate. The intrinsic function and metabolic branching of AMC have been shown to lead to the production of some bacterial metabolites (e.g., quorum-sensing molecules, etc.) that can have a profound impact on the bacterial physiology and surrounding microbial community. SAA metabolism is also connected with aspartate metabolism via homoserine. However, most *Streptomyces* spp. and other actinobacteria do not encode a MetA enzyme, which acylates the hydroxyl of homoserine with succinyl coenzyme A (succinyl-CoA). Instead, a direct sulfhydrylation pathway comprising *metX* and *metY* is found in some actinomycetes (Fig. 1) (15, 16). Furthermore, *Streptomyces coelicolor* apparently lacks not only *metA* but also *metXY* (17); this aspect of Met synthesis in what is often considered the model *Streptomyces* organism remains unsolved. Most actinobacterial genomes also do not appear to have a canonical *metC* that codes for a cystathionine- $\beta$ -lyase (14); it has been suggested that the *maly* gene product may perform this function (18). The genetically amenable ABM-producing species *S. griseus* is proposed as an excellent system for investigation into *Streptomyces* SAA metabolism and the interplay of primary and specialized metabolism, since the results may have a direct impact on industrial production of the ABM antibiotic.

We initiated research on ABM biosynthesis with one aim: to improve ABM production in a native producer, *S. griseus* ATCC 700974 (19–21). A draft genome sequence of *S. griseus* was obtained, and the ABM biosynthetic gene cluster (*abm*) was identified. Based on previous reports (22, 23), the purification and analytical determination of ABM were established, and a robust genetic system within *S. griseus* was developed to link the genetic information with ABM production. In collaboration with others, we also developed an *in vitro* enzyme assay for SerRS to show that SB-217452 cleaved from purified ABM is indeed a potent inhibitor of the enzyme activity. Interestingly, the *abm* gene cluster lacks an obvious pathway-specific regulatory gene, suggesting that ABM production might be controlled by the availability of a sulfur-containing primary metabolite. The protein encoded by *abmD* was identified as the best candidate to catalyze the first committed step in the biosynthesis of the nucleoside component of ABM using one of the known sulfur-containing primary metabolites. Here, we present experimental evidence to support this hypothesis, obtained by manipulating the newly identified genes of *Streptomyces* SAA metabolism, measuring the intracellular Hcy/Cys ratio, and performing fermentation optimization studies. The level of ABM production by *S. griseus* was increased 4-fold, and cell level analysis indicated that Hcy is directly involved in ABM biosynthesis.

TABLE 1 Bacterial strains and plasmids used or constructed in this study

Strain or plasmid	Description <sup>a</sup>	Source or reference <sup>b</sup>
<b>Strains</b>		
<i>E. coli</i>		
JM109	Strain used as a host for cloning and bioassay tester organism	ATCC
ET12567	Strain harboring pUZ8002 plasmid; used for conjugating plasmid into <i>Streptomyces</i>	24
<i>S. griseus</i>		
ATCC 700974	Wild type	ATCC
SCAK1	WT-derived strain containing pSET and <i>hrdBp-metYXSO</i> at <i>attB</i>	This study
SCAK2	WT-derived strain containing pSET and <i>hrdBp-abmD</i> at <i>attB</i>	This study
SCAK3	WT-derived strain containing pSET and <i>hrdBp-metYXSO-hrdBp-abmD-hrdBp-malY-metB</i> at <i>attB</i>	This study
SCAK4	WT-derived strain containing pSET and <i>hrdBp-ask-asd</i> at <i>attB</i>	This study
SCAK5	WT-derived strain containing pSET and <i>hrdBp-metYXSO-hrdBp-ask-asd</i> at <i>attB</i>	This study
<i>S. albulus</i>		
NBRC 14147	Genome used to amplify the genes for feedback-insensitive Ask and Asd	NBRC
<b>Plasmids</b>		
pDrive	Used for subcloning (Kan <sup>r</sup> Amp <sup>r</sup> )	Qiagen
pSET(GUS)	Used for subcloning (Apr <sup>r</sup> )	25
pSE34-oriT	Multicopy plasmid in <i>Streptomyces</i> (Ts <sup>r</sup> )	19
pA10	pSE34 derivative control	19
pCK1	pSE34 derivative for <i>ermE*</i> p expression of <i>metYXSO</i>	This study
pCK2	pSE34 derivative for <i>ermE*</i> p expression of <i>malY-metB</i>	This study
pCK3	pSE34 derivative for <i>ermE*</i> p expression of <i>mtcC-mtcB</i>	This study
pCK4	pSE34 derivative for <i>ermE*</i> p expression of <i>sahH</i>	This study
pCK5	pSE34 derivative for <i>ermE*</i> p expression of <i>metK-sahH</i>	This study
pCK6	pSE34 derivative for <i>ermE*</i> p expression of <i>metE</i>	This study
pCK7	pSE34 derivative for <i>ermE*</i> p expression of <i>metK-sahH-metE</i>	This study

<sup>a</sup> Ts<sup>r</sup>, thiostrepton resistant; *ermE\**p, mutated *ermE* promoter.

<sup>b</sup> ATCC, American Type Culture Collection; NBRC, Japanese NITE Biological Resource Center.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, reagents, and growth media.** The bacterial strains and plasmids used are listed in Table 1. Primers for PCR amplification are in Table 2. Chemicals were purchased from standard commercial sources. General *Streptomyces* microbiological procedures were followed (24). The original ABM production broth (APB) consisted of the following (23): 20 g of starch, 5 g of L-ornithine HCl, 1.8 g of KH<sub>2</sub>PO<sub>4</sub>, 10.2 g of Na<sub>2</sub>HPO<sub>4</sub>, 2 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g of NaCl, 2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.8 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.28 g of FeSO<sub>4</sub>·7H<sub>2</sub>O, and 0.02 g of ZnSO<sub>4</sub>·7H<sub>2</sub>O per liter of distilled water. To simplify ABM purification here, APB was modified to contain 20 g of glycerol as the sole carbon source, and ornithine and starch were omitted. *Streptomyces* spores and mycelia were routinely maintained on mannitol-soya flour (MS) agar plates. *Streptomyces* seed culture was in tryptic soy broth (TSB). *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or agar.

**Standard DNA manipulation and *Streptomyces* genetic techniques.** Plasmids and DNA fragments were purified according to the provided protocol (Qiagen). Sequence analysis for cloning was performed with CLC Genomics Workbench 4.0. All restriction enzymes, DNA ligase, and other molecular biology reagents were from New England BioLabs (NEB) and used by following the provided instructions. PCR was performed with Q5 high-fidelity DNA polymerase from NEB. The annealing temperature was calculated based on the supplier's information (Integrated DNA Technologies). DNA was introduced into *S. griseus* ATCC 700974 by conjugation using *E. coli* ET12567/pUZ8002. Briefly, while *E. coli* in 10 ml of LB was grown to an optical density at 600 nm (OD<sub>600</sub>) of ~0.4, 10 μl of *S. griseus* spores in 0.5 ml of 2× yeast extract-tryptone (YT) medium was subjected to heat shock for 10 min at 50°C and then allowed to cool to room temperature. After a washing with fresh LB, 0.5-ml quantities each of *E. coli* and *Streptomyces* were mixed, centrifuged at high speed, and resuspended in 50 μl of 2× YT. The resuspension was diluted with sterile water from 10<sup>-1</sup> to 10<sup>-4</sup>; 100 μl of each dilution was plated onto an MS

plate. After 16 to 20 h, the plates were overlaid with 25 μg/ml of nalidixic acid and 12 μg/ml of thiostrepton for replicating plasmids like pSE34 or with 50 μg/ml of apramycin for integration mutants. After an additional 5 days, individual colonies were picked for bioassay and PCR verification. Spores from at least three genetically confirmed transformants were pooled to create a strain for biochemical analysis.

**Construction of pCK for plasmid-based gene overexpression.** The pCK plasmids were constructed from pSE34-oriT, which has a mutated *ermE* promoter (*ermE\**p) to drive the expression of genes cloned between XbaI and HindIII. Because XbaI and NheI cuts generate compatible cohesive ends and a religated sequence will no longer be recognized by the two enzymes, PCR fragments restricted with XbaI and NheI at the 5' and 3' ends, respectively, can be sequentially inserted into the XbaI site in the pSE34-derived vector. Genes of interest were amplified using *S. griseus* ATCC 700974 genomic DNA and a pair of primers, the names of which indicate the target, direction, and a built-in restriction site for cloning (Table 2). In some cases, the ribosomal binding site (RBS) of a target gene was included within the 3' end of a fragment following the restriction site. Control plasmid pA10 is the pSE34-oriT vector containing *abmK* (19). The direct sulfhydrylation genes *metYXSO* were amplified as a single 4-kb DNA fragment and cloned into pSE34-oriT to obtain pCK1. To assemble the 2.4-kb *malY-metB* DNA fragment, PCR products were first assembled in pDrive (Qiagen) and then moved into pSE34-oriT to yield pCK2. To assemble the 2.6-kb *mtcC-mtcB* DNA fragment, PCR products were directly cloned into pSE34-oriT to yield pCK3. Cloning of the PCR-amplified 1.4-kb *sahH* and 2.3-kb *metE* DNA fragments into pSE34-oriT yielded the pCK4 and pCK6 plasmids. The 1.2-kb *metK* DNA fragment was first assembled with *sahH* as a two-gene operon in pDrive; then the two-gene fragment was cloned into pSE34-oriT to obtain pCK5. A *metE* fragment was also used to construct an ~3.9-kb XbaI-HindIII *sahH-metE* DNA fragment, which was then cloned downstream of the XbaI-NheI *metK* DNA fragment to obtain a 5.1-kb XbaI-HindIII *metK-sahH-metE*

TABLE 2 DNA primers used for PCR in this study

Primer	Sequence (5' to 3')	Purpose
DirectS-F-XbaI	GAGGTCTAGAATGAGCCAGCCCCTCGACTCCGTAC	Cloning <i>metYXSO</i> into pSE34
DirectS-R-HindIII	AAACCAAGCTTTTCGGTCCCCTGCCGGTGGCCG	
CGL-F-XbaI	AAAAAATCTAGATGAGCACCATGGGCGACGGAAACAG	Cloning <i>mtcC</i> into pSE34
CGL-R-RBS-NheI	AAAAAAGCTAGCTCTATCTACCGGACCGCCGCTCCAGCGCCTG	
CBS-F-XbaI	AAAAAATCTAGAGTGCAATTCACGATTTCGATGATCAG	Cloning <i>mtcB</i> into pSE34
CBS-R-HindIII	AAAAAAAAGCTTTTCAGGCCTTGCCCGTCCCGGCTCCT	
CBL-F-XbaI	AAAAAATCTAGAGTGGGCGCGCCCTACGACTTCGATAC	Cloning <i>maly</i> into pSE34
CBL-R-RBS-NheI	AAAAAAGCTAGCTCTATTCATTCGCCGAGGGGCTTGTTCAG	
CGS-F-XbaI	AAAAAATCTAGAGTGGCCGGTAAGGCCCTTGGCAGC	Cloning <i>metB</i> into pSE34
CGS-R-HindIII	AAAAAAAAGCTTTTCAGCCAGCGCCTGGGTGAGGTCG	
metK-F-XbaI	AGGTCTAGAGTGTCCCGCCGCTTTTCACCTCGG	Cloning <i>metK</i> into pSE34
metK-R-HindIII	CCTCCGAAGCTTTGTCTGTGCTTGTACGGCCCTTACAGACC	
metE-F-XbaI	AAAAAATCTAGAGTGACAGCGAAGCCCGCAGCC	Cloning <i>metE</i> into pSE34
metE-R-HindIII	AAAAAAAAGCTTCGGATCACGCCGCTTCGGTGGGC	
sahH-F-XbaI	CAGTCTAGAATGACGACGACGACCAACCCGT	Cloning <i>sahH</i> into pSE34
sahH-R-RBS-NheI	CTTCCGGCTAGCGTCTCTGGATCAGTAGCGGTAGTGGTC	
hrdBp-F-BamHI	AAAAAAGGATCCCATGCGTCACTGCTCGTCTCCATC	Cloning <i>hrdBp</i> -linked constructs into pSET
hrdBp-F-EcoRI	AAAAAAGAATTCCATGCGTCACTGCTCGTCTCCATC	Cloning <i>hrdBp</i> promoter into pSE34
hrdBp-R-XbaI	AAAAAATCTAGAACCTCTCGGAACGATGAAACGGC	Cloning <i>hrdBp</i> promoter into pSE34
DirectSinbet-F-BamHI	AAAAAAGGATCCGCCCGGTCGTCGCCGCTGGGC	mutating the BamHI within <i>metYXSO</i>
DirectSinbet-R-BglII	AAAAAAGATCTCCCTCCCGTCCGCCACGGAGGCCGGC	mutation and for PCR verification
DirectS-R-BglII	AAAAAAGATCTTTTCAGTCACTGCTCCCGGTCGCCGAC	Cloning <i>metYXO</i> into pSET
abmD-F-XbaI	AAAAAATCTAGAATGACGGTCTTCCCT	Cloning <i>abmD</i> into pSE34
abmD-R-HindIII	AAAAAAAAGCTTGTCTCCTCTCGGGTAC	Cloning <i>abmD</i> into pSE34
abmD-R-NheI	AAAAAAGCTAGCGACGGGCGGTGGGC	Cloning <i>abmD</i> into pSET
ask- <i>asd</i> -F-XbaI	AAAAAATCTAGAGTGGGCCTTGTCTGTCGAGAAGTACGGC	Cloning <i>ask-<i>asd</i></i> into pSE34
ask-D-Rm	CCGCGCGGACGGATCCGCGCCGCGCACCCGTTGACCTC	BamHI mutation primer
ask-D-Fm	CGCGCGCGGCATCCGTCGGCGGCGGCATCATCGCCAAC	BamHI mutation primer
ask- <i>asd</i> -R-RBS-HindIII	AAAAAAAAGCTTTCTATCCGGAGAATCAGCCGCCCCCTAAGAGC	Cloning <i>ask-<i>asd</i></i> from into pSE34
hrdBp-F-HindIII	AAAAAAAAGCTTCTCTCGTCTCCATC	Cloning <i>hrdBp-ask-<i>asd</i></i> from pSCAK2 to pSET
ask- <i>asd</i> -R-RBS-NheI	AAAAAAGCTAGCTCTATCCGGAGAATCAGCCGCCCCCTAAGAGC	

DNA fragment in pDrive. The three-gene assembly was cloned into pSE34-oriT to obtain pCK7.

#### Construction of SCAK strains for integrative gene overexpression.

All pSCAK plasmids have a pSETGUS (25) vector backbone, and DNA fragments were cloned using either BamHI or XbaI or both sites. The *hrdBp* promoter was amplified from the genome in the form of an ~0.3-kb EcoRI-XbaI DNA fragment to replace the *ermE*\*p sequence in the pCK plasmids mentioned above. Since the ~4.3-kb *hrdBp-metYXSO* DNA fragment of the pCK1 derivative has a BamHI located in the intergenic region of *metY-metX*, a 1.4-kb XbaI-BglII DNA fragment from *hrdBp* to the BamHI site was amplified using primers DirectS-F-XbaI and DirectSinbet-R-BglII and used to replace the same-size XbaI-BamHI fragment in the pCK1 derivative. The original BamHI was thus mutated due to the religatable sticky ends from BamHI and BglII restriction. Using this plasmid as a template and primers hrdBp-F-BamHI and DirectS-R-BglII, the 4.3-kb BamHI-BglII DNA fragment was amplified and cloned into pSET to obtain pSCAK1. Gene *abmD* was amplified as an ~1-kb XbaI-HindIII DNA fragment with primers abmD-F-XbaI and abmD-R-HindIII and cloned into the *hrdBp*-containing pSE34 vector. Using this plasmid as the template, a 1.3-kb BamHI-NheI *hrdBp-abmD* DNA fragment was amplified with primers hrdBp-F-BamHI and abmD-R-NheI and cloned into pSET to obtain pSCAK2. The pDrive vector was used to first assemble a fragment with genes from multiple pathways. A 4.3-kb BamHI-BglII *hrdBp-metYXO* fragment and a 1.3-kb EcoRI-HindIII *PhrdB-abmD* fragment were amplified from pSCAK1 and pSCAK2, respectively, using primer pairs hrdBp-F-BamHI/DirectS-R-BglII and hrdBp-F-EcoRI/abmD-R-HindIII. A 2.7-kb HindIII-NheI *hrdBp-maly-metB* DNA fragment was amplified from a pCK2 derivative. These fragments were sequentially cloned into the pDrive vector to generate a six-

gene construct. The ~8.3-kb DNA insert was recovered as a BamHI-NheI fragment and cloned into pSET to obtain pSCAK3. The *ask-*asd** operon has an internal BamHI site at position 416 of *asd*. Site-directed mutagenesis was performed to make a G416C mutation of the BamHI while switching to a synonymous codon. It involved overlapping of two PCR fragments, 1.68-kb and 0.753-kb DNA, amplified from the left and right sides of the mutation site, using primers ask-*asd*-F-XbaI/ask-D-Rm and ask-D-Fm/ask-*asd*-R-RBS-HindIII, respectively. The fragments were then mixed and used as a template for another round of PCR using the two inward primers. The amplified 2.4-kb XbaI-HindIII DNA fragment was coligated with the 0.3-kb EcoRI-XbaI *hrdBp* DNA fragment into pSE34. The resulting plasmid was used to amplify a 2.7-kb BamHI-NheI *hrdBp-ask-*asd** DNA fragment using primers hrdBp-F-BamHI and ask-*asd*-R-RBS-NheI, which was then cloned into pSET to obtain pSCAK4. A 2.7-kb HindIII-NheI *hrdBp-ask-*asd** DNA fragment was amplified from pSCAK2 using hrdBp-F-HindIII and ask-*asd*-R-RBS-NheI. This fragment and the 4.3-kb BamHI-BglII *hrdBp-metYXOS* DNA fragment from pSCAK4 were sequentially cloned into pDrive to generate a five-gene construct. The BamHI-NheI 7.1-kb DNA insert was recovered from the pDrive plasmid and cloned into pSET to obtain pSCAK5. The pSCAK plasmids were introduced into *S. griseus* ATCC 700974 by conjugation. Chromosomally integrated SCAK mutants, selected by apramycin resistance and verified by PCR with purified genomic DNA, were confirmed to be stable through several generations and minimally 7 months.

**Growth of the wild type in PPGL-supplemented or iron-deficient medium and CDW.** *S. griseus* ATCC 700974 spores were grown in tryptic soy broth (Bacto) for 2 days. Two milliliters of the seed culture was used to inoculate 50 ml of APB in a 300-ml flask with extradeep baffles. The flasks were shaken at 250 rpm and 28°C for 4 days and sampled at the desired

time point. Propargylglycine (PPGL; Sigma) was added to a final concentration of 9 mM at the time of inoculation. Iron-deficient medium was prepared by excluding ferrous sulfate from the production medium. The cell mass of the bacterial culture was measured by a modified method (26). A bacterial culture (50 ml) was centrifuged to sediment the cells. The cell pellets were resuspended in 3 ml of water, transferred onto a preweighed glass dish, and then dried in a chamber at 80°C overnight. The weight difference was reported as the cell dry weight (CDW).

**Bioassay for determination of ABM production and titer.** Agar diffusion bioassay is a standard method for detecting microbial antagonism. Since it was previously shown that ABM from *S. griseus* ATCC 700974 in the defined medium APB is the sole agent responsible for the specific bioactivity (19, 23), a bioassay was used to detect the production during fermentation. An *E. coli* JM109 culture grown overnight was diluted to an OD<sub>600</sub> of ~0.1. A 100- $\mu$ l quantity of the diluted culture was pipetted onto a Mueller-Hinton (MH) agar plate and spread evenly over the surface of the agar with a sterile cotton swab. Wells were made in MH agar using the back end of a 20- to 200- $\mu$ l tip, to which *Streptomyces* culture (20  $\mu$ l) was added. The plate was incubated at 37°C, and a zone of inhibition which formed in the *E. coli* lawn was read at 18 h. A bioassay plate was loaded with serially diluted cultures or solutions for comparing and estimating ABM concentrations (7, 23). A standard curve was generated with freshly purified ABM, showing that the assay limit is about 2 ng in 20  $\mu$ l of solution, which is 100 times more sensitive than high-performance liquid chromatography (HPLC) quantitation. A linear regression between the logarithm of the ABM concentration and the diameter of the zone was identical to that reported previously (23) (see Fig. S1 in the supplemental material).

**HPLC-fluorescence detection of intracellular thiol amino acids.** The major intracellular thiols were extracted by a modification of a previously reported method (27). Cells from a 1-ml culture were pelleted by centrifugation and then quickly washed twice with 1 ml of 2.63% NaCl solution. HPLC-grade methanol prechilled at -20°C (1 ml of 60% solution) was added to the tube. The pellet was resuspended, frozen in liquid nitrogen, and stored at -80°C until extraction. A 20- $\mu$ l quantity of 2 mM monobromobimane (Life Technologies), dissolved in acetonitrile (ACN), was added to the suspension after thawing of the cells. The mixture was vortexed for 1 min and then immediately frozen in liquid nitrogen for 5 min. This sequence of steps was repeated three times. The tube was centrifuged at 5,000  $\times$  g for 10 min at 4°C, and the supernatant was transferred to a new tube and mixed with an equivalent amount of chloroform. After centrifugation for 10 min at 13,000  $\times$  g, the aqueous phase was recovered and 10  $\mu$ l was analyzed by HPLC. As a negative control, 20  $\mu$ l of 2 mM *N*-ethylmaleimide (NEM) in ethanol was added to the tube during cell lysis. The data were collected with cultures grown for 4 days. HPLC was performed using a Shimadzu Prominence system that included a photodiode array detector (SPD-M20A) and a fluorescence detector (RF-20A). The HPLC system was equipped with an Alltech ALLSPHERE ODS-2 5 $\mu$  250- by 4.6-mm column, and the flow rate was maintained at 1.5 ml/min. The gradient program from solvent A (H<sub>2</sub>O) to solvent B (ACN) was 0 to 40% B in 10 min, 40% to 80% solvent B in 5 min, 80% to 100% solvent B in 2 min, 100% to 0% solvent A in 2 min, and 0% solvent A for 4 min.

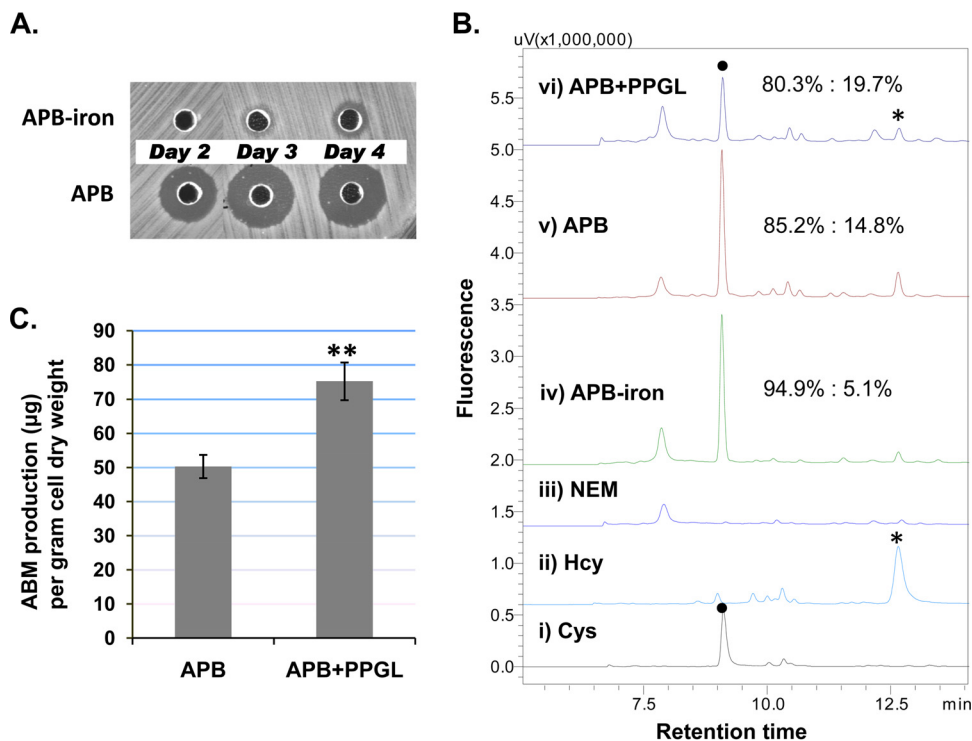
**ABM isolation and HPLC analysis of ABM production.** The procedures were modified from previous methods (19, 23). Cleared culture (50 ml) was mixed with 5 to 10 g of XAD-4 resin (Sigma). The resin was first washed with 100 ml of water and then washed with 100 ml of 15% methanol. ABM was eluted with 100 ml of 50% methanol, and the methanol was removed with a rotary evaporator. The extract was lyophilized to dryness and reconstituted in 1 ml of water. The extract (100  $\mu$ l) was used for HPLC analysis in two steps. The first HPLC separation used a Phenomenex Spherclone C<sub>18</sub> ODS-2 10 $\mu$  column (250 by 4.6 mm) with a flow rate of 1.5 ml/min. The gradient program from solvent A (H<sub>2</sub>O in 0.01% formic acid) to solvent B (ACN in 0.01% formic acid) was 0 to 40% solvent B in 15 min, 40% to 95% solvent B in 2 min, 95% solvent B for 3 min, 95% to 0% solvent B in 2 min, and 0% solvent B for 3 min. A

Shimadzu FRC-10A fraction collector was used to collect 0.5-ml fractions, of which 20  $\mu$ l was used in each bioassay. The active fractions (~9.3 min) were combined, lyophilized, and reconstituted in 100  $\mu$ l of water. ABM was further purified using HPLC with a Phenomenex Kinetex XB-C-18 2.6 $\mu$  column (75 by 4.6 mm). The solvents were the same as described above, and the flow rate was maintained at 1 ml/min. The gradient was from 0 to 40% solvent B in 15 min, 40% to 95% solvent B in 2 min, 95% solvent B for 3 min, 95% to 0% solvent B in 3 min, and 0% solvent B for 7 min. ABM was eluted at ~6.5 min and verified by high-resolution mass spectrometry, UV-visible (UV-VIS) absorption spectrum, and the corresponding bioactivity. A standard curve of HPLC peak area versus the amount of pure ABM analyzed was prepared (see Fig. S2 in the supplemental material).

## RESULTS AND DISCUSSION

**Intracellular flux of free-thiol amino acids in relation to ABM production.** Gene *abmD* was predicted to encode a pyridoxal phosphate (PLP)-dependent enzyme that is similar to ACCD (1-aminocyclopropane-1-carboxylic acid) deaminase (E value, 2e-55) (19) through sequence alignment or cystathionine- $\beta$ -synthase (CBS; E value, 4e-67) through structural predictions. It also has weak homology to D-cysteine desulfhydrases. AbmD produced and purified from *E. coli* showed the expected absorbance maximum at ~410 nm at pH 8.0 (see Fig. S3 in the supplemental material), which is indicative of a PLP-containing enzyme. AbmD is the only ABM biosynthetic enzyme that displayed absorption, suggesting that AbmD catalyzes a reaction that can be a key link between primary and secondary metabolism by utilizing one of the two free-thiol amino acids to perform the first committed step of the pathway. While functional investigation of AbmD is ongoing, we tested whether ABM biosynthesis would be controlled by the *abmD* gene product in a productive manner (28).

We examined the intracellular flux of Hcy and Cys in relation to ABM production (Fig. 2). An HPLC-fluorescence method was used to monitor the relative concentrations of Hcy and Cys since the two metabolites were the only major free-thiol amino acids detected in the cells under various fermentation conditions. It was previously reported that iron (Fe<sup>2+</sup> or Fe<sup>3+</sup>) supplementation in the growth medium (up to 1 mM) significantly increased ABM production, and this increase was positively correlated (23). We therefore asked whether iron would also affect intracellular levels of Hcy and Cys. When wild-type (WT) *S. griseus* was grown in glycerol-based ABM production broth without iron supplementation (APB-iron), the residual iron in other medium components was sufficient to support growth to ~70% of the cell dry weight (CDW) of cultures from normal APB (2.75 g versus 3.92  $\pm$  0.08 g/liter), but very little ABM production was observed after a 4-day incubation (Fig. 2A). Intracellular Hcy was barely detected in the iron-deficient cells (Fig. 2B, HPLC trace iv), while the amount of Hcy was significantly greater in iron-supplemented cells (Fig. 2B, HPLC trace v). Hcy in the APB sample was three times that in the APB-iron sample when it was normalized to the Cys detected with the same method. We next examined the effect of supplementing the culture with propargylglycine (PPGL), which is known to inhibit SAA metabolism *in vivo*. PPGL functions as a mechanism-based inhibitor of the transsulfuration reactions, targeting both CGS and CGL proteins (Fig. 1) (29, 30), but it is less effective on direct sulphydrylation (31). PPGL was previously shown to inhibit the synthesis of a cysteine-containing antibiotic, thienamycin, produced by *Streptomyces cattleya* NRRL 8057 (30). The WT *S. griseus* strain was grown in APB in the



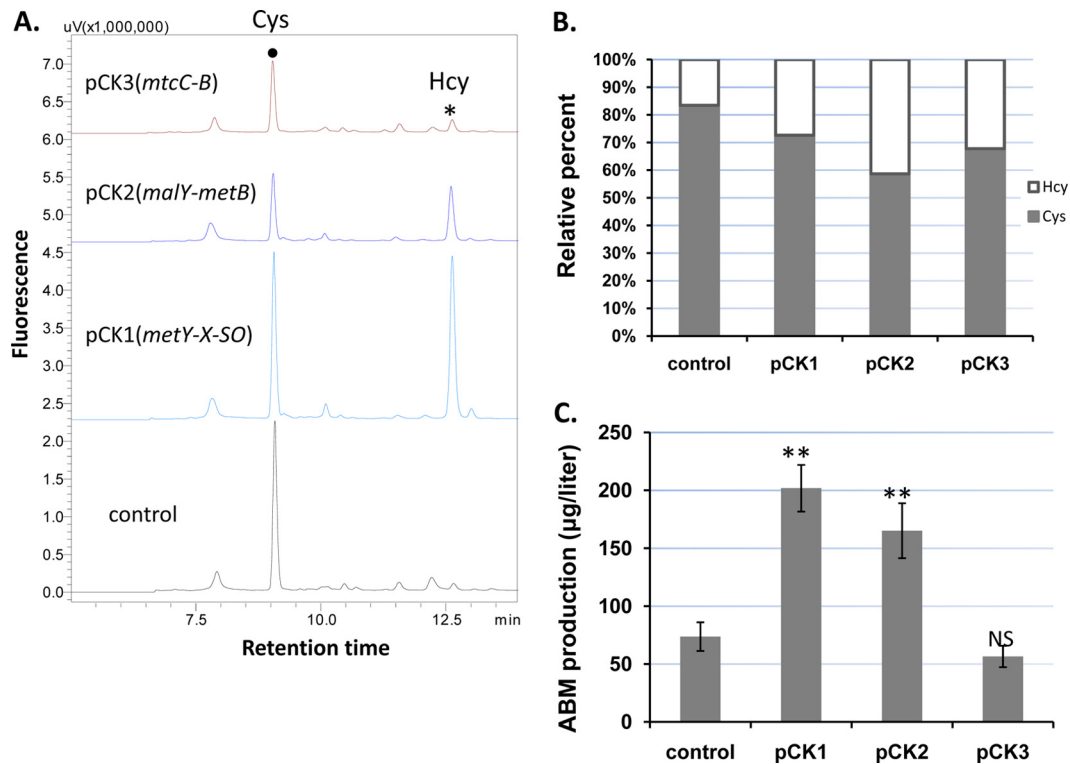
**FIG 2** Relationship of ABM production and intracellular free-thiol amino acids of WT *S. griseus*. (A) Bioassay with cells grown in the absence (APB-iron) or presence (APB) of 1 mM iron in a glycerol-based ABM production medium (APB). The size of the inhibition zone is proportional to the amount of ABM in the culture assayed (see Materials and Methods). (B) HPLC-fluorescence detection of monobromobimane-derived intracellular amino acids. Cysteine (●) and homocysteine (\*) are the only two free-thiol amino acids detected under these conditions. The chromatograms show (i) and (ii) standards, (iii) *N*-ethylmaleimide (NEM)-treated intracellular metabolic extracts (the negative control), (iv) cells in APB without iron, (v) cells in normal APB with 1 mM iron, and (vi) cells in APB supplemented with 9 mM propargylglycine (APB+PPGL). Percentage ratios of the peak areas of the two thiols are shown for traces iv, v, and vi. The standard error of three replicates for each was  $\leq 0.2$ . (C) ABM production of cells grown in APB or APB+PPGL. ABM was quantified by isolation and HPLC analysis. The statistical significance was derived from nine experimental replicates; the standard error is presented by an error bar. \*\*,  $P < 0.01$  (*t* test).

presence of PPGL (APB+PPGL). While the cells grew to a CDW ( $3.20 \pm 0.17$  g/liter) that was  $\sim 81\%$  of normal growth, the PPGL-treated culture produced 30 to 50% more ABM on day 4, as determined by both bioassay and HPLC quantification of the purified ABM (Fig. 2C; see also Fig. S4 in the supplemental material). Thus, unlike thienamycin production, albomycin production does not seem to depend upon the transsulfuration pathways. Furthermore, in the profile of SAA, both Cys and Hcy peaks decreased but the relative amount of Hcy increased to 19.7% (Fig. 2B, HPLC traces v and vi). Taking into consideration the three independent growth conditions, the results suggest that an increase in ABM production is correlated with an increase in the relative intracellular concentration of Hcy.

**Key SAA metabolic genes in *S. griseus* and functional assignment.** We previously reported that the genome of the ABM producer is very similar to that of the well-studied *S. griseus* NBRC 13350 (19). A preliminary genome-scale comparison is presented in Fig. S5 in the supplemental material. Since there are few reports concerning SAA metabolic genes in *Streptomyces* (32), we used the RAST genome annotation server (33) to identify candidates in the whole genome of NBRC 13350 (34). Ten major SAA metabolic genes or open reading frames (ORFs) were identified (Fig. 1), suggesting that *S. griseus* is Cys and Met prototrophic. Their homologs were subsequently identified in the *S. griseus* ATCC 700974 genome. Nucleotide identity between the genes of NBRC 13350 and ATCC 700974 (see Table S1 in the supplemental mate-

rial) is 93.7% on average, with the highest at 97% (*sahH*) and the lowest at 89% (*malY*). The two closely related *S. griseus* strains can be used to perform genomic research of *Streptomyces*, such as on housekeeping genes and the conserved functions (35), as well as small noncoding RNAs (36).

Several subclasses of PLP-dependent enzymes are involved in the formation of thiol amino acids (14, 37), and the functions of the assigned ORFs were interrogated. The amplified ORFs of each pathway were assembled into a cassette for expression under the control of the *ermE*\* promoter in *Streptomyces* multicopy plasmid pSE34-oriT (19). It should be noted that direct sulfhydrylation ORFs *sgr6647* and *sgr6646* (*metY* and *-X*) appear in both genomes and are located in a genetic operon with a third ORF, *sgr6645* (*metSO*), encoding a putative sulfite oxidase, immediately downstream of *sgr6646*. The three ORFs were amplified and cloned as one DNA fragment for expression. The different plasmids were separately introduced into *S. griseus*, and the fermentation cultures of the positive transformants were analyzed for ABM production and Hcy/Cys levels. Overexpression of the direct sulfhydrylation ORFs in pCK1 and transsulfuration ORFs *sgr3417* and *sgr2579* (*malY* and *metB*) in pCK2 caused an increase of Hcy relative to Cys, while the reverse transsulfuration ORFs *sgr3660* and *sgr4452* (*mtcC* and *mtcB*) in pCK3 caused a reduction of intracellular Hcy, although the effect was less pronounced (Fig. 3A and B). These results are consistent with expectations for SAA metabolism. Overexpression of *metYXSO* increased overall intra-



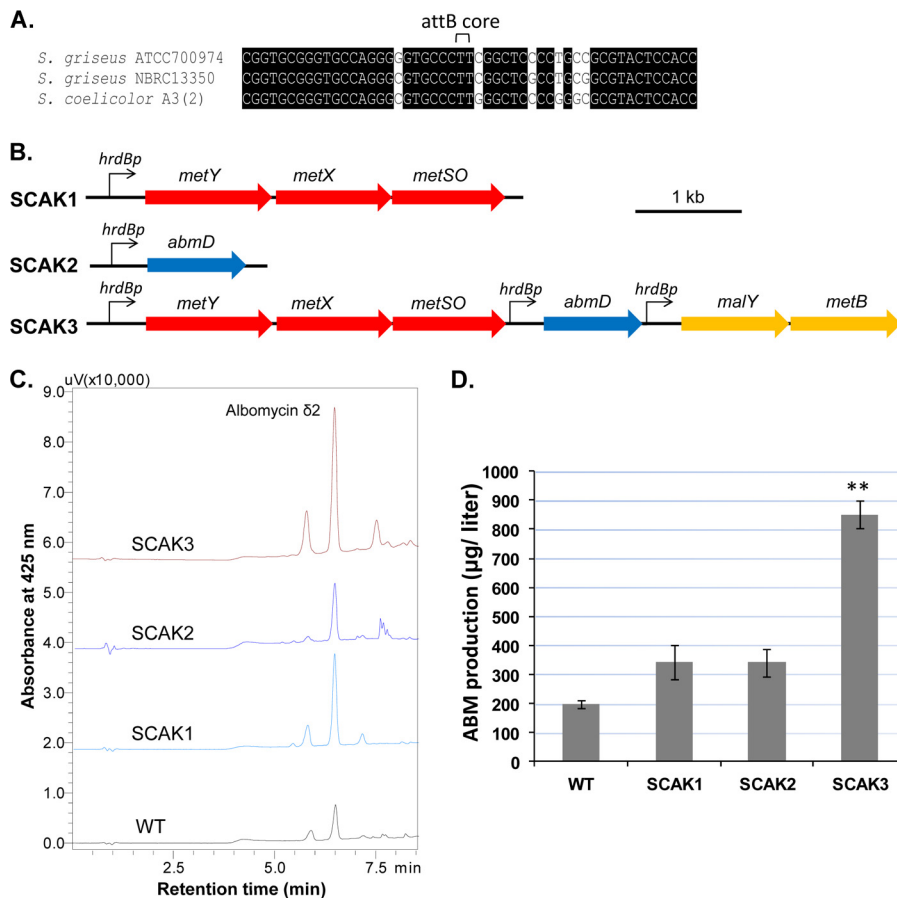
**FIG 3** Effect of plasmid-based overexpression of the identified sulfur amino acid metabolic genes in WT *S. griseus*. (A) HPLC-fluorescence chromatograms of intracellular Cys and Hcy detected in the transformed strains. (B) Relative percentages of intracellular Cys and Hcy. For each strain that is a mixture of 3 to 5 individual colonies, the percentage is an average of three independent measures. (C) ABM production level. The introduced plasmids are described in Table 1. Each production level is the average of at least three experimental replicates; the standard error is represented by an error bar. Statistical significance was determined by comparing the level of a specific strain to the control. \*\*,  $P < 0.01$ ; NS, not significant.

cellular thiol amino acid concentrations, and this increase was significantly greater than for recombinant strains expressing *malY-metB* (Fig. 3A). The recombinant strains also produced ABM at different levels (Fig. 3C). The additional *metYXSO* in pCK1 enabled the highest ABM production compared to the control plasmid without the genes, while the addition of *mtcC-mtcB* (pCK3) led to comparable levels of ABM production relative to the control. No difference in the CDWs of these strains was observed. The results agreed in general with the functional assignment of the SAA metabolic genes. Moreover, the results revealed that a higher level of Hcy caused by the gene expression is not toxic to *S. griseus* and, importantly, that increased Hcy improves ABM production. The overflow of Hcy could be redirected to the ABM pathway by overexpressing the gate-keeping enzyme *AbmD*. A comparable engineering strategy leading to improved production of microbial metabolites has been reported for other systems, such as cephamycin C-producing *Streptomyces* (28).

**Overexpression of Hcy synthesis genes and *abmD* for improved and stable ABM production.** Hcy concentration can be increased by inserting genetic constructs directly into the chromosome. The *S. griseus* draft genome was searched for two genetic elements for chromosomal incorporation of exogenous DNA. The first element was a phage  $\Phi$ C31 *attB* site that allows the integration of pSET vector-carried DNA into a host chromosome (38). A 51-bp sequence in a hypothetical protein gene (39), highly similar to the *attB* sequences found in other *Streptomyces* genomes, was identified (Fig. 4A). The second genetic element is a

constitutive promoter, such as *hrdBp*, often used in *Streptomyces* genetic engineering (26). *HrdB* is a principal sigma factor that has been studied in *S. griseus* (40). The genes encoding *HrdB* (*sgr1701*) in the two *S. griseus* strains are 96% identical. The promoter *hrdBp* was defined as an ~350-bp DNA upstream sequence. We initially constructed a chromosomally integrated mutant, SCAK1, that has the direct sulfhydrylation genes *metYXSO* under the control of the *hrdBp* promoter sequence (Fig. 4B; see also Fig. S6 in the supplemental material). As expected, the intracellular concentration of Hcy of SCAK1 increased to a level almost equal to that of Cys (see Fig. S7 in the supplemental material). While ABM production by SCAK1 increased (Fig. 4C and D), the CDW of SCAK1 at day 4 decreased to  $2.22 \pm 0.27$  g/liter, compared to the CDW of the WT,  $3.92 \pm 0.08$  g/liter. We also made an *abmD*-integrated mutant, SCAK2 (Fig. 4B), that has *abmD* under the control of the *hrdBp* promoter, and the exconjugants (*attB*::pSCAK2) were obtained at a much lower frequency than for SCAK1 generation and the double-crossover mutant previously prepared (19). After several trials, we managed to isolate one such mutant, SCAK2 (see Fig. S6 in the supplemental material), from the  $10^{-2}$  to  $10^{-3}$  dilution of a conjugation mixture. ABM production by SCAK2 was nearly double that of the wild-type strain (Fig. 4C and D).

To demonstrate that ABM production is jointly controlled by Hcy flux and *abmD* expression, the transsulfuration genes (*malY-metB*) under the control of *hrdBp* were included with *abmD* and the direct sulfhydrylation genes (*metYXSO*) to generate the integrated mutant strain SCAK3 (Fig. 4B). The transformed colonies



**FIG 4** Chromosomal integration of genetic constructs for increasing sulfur-carbon flow toward Hcy and the improved ABM production. (A) Comparison of *attB* sequences identified in the *Streptomyces* genomes. (B) Diagram of the major chromosomally integrated constructs. (C) HPLC chromatograms of purified albomycins of *S. griseus* ATCC 900794 WT and the mutants. Albomycin  $\delta_2$  is the major product. Other albomycin congener (ferrichrome) or variants ( $\delta_1$ ) were eluted from the HPLC column before or after the  $\delta_2$ . (D) ABM production of the analyzed chromosomal mutants. Each production level is the average of at least three experimental replicates; the standard error is represented by an error bar. \*\*,  $P < 0.01$ .

were readily prepared and verified by PCR (see Fig. S6 in the supplemental material). Thirty colonies were screened using the antibacterial bioassay, and  $\sim 27$  colonies gave a larger zone of inhibition than that of the WT strain. These colonies all produced similarly high levels of ABM. Several colonies were pooled to create strain SCAK3 for the subsequent metabolic analyses. The Hcy level in the SCAK3 cells was steadily low, and the CDW of SCAK3 was reproducibly  $4.25 \pm 0.24$  g/liter, comparable to or better than that of the WT, suggesting that the growth problem of the two early mutants can be overcome by a combined action of the precursor supply pathways and overexpression of rate-limiting *abmD*. ABM production by SCAK3 was  $\sim 400\%$  that for the WT strain and  $\sim 200\%$  that for the SCAK1 or SCAK2 strain (Fig. 4C), indicating that the co-overexpression of *abmD* has efficiently diverted the augmented Hcy flux into ABM biosynthesis. Furthermore, no antibiotics were needed to maintain the ABM overproduction phenotype, although the SCAK3 strain was repeatedly passed through medium without selection. Thus, a stable, high-level ABM producer was generated. Although it is known that some bacteria cannot tolerate an elevation of intracellular Hcy (41), this strategy has the potential to be used to discover ABM-like molecules potentially produced by other actinomycetes, such as *Streptomyces* C (19).

**Exploring metabolic switches and sulfur source for increasing ABM production.** Biosynthesis of methionine in bacteria is linked to central carbon metabolism and whole-cell physiology via additional metabolic pathways: one is the aspartate-to-homoserine path of  $C_4$  metabolism that starts with aspartokinase (Ask) and aspartate semialdehyde dehydrogenase (Asd), and another is the activated methyl cycle (AMC) of  $C_1$  metabolism (Fig. 1). The  $C_4$  carbon flow is controlled by a feedback inhibition mechanism of Ask and Asd. *Streptomyces albus* NBRC 14147 encodes an inhibition-resistant Ask in the *ask-asd* operon, which is a major reason that it produces an aspartate-derived antibiotic product at a gram/liter level (42). AMC has two metabolic controlling elements as well. First, S-adenosyl-L-methionine (SAM)-dependent methyltransferases are subject to S-adenosyl-L-homocysteine (SAH) product inhibition. Without sufficient SAH hydrolase to degrade the coproduct generated by SAM-dependent enzymes, including AbmI (19), a cell's primary metabolism is impeded. Second, SAM is shared by many biological pathways, and the supply is limited. Thus, an exogenous supply of SAM or overexpression of SAM synthetase (MetK) has been reported to increase the production of antibiotics in *Streptomyces* (43). We attempted to explore these master metabolic switches for increasing ABM production by genetic manipulation. Single- or multiple-gene constructs were



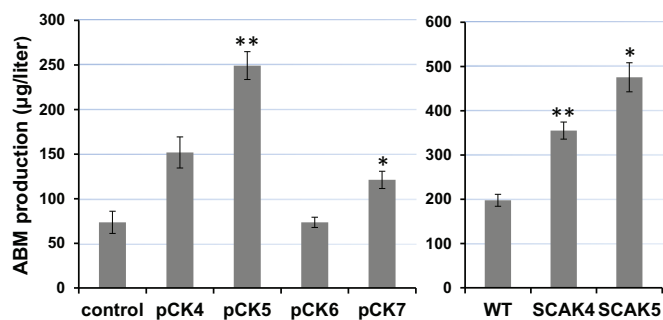


FIG 5 ABM production of the constructed strains. pCK series are strains containing replicating plasmids. SCAKs are strains with chromosomally integrated mutations. The genes cloned in each construct for overexpression are as follows: for pCK4, *sahH*; for pCK5, *metK-sahH*; for pCK6, *metE*; for pCK7, *metK-sahH-metE*; for SCAK4, *ask-asd*; and for SCAK5, *metY-X-SO-ask-asd*. Each production level is the average of at least three experimental replicates; the standard error is represented by an error bar. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

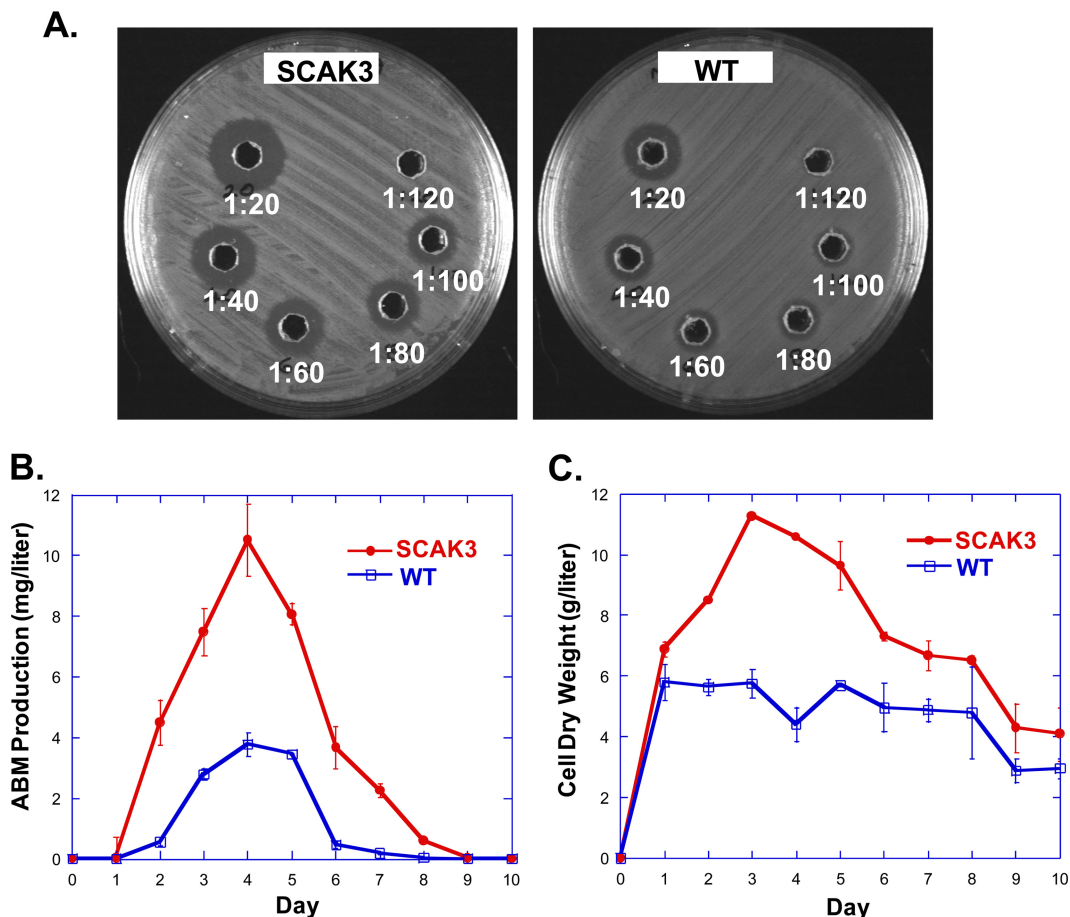
cloned into pSE34-oriT under the control of *ermE*\*p for expression in WT *S. griseus*. As shown in Fig. 5, *sahH* overexpression with pCK4 improved ABM production, but the effect was less than with *metK-sahH* coexpression with pCK5. Overexpression of *metE* with pCK6 did not cause a change of ABM production, while the coexpression of the three genes with pCK7 only slightly increased ABM production levels (Fig. 5). The overexpression of *metE* was unable to compete with natural *abmD* but reduced the positive effect of *metK-sahH* coexpression. CDWs of strains carrying pCK4 and pCK5 were ~15% more than for the control strain, indicating the important role of *metK* and *sahH* in cell fitness rather than having a specific effect on ABM biosynthesis. We finally made two more chromosomally integrated mutants, SCAK4 (*hrdBp-ask-asd*) and SCAK5 (*hrdBp-metYXSO-hrdBp-ask-asd*). Both of them showed a modest but reproducible increase of ABM production (Fig. 5). We have yet to confirm whether Ask/Asd enzyme activities are elevated in SCAK4 and SCAK5. However, in glycerol-based medium, the levels of intracellular Cys and Hcy were nearly identical, suggesting that the increased Hcy flux caused by the *ask-asd* played a direct role in the increase in ABM production. Future engineering efforts involving  $C_1$  and  $C_4$  metabolism for optimizing ABM production will likely need to consider the major carbon source of fermentation.

In addition to engineering the metabolic switches related to SAA metabolism, we also thought that a more reduced sulfur source in the medium might increase ABM production. Sodium sulfide cannot be used because it reacts immediately with iron to form a precipitate; thus, thiosulfate supplementation was examined. SCAK3 grew normally in thiosulfate-based medium, but ABM production was not increased. It remains unknown whether *S. griseus* has a thiosulfate reductase activity. Overall, the results are consistent with the higher-order metabolic regulatory circuits such as AMC, Ask/Asd, and sulfur assimilation in *S. griseus* having an indirect role in ABM production by altering Hcy flux.

**SCAK3's growth and ABM production level in an optimized medium.** It was reported that WT *S. griseus* produced ABM at 1 to 2 mg/liter during standard fermentation (23). In addition to iron and buffering agents, ornithine and specific carbon sources in the medium were shown to be important for high-level ABM production by selected clones. Nonetheless, high-level-pro-

ducing strains could not be preserved for a long period. At the outset of our experiments that lasted >8 months, the strains were grown up in glycerol-APB medium without ornithine, and ABM was isolated and quantified with HPLC. The yield was  $0.20 \pm 0.013$  mg/liter or  $0.85 \pm 0.046$  mg/liter for WT or SCAK3, respectively, when fermented in flasks with rigorous shaking. Adding ornithine alone to the glycerol-APB medium did not improve ABM production in SCAK3. The previously optimized medium, which contains ornithine and 2% starch, supported a higher ABM production by SCAK3 with the shake flask method; however, the ABM purification process became significantly more complex under those conditions. We then used the bioassay to determine the ABM titer and estimated the production by SCAK3 in the optimized medium despite this problem (Fig. 6A). ABM production peaked at day 4, when the level for SCAK3 (~11 mg/liter) was three times as much as for the WT (~3.7 mg/liter) (Fig. 6B). The CDWs of both cultures reached the highest level at day 3 (Fig. 6C), when SCAK3's mass was about twice that of the WT in flasks. Starting at day 4, the CDW of SCAK3 decreased toward the end, while the CDW of the WT changed less even after ABM production ceased. This observation indicated that more SCAK3 cells might have lysed once it produced a large amount of ABM in the shake flask fermentation. Nonetheless, the SCAK3 strain produced more ABM than the WT strain in the optimized medium at a given time. Since ABM production by the WT could be increased up to 25 mg/liter in a fed-batch fermentor (23), we estimate that ABM production by SCAK3 will approach ~100 mg/liter. The purification of highly water-soluble small-peptide ABM and ABM derivatives remains challenging but solvable. A more efficient ABM purification process will be needed to improve the yields for further preclinical studies or for generating ABM analogs by mutasynthesis or semisynthesis.

In summary, ABM is an antibacterial agent that was chosen for pharmaceutical development long before the genomic era (6, 12, 13, 44). A rational metabolic engineering strategy for increasing ABM production was adopted in this study. We analyzed two free-thiol amino acids, Cys and Hcy, in the model producing strain, *S. griseus* ATCC 700974, and correlated the levels to ABM production. We showed that the intracellular concentration of Hcy relative to that of Cys could be dramatically increased by overexpression of genes involved in direct sulfhydrylation and transsulfuration pathways. Refactoring of the primary metabolic genes for constitutive expression and integration into the chromosome, along with providing a duplicate *abmD* gene for the proposed initial step of the biosynthesis of the nucleoside component of ABM, resulted in a genetically stable, high-level ABM producer. In a glycerol-based medium, the ABM production level of the resulting strain, SCAK3, quantified by isolation and HPLC, was three times more than that of the WT. Thus, although ABM biosynthesis in this strain is apparently not controlled by a pathway-specific regulatory gene, ABM production can be manipulated by genetic engineering of the Hcy branch point of SAA metabolism in *Streptomyces*. The present research has only focused on limited principles of metabolic engineering for increasing ABM production. Further improvement is expected upon a more thorough metabolomic analysis of *S. griseus* as well as whole-cell modeling of the production event. At the enzyme level, AbmD is proposed to catalyze S-C bond formation using two building blocks, a hydroxyl-activated ser-



**FIG 6** (A) Bioassay plates for determining the ABM titer of SCAK3 and WT strains grown in starch- and ornithine-based APB medium. Cleared fermentation culture was diluted with water from 1:20 to 1:120, as indicated. (B) ABM production of SCAK3 and WT over time. Twenty milliliters of TSB seed culture grown for 2 days was equally distributed to 10 300-ml baffled flasks, each containing 50 ml of starch-ornithine-APB medium. The flasks were incubated at 28°C and 250 rpm. One flask was harvested every day for determining bioactivity and CDW. The experiment was performed in duplicates; the standard error of each data point is represented by an error bar. (C) Cell dry weight of the cultures.

ine and an Hcy (see Fig. S8 in the supplemental material). The reaction path should be interesting due to its deviation from the course of plant ACCD (45) or human CBS (46), offering yet another microbial example of the diversity of the PLP-dependent enzyme superfamily.

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#### REFERENCES

- Waksman SA, Henrici AT. 1943. The nomenclature and classification of the actinomycetes. *J Bacteriol* 46:337–341.
- Waksman SA, Reilly HC, Harris DA. 1948. *Streptomyces griseus* (Krain-sky) Waksman and Henrici. *J Bacteriol* 56:259–269.
- Jones D, Metzger HJ, Schatz A, Waksman SA. 1944. Control of Gram-negative bacteria in experimental animals by streptomycin. *Science* 100:103–105. <http://dx.doi.org/10.1126/science.100.2588.103>.
- Gause GF. 1955. Recent studies on albomycin, a new antibiotic. *Br Med J* 12:1177–1179.
- Stapley EO, Ormond RE. 1957. Similarity of albomycin and grisein. *Science* 125:587–589. <http://dx.doi.org/10.1126/science.125.3248.587>.
- Kuehl FA, Bishop MN, Chaiet L, Folkers K. 1951. Isolation and some chemical properties of grisein. *J Am Chem Soc* 73:1770–1773. <http://dx.doi.org/10.1021/ja01148a099>.
- Reynolds DM, Waksman SA. 1948. Grisein, an antibiotic produced by certain strains of *Streptomyces griseus*. *J Bacteriol* 55:739–752.
- Pramanik A, Stroehrer UH, Krejci J, Standish AJ, Bohn E, Paton JC, Autenrieth IB, Braun V. 2007. Albomycin is an effective antibiotic, as exemplified with *Yersinia enterocolitica* and *Streptococcus pneumoniae*. *Int J Med Microbiol* 297:459–469. <http://dx.doi.org/10.1016/j.ijmm.2007.03.002>.
- Waksman SA. 1957. Penalty of isolationism. *Science* 125:585–587. <http://dx.doi.org/10.1126/science.125.3248.585>.
- Anonymous. 1958. Albomycin and grisein. *Br Med J* 1:391.
- Braun V, Pramanik A, Gwinner T, Koberle M, Bohn E. 2009. Sideromycins: tools and antibiotics. *Biometals* 22:3–13. <http://dx.doi.org/10.1007/s10534-008-9199-7>.
- Stefanska AL, Fulston M, Houge-Frydrych CSV, Jones JJ, Warr SR. 2000. A potent seryl tRNA synthetase inhibitor SB-217452 isolated from a *Streptomyces* species. *J Antibiot* 53:1346–1353. <http://dx.doi.org/10.7164/antibiotics.53.1346>.

13. Paulsen H, Brieden M, Benz G. 1987. Synthese des Sauerstoffanalogs der Desferriform von  $\delta$ 1-Albomycine. *Liebigs Ann Chem* 1987:565–575.
14. Ferla MP, Patrick WM. 2014. Bacterial methionine biosynthesis. *Microbiology* 160:1571–1584. <http://dx.doi.org/10.1099/mic.0.077826-0>.
15. Lee HS, Hwang BJ. 2003. Methionine biosynthesis and its regulation in *Corynebacterium glutamicum*: parallel pathways of transsulfuration and direct sulphydrylation. *Appl Microbiol Biotechnol* 62:459–467. <http://dx.doi.org/10.1007/s00253-003-1306-7>.
16. Nagasawa T, Kanzaki H, Yamada H. 1984. Cystathionine gamma-lyase of *Streptomyces phaeochromogenes*. The occurrence of cystathionine gamma-lyase in filamentous bacteria and its purification and characterization. *J Biol Chem* 259:10393–10403.
17. Kim SH, Lee B-R, Kim J-N, Kim B-G. 2012. NdgR, a common transcriptional activator for methionine and leucine biosynthesis in *Streptomyces coelicolor*. *J Bacteriol* 194:6837–6846. <http://dx.doi.org/10.1128/JB.00695-12>.
18. Zdych E, Peist R, Reidl J, Boos W. 1995. MalY of *Escherichia coli* is an enzyme with the activity of a beta C-S lyase (cystathionase). *J Bacteriol* 177:5035–5039.
19. Zeng Y, Kulkarni A, Yang Z, Patil P, Zhou W, Chi X, Van Lanen S, Chen S. 2012. Biosynthesis of albomycin  $\delta_2$  provides a template for assembling siderophore and aminoacyl-tRNA synthetase inhibitor conjugates. *ACS Chem Biol* 7:1565–1575. <http://dx.doi.org/10.1021/cb300173x>.
20. Zeng Y, Roy H, Patil PB, Ibba M, Chen S. 2009. Characterization of two seryl-tRNA synthetases in albomycin-producing *Streptomyces* sp. strain ATCC 700974. *Antimicrob Agents Chemother* 53:4619–4627. <http://dx.doi.org/10.1128/AAC.00782-09>.
21. Kim JY, Carlson BA, Xu X-M, Zeng Y, Chen S, Gladyshev VN, Lee BJ, Hatfield DL. 2011. Inhibition of selenocysteine tRNA<sup>[Ser]<sup>Sec</sup></sup> aminoacylation provides evidence that aminoacylation is required for regulatory methylation of this tRNA. *Biochem Biophys Res Commun* 409:814–819. <http://dx.doi.org/10.1016/j.bbrc.2011.05.096>.
22. Benz G, Schröder T, Kurz J, Wünsche C, Karl W, Steffens G, Pfützner J, Schmidt D. 1982. Constitution of the Deferrri form of the albomycins  $\delta$ 1,  $\delta$ 2 and  $\epsilon$ . *Angew Chem Int Ed Engl* 21:527–528. <http://dx.doi.org/10.1002/anie.198205271>.
23. Fiedler HP, Walz F, Dohle A, Zahner H. 1985. Albomycin: studies on fermentation, isolation and quantitative determination. *Appl Microbiol Biotechnol* 21:341–347.
24. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. Practical *Streptomyces* genetics. John Innes Centre, Norwich Research Park, Colney, Norwich, England.
25. Myronovskiy M, Welle E, Fedorenko V, Luzhetskyy A. 2011.  $\beta$ -Glucuronidase as a sensitive and versatile reporter in actinomycetes. *Appl Environ Microbiol* 77:5370–5383. <http://dx.doi.org/10.1128/AEM.00434-11>.
26. Du D, Zhu Y, Wei J, Tian Y, Niu G, Tan H. 2013. Improvement of gougouerin and nikkomycin production by engineering their biosynthetic gene clusters. *Appl Microbiol Biotechnol* 97:6383–6396. <http://dx.doi.org/10.1007/s00253-013-4836-7>.
27. Newton GL, Fahey RC, Cohen G, Aharonowitz Y. 1993. Low-molecular-weight thiols in streptomycetes and their potential role as antioxidants. *J Bacteriol* 175:2734–2742.
28. Malmberg LH, Hu WS, Sherman DH. 1993. Precursor flux control through targeted chromosomal insertion of the lysine epsilon-aminotransferase (lat) gene in cephamycin C biosynthesis. *J Bacteriol* 175:6916–6924.
29. Johnston M, Jankowski D, Marcotte P, Tanaka H, Esaki N, Soda K, Walsh C. 1979. Suicide inactivation of bacterial cystathionine  $\gamma$ -synthase and methionine  $\gamma$ -lyase during processing of L-propargylglycine. *Biochemistry* 18:4690–4701. <http://dx.doi.org/10.1021/bi00588a033>.
30. Williamson JM, Meyer R, Inamine E. 1985. Reverse transsulfuration and its relationship to thienamycin biosynthesis in *Streptomyces cattleya*. *Antimicrob Agents Chemother* 28:478–484. <http://dx.doi.org/10.1128/AAC.28.4.478>.
31. Piotrowska M, Paszewski A. 1986. Propargylglycine as a fungal inhibitor: effect on sulphur amino acid metabolism. *J Gen Microbiol* 132:2753–2760.
32. Chang Z, Vining LC. 2002. Biosynthesis of sulfur-containing amino acids in *Streptomyces venezuelae* ISP5230: roles for cystathionine  $\gamma$ -synthase and transsulfuration. *Microbiology* 148:2135–2147. <http://dx.doi.org/10.1099/00221287-148-7-2135>.
33. Aziz R, Bartels D, Best A, DeJongh M, Disz T, Edwards R, Formsma K, Gerdes S, Glass E, Kubal M, Meyer F, Olsen G, Olson R, Osterman A, Overbeek R, McNeil L, Paarmann D, Paczian T, Parrello B, Pusch G, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST server: Rapid Annotations using Subsystems Technology. *BMC Genomics* 9:75. <http://dx.doi.org/10.1186/1471-2164-9-75>.
34. Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, Yamashita A, Hattori M, Horinouchi S. 2008. Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J Bacteriol* 190:4050–4060. <http://dx.doi.org/10.1128/JB.00204-08>.
35. Rong X, Huang Y. 2010. Taxonomic evaluation of the *Streptomyces griseus* clade using multilocus sequence analysis and DNA-DNA hybridization, with proposal to combine 29 species and three subspecies as 11 genomic species. *Int J Syst Evol Microbiol* 60:696–703. <http://dx.doi.org/10.1099/ijs.0.012419-0>.
36. Moody M, Young R, Jones S, Elliot M. 2013. Comparative analysis of non-coding RNAs in the antibiotic-producing *Streptomyces* bacteria. *BMC Genomics* 14:558. <http://dx.doi.org/10.1186/1471-2164-14-558>.
37. Aitken SM, Kirsch JF. 2005. The enzymology of cystathionine biosynthesis: strategies for the control of substrate and reaction specificity. *Arch Biochem Biophys* 433:166–175. <http://dx.doi.org/10.1016/j.abb.2004.08.024>.
38. Baltz RH. 2012. *Streptomyces* temperate bacteriophage integration systems for stable genetic engineering of actinomycetes (and other organisms). *J Ind Microbiol Biotechnol* 39:661–672. <http://dx.doi.org/10.1007/s10295-011-1069-6>.
39. Combes P, Till R, Bee S, Smith MCM. 2002. The *Streptomyces* genome contains multiple pseudo-attB sites for the  $\phi$ C31-encoded site-specific recombination system. *J Bacteriol* 184:5746–5752. <http://dx.doi.org/10.1128/JB.184.20.5746-5752.2002>.
40. Shinkawa H, Hatada Y, Okada M, Kinashi H, Nimi O. 1995. Nucleotide sequence of a principal sigma factor gene (hrdB) of *Streptomyces griseus*. *J Biochem* 118:494–499.
41. Roe AJ, O'Byrne C, McLaggan D, Booth IR. 2002. Inhibition of *Escherichia coli* growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. *Microbiology* 148:2215–2222. <http://dx.doi.org/10.1099/00221287-148-7-2215>.
42. Hamano Y, Nicchu I, Shimizu T, Onji Y, Hiraki J, Takagi H. 2007.  $\epsilon$ -Poly-L-lysine producer, *Streptomyces albulus*, has feedback-inhibition resistant aspartokinase. *Appl Microbiol Biotechnol* 76:873–882. <http://dx.doi.org/10.1007/s00253-007-1052-3>.
43. Zhao XQ, Gust B, Heide L. 2010. S-Adenosylmethionine (SAM) and antibiotic biosynthesis: effect of external addition of SAM and of overexpression of SAM biosynthesis genes on novobiocin production in *Streptomyces*. *Arch Microbiol* 192:289–297. <http://dx.doi.org/10.1007/s00203-010-0548-x>.
44. Maehr H, Berger J. 1969. The production, isolation and characterization of a grisein-like sideromycin complex. *Biotechnol Bioeng* 11:1111–1123. <http://dx.doi.org/10.1002/bit.260110608>.
45. Thibodeaux CJ, Liu H-W. 2011. Mechanistic studies of 1-aminocyclopropane-1-carboxylate deaminase: characterization of an unusual pyridoxal 5'-phosphate-dependent reaction. *Biochemistry* 50:1950–1962. <http://dx.doi.org/10.1021/bi101927s>.
46. Miles EW, Kraus JP. 2004. Cystathionine  $\beta$ -synthase: structure, function, regulation, and location of homocystinuria-causing mutations. *J Biol Chem* 279:29871–29874. <http://dx.doi.org/10.1074/jbc.R400005200>.