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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Increased valinomycin production in mutants of *Streptomyces* sp. M10 defective in bafilomycin biosynthesis and branched-chain α-keto acid dehydrogenase complex expression

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Abstract *Streptomyces* sp. M10 is a valinomycin-producing bacterial strain that shows potent bioactivity against Botrytis blight of cucumber plants. During studies to increase the yield of valinomycin (a cyclododecadepsipeptide) in strain M10, additional antifungal metabolites, including bafilomycin derivatives (macrolide antibiotics), were identified. To examine the effect of bafilomycin biosynthesis on valinomycin production, the bafilomycin biosynthetic gene cluster was cloned from the genome of strain M10, as were two branched-chain α-keto acid dehydrogenase (BCDH) gene clusters related to precursor supply for bafilomycin biosynthesis. A null mutant (M10bafm) of one bafilomycin biosynthetic gene (*bafV*) failed to produce bafilomycin, but resulted in a 1.2- to 1.5-fold increase in the amount of valinomycin produced. In another null mutant (M10bkdFm) of a gene encoding a subunit of the BCDH complex (*bkdF*), bafilomycin production was completely abolished and valinomycin production increased fourfold relative to that in the wild-type M10 strain. The higher valinomycin yield was likely the result of redistribution of the metabolic flux from bafilomycin to valinomycin biosynthesis, because the two antibiotics share a common precursor, 2-ketoisovaleric acid, a deamination product of valine. The results show that directing precursor flux toward active ingredient biosynthesis could be used as a prospective tool to increase the competence of biofungicides.

Keywords Antifungal metabolite · Bafilomycin biofungicide · Biosynthetic gene cluster · Branched-chain α-keto acid dehydrogenase · *Streptomyces* · Valinomycin

Introduction

The secondary metabolites of *Streptomyces* spp. represent a promising reservoir from which new and improved biofungicides can be developed for plant disease control [6]. However, wild-type strains of natural origin usually produce active components at low titers during fermentation, which make strain improvement an indispensable procedure during the commercialization [4]. Recent studies on biosynthetic pathways, precursor supplies, and genetic regulation of antimicrobials have provided important clues about how metabolic engineering approaches can be employed to enhance the production of metabolites with antimicrobial activity [17, 22, 23, 27, 28].

A culture extract of *Streptomyces* sp. M10 isolated from a soil environment showed potent bioactivity against Botrytis blight [29]. The active antifungal ingredient against *Botrytis cinerea* was identified as valinomycin, a non-ribosomally synthesized cyclododecadepsipeptide consisting of a triplicated tetrapeptide unit made up of D-, L-valine, D-α-hydroxyisovaleric acid, and L-lactic acid [5, 12]. Recently, we found that additional antifungal metabolites besides valinomycin are produced by strain M10. The antifungal compounds were identified as bafilomycin derivatives,

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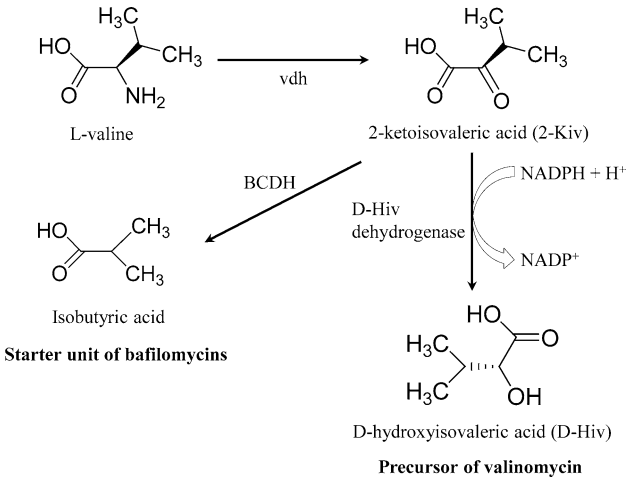


Fig. 1 Valine catabolism provides precursors for bafilomycin and valinomycin production. *vdh* valine dehydrogenase, *BCDH* branched-chain α-keto acid dehydrogenase

as described below. Bafilomycins comprise a group of 16-membered ring macrolide antibiotics that were first isolated from *Streptomyces griseus* and are well-known as vacuolar-type H^+-ATPase inhibitors. These antibiotics were reported to have antibacterial, antifungal, antineoplastic, and immunosuppressive activities but exhibited weak antifungal activity on *B. cinerea* [2, 13, 35, 38]. The macrolactone and hemiacetal ring are synthesized by bafilomycin polyketide synthases (PKSs) using one isobutyric acid as a starter unit, and seven methylmalonic acids, two malonic acids, and two methoxymalonic acids as extender units [31, 39].

While the cyclic peptide structure of valinomycin is composed of D-valine, L-valine, D-α-hydroxyisovaleric acid, and L-lactic acid, the macrolactone core of bafilomycin is assembled by Claisen condensations of short-chain keto acids (e.g., malonic acid, methylmalonic acid, and methoxymalonic acid), starting from the incorporation of isobutyrate on the loading module of the bafilomycin PKS [31]. Although valinomycin and bafilomycins are generated by two different biosynthetic routes, they possibly share a common precursor pool of valine, a branched-chain amino acid. L-valine is reportedly incorporated into the D-valyl and L-valyl portions of valinomycin in equal amounts [24]. The D-α-hydroxyisovaleryl portion of the antibiotic is also supplied to lesser extent by the valine pool by D-hydroxyisovalerate (D-Hiv) dehydrogenase via 2-ketoisovaleric acid (2-Kiv) and D-Hiv (Fig. 1) [21]. The starter unit of bafilomycin biosynthesis, isobutyrate, is also provided by the catabolism of valine [31]. The branched-chain carboxylic acid precursors for polyketide biosynthesis are mainly supplied by the degradation of branched-chain amino acids. In the studies on avermectin biosynthesis in *Streptomyces avermitilis*, valine dehydrogenase catalyzed the deamination of valine to yield 2-Kiv, which is biotransformed to isobutyryl-CoA by a branched-chain α-keto acid dehydrogenase complex (BCDH) [10, 15, 32].

The identification of bafilomycins as additional antifungal metabolites to valinomycin in *Streptomyces* sp. M10 led us to pursue a study of the effects of bafilomycin biosynthesis on valinomycin titers, because the M10 strain is under commercialization as a biocontrol agent against Botrytis blight. In this study, we confirmed bafilomycin production in the M10 strain and cloned the bafilomycin biosynthetic gene clusters, as well as two BCDH gene clusters. In addition, the effects of the BCDH-catalyzed reaction on the titers of valinomycin and bafilomycin were examined by performing mutation and precursor supply analyses.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Escherichia coli strains used in this study were grown according to standard protocols [30]. *Escherichia coli* DH5α was employed for routine gene manipulation. The pTOP TA V2 vector (Macrogen, Seoul, Korea) was used for polymerase chain reaction (PCR) fragment cloning. *Escherichia coli* ET12567 harboring pUZ8002 was used for conjugal transfer to *Streptomyces* [1]. *Streptomyces* sp. M10 was grown on ISP3 agar medium (20 g oatmeal, 18 g agar, and 1000 mL distilled water) as basal medium. For the production analyses of bafilomycin and valinomycin, wild-type or mutant strains were grown on SGGP agar medium (10 g glucose, 4 g peptone, 4 g yeast extract, 4 g casamino acids, 2 g glycine, 0.5 g $MgSO_4 \cdot H_2O$, 1.3 g K_2HPO_4, 15 g agar, and 1000 mL distilled water) at 28 °C for 3 days.

Analysis of bafilomycin and valinomycin production

The methanol extracts of the bacterial cultures were centrifuged at 8000 rpm for 30 min to remove cells and then concentrated in vacuo by using a rotary evaporator (Büchi, Flawil, Switzerland). The concentrates were suspended in distilled water and loaded onto a column filled with Diaion HP-20 resin (Mitsubishi Chemical Co., Tokyo, Japan). After elution via stepwise gradients of methanol–water, the active fractions (80–100 % methanol, v/v) were pooled in ethylacetate and further chromatographed on a silica gel column (silica gel 60, 23–660-μm mesh; Merck, Kenilworth, NJ, USA) with stepwise gradients of ethylacetate-methanol. The eluates of ethylacetate-methanol (100:0–70:30, v/v) retained antifungal activity. The active fractions were pooled and further purified by

using a semi-preparative Varian Prostar 210 HPLC (high-performance liquid chromatography) System (Varian Inc., Palo Alto, CA, USA) equipped with a C18 reversed-phase column (ODSH80, 1 × 25 cm, 4 μm; YMC Co., Kyoto, Japan), and an elution system of linear gradients from 70 to 95 % methanol in distilled water (supplemented with 0.1 % aqueous formic acid) for 20 min, followed by isocratic elution for 40 min (2 mL/min; ultraviolet (UV) detection at 210 nm). Titers of bafilomycins and valinomycin produced from wild-type and mutant strains were calculated using a standard curve created with known amounts of bafilomycin A1 (Sigma-Aldrich, USA), bafilomycin derivatives (purified in this study), and valinomycin (Sigma-Aldrich, USA), respectively. HPLC analyses for quantifications were performed using the same method described above. The sum of the titers of six bafilomycin derivatives was indicated as the titers of bafilomycins. High-resolution mass spectra of the purified compounds were recorded on a quadruple-time of flight tandem mass spectrometer (Waters, Manchester, UK) by using the electrospray ionization-mass spectrometry (ESI–MS) method. Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a Varian 500 NMR spectrometer (Varian Inc.). ^{1}H-NMR spectra (500 MHz) of compounds were measured in $CDCl_3$ (99.96 atom % D; Sigma-Aldrich Corporation, St. Louis, MO, USA). Chemical shifts are given in δ values (ppm) referenced to the proton of the solvent at 7.26 ppm, and coupling constants (J) are given in Hz. ^{13}C NMR spectra (125 MHz) were recorded in $CDCl_3$ by using broad-band proton decoupling. The structure of the compounds was further deduced by 2D-NMR analyses, including ^{1}H–^{1}H gradient-selected correlation spectra and hetero-nuclear shift correlations via multiple bond correlation experiments. Data were processed by using Mestre Nova version 6.0.2-5475 software (Mestrelab Research, Santiago de Compostela, Spain).

Construction of a genomic library and screening of the bafilomycin biosynthetic gene cluster

Streptomyces sp. M10 genomic DNA was extracted and purified as described previously [16]. A strain M10 genomic DNA library was constructed by using the Copy-Control Fosmid Library Production Kit (Epicentre, Madison, WI, USA). For PCR-based screening of the fosmid clones harboring the bafilomycin biosynthetic gene cluster, a primer set was designed from a conserved region of the ketosynthase domain of the type I PKSs, as follows: KSLF forward primer (5′-CCSCAGSAGCGCSTSYTSCTSGA-3′) and KSLR reverse primer (5′-GTSCCSGTSCCGTGSGYSTCSA-3′). The PCR amplification conditions comprised an initial denaturation step at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 3 min. All PCR amplifications were performed by using 2X BluePreMix (8-Strips)-MG™ *Taq* PCR Premix (Macrogen, Seoul, Korea) according to the manufacturer's instructions. The fosmid clones generating the DNA fragments (~700 bp) that were homologous to known KS domains were selected for further sequencing.

Screening of BCDH gene clusters

The primer sets for the two BCDH gene clusters included bkd-1F/R [32] and bkdF-F/R [10], which are both derived from the *S. avermitilis* genomic region containing the E1α and E1β open reading frames (ORFs). The primer sequences are listed in Supplementary Table S1. The PCR products were confirmed by DNA sequencing and then used as probes for fosmid library screening.

Targeted disruption of genes related to bafilomycin biosynthesis

To confirm that the fosmid clone 11E-1013 contained the genes involved in bafilomycin biosynthesis, one insertional mutant of *Streptomyces* sp. M10 was generated by replacing a part of the thioesterase domain with an apramycin resistance cassette via the PCR-targeted gene replacement method [14]. The *aac(3)IV* apramycin resistance gene and *oriT* were amplified from the pIJ773 disruption cassette. The PCR product resulting from the bafTE AprF and bafTE AprR primer set was introduced into *E. coli* pRedET/p11E-1013, and replacement of the thioesterase domain was confirmed by PCR with the bafTE-F and bafTE-R primer set, designed to amplify 100-bp upstream and downstream of the replaced region.

In addition, the *bkdA* and *bkdF* genes were disrupted by using the PCR-targeted *Streptomyces* gene replacement method [14]. The *aac(3)IV* apramycin resistance gene and *oriT* were amplified from the pIJ773 disruption cassette. The PCR product of the primer set (bkdA AprF and bkdA AprR) containing apramycin resistance gene was introduced into *E. coli* pRedET/p1E-52. The PCR product of bkdF AprF and bkdF AprR primers was introduced to *E. coli* pRedET/p4A-292. The replacement mutants were selected by PCR (using bkdA-F/R and bkdF-F/R primer sets) and used for transformation of the strain M10. The primer sequences used for disruption are listed in Supplementary Table S1.

The conjugation procedure was performed following the standard method [19]. Double-crossover allelic exchange was confirmed by PCR and southern blotting analysis. For southern blotting analysis, genomic DNAs from wild-type and mutant strains were digested with an appropriate

enzyme (*SmaI*; Roche, Indianapolis, IN, USA) and then probed by using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Manheim, Germany).

Gene expression analysis by RT-PCR

The mycelia from wild-type and mutant strains were harvested by centrifugation (13,000 rpm, 5 min) after 16, 24, 36, 48, and 60 h of growth in SGGP broth culture, and stored at −80 °C for RNA extraction. The whole-cell RNA was extracted by using the NucleoSpin® RNA Midi Kit (Macherey–Nagel, Bethlehem, PA, USA), and the cDNA derived from the whole-cell RNA was generated by using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR of target genes was performed by using the iCycler™ Real-Time PCR Detection System and the iQ™ SYBR Green Supermix Kit (Bio-Rad). Real-time PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 30 s, and extension at 68 °C for 35 s. The amplification was internally normalized to 16S rDNA levels. Each primer pair was designed to generate a fragment of ~300 bp (Supplementary Table S1).

Complementation of the *bkdR* gene disruption mutant

For the complementation analysis, the complete *bkdR* DNA fragment was amplified from *Streptomyces* sp. M10 genomic DNA by PCR with bkdRC-F/R primer sets (Supplementary Table S1). The amplified fragment was subsequently inserted into the *Eco*RV site of a pSET152 derivative integrative plasmid containing a strong constitutive promoter, *ermE** [37]. The resulting plasmid was passed through *E.coli* ET12567/pUZ8002 and then introduced into a *bkdR* gene disruption mutant (M10bkdRm) by conjugal transfer. The resulting transformants were selected by apramycin resistance and confirmed by PCR.

Precursor feeding experiment

SGGP broth medium supplemented with L-valine (10, 20, 40, 80, and 100 mM), L-leucine (20 mM), L-isoleucine (20 mM), α-methyl-butyric acid (5 mM), isobutyric acid (5 mM), or isovaleric acid (5 mM) was used to analyze the feeding effect of branched-chain amino acids and their deamination products on bafilomycin and valinomycin production. The reagents used in this experiment were purchased from Sigma-Aldrich Corporation. Broth cultures were incubated at 28 °C for 3 days using a 1-L baffled flask, followed by extraction of the antibiotics. The dry cell weight of all treatments tested was measured by weight of freeze-drying cells. The antibiotics were analyzed as described above.

Results and discussion

During studies to increase the titer of valinomycin, a mutant showing defects in valinomycin production still exhibited weak antifungal activity against *B. cinerea* and moderate activity against other plant pathogenic fungi, such as *Colletotrichum orbiculare*. We therefore speculated that additional antifungal metabolites besides valinomycin might be produced by strain M10, and thus we set out to identify these metabolites.

Identification of antifungal compounds produced by the M10 strain

Additional antibiotic compounds M10-1, 2, 3, 4, 5, and 6 were isolated from agar cultures of *Streptomyces* sp. M10 through a series of extractions and column chromatographies. In a paper disk assay of eluates collected via semi-preparative HPLC (Fig. 2a), peaks corresponding to M10-1, 2, 3, 4, 5, and 6 exhibited antifungal activity against *C. orbiculare*, while only valinomycin (compound CN1) exerted antifungal activity against *B. cinerea* (Fig. 2b, c).

M10-1, 2, 3, 4, 5, and 6 were obtained as colorless or yellow amorphous powders by preparative HPLC (Fig. 2a). The UV-absorption spectra of the compounds showed similar patterns, with maximum absorption wavelengths at 208, 211, 208, 214, 212, and 251 nm, respectively, and additional shoulders between 250 and 260 nm (Supplementary Fig. S1). The structures of these compounds were identified on the basis of high-resolution (HR) ESI–MS, one-dimensional (1D) NMR, and two-dimensional (2D) NMR (in $CDCl_3$) spectra (Supplementary Table S2). Compounds M10-1, 2, 3, 4, 5, and 6 yielded $[M–H]^-$ data at *m/z* 621.4013, *m/z* 635.4155, *m/z* 719.4002, *m/z* 733.4153, *m/z* 814.4380, and *m/z* 828.4525, respectively, indicating molecular formulas of $C_{35}H_{58}O_9$ (calculated $[M–H]^-$ ion at *m/z* 621.4003), $C_{36}H_{60}O_9$ (calculated $[M–H]^-$ ion at *m/z* 635.4159), $C_{39}H_{60}O_{12}$ (calculated $[M–H]^-$ ion at *m/z* 719.4007), $C_{40}H_{62}O_{12}$ (calculated $[M–H]^-$ ion at *m/z* 733.4163), $C_{44}H_{65}NO_{13}$ (calculated $[M–H]^-$ ion at *m/z* 814.4378), and $C_{45}H_{67}NO_{13}$ (calculated $[M–H]^-$ ion at *m/z* 828.4534). The ^{1}H-NMR spectrum showed signals for nine methyl groups at δ H (1.99, 1.94, 1.07, 1.02, 0.99, 0.93, 0.86, 0.83, and 0.77, 3H each), and two methoxy groups at δ H (3.64, 1H) and δ H (3.24, 3H). The ^{13}C-NMR spectrum also revealed nine methyl groups at δ C (21.8, 21.4, 20.3, 17.4, 14.4, 14.2, 12.3, 10.0, and 7.2), and two methoxy groups at δ C 60.1 and δ C 55.7.

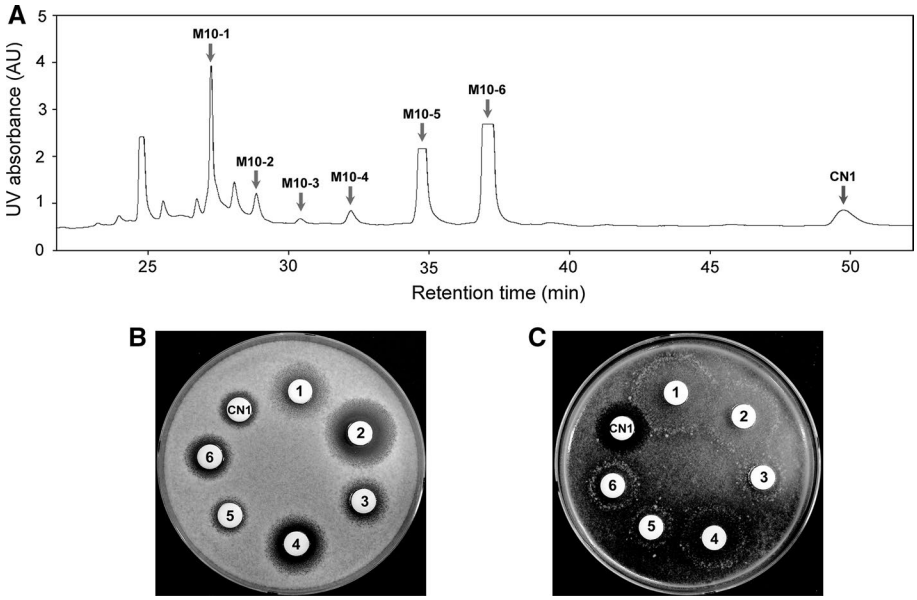
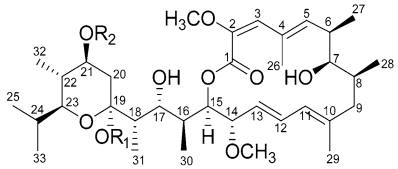


Fig. 2 **a** High-performance liquid chromatogram of strain M10 crude extracts obtained via silica gel column chromatography. **b**, **c** Antifungal activity of M10 peaks against *C. orbiculare* (**b**) and *B. cinerea* (**c**). The peaks of bafilomycin (M10-1 to 6) and valinomycin (CN1) are indicated by *black arrows*. *AU* absorbance units



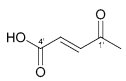
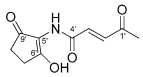
Compounds	Molecular weight	Bafilomycin	R_1	R_2
M10-1	622	A1	H	H
M10-2	636	A2	CH_3	H
M10-3	720	C1	H	
M10-4	734	C2	CH_3	
M10-5	815	B1	H	
M10-6	829	B2	CH_3	

Fig. 3 Structure and mass spectra of bafilomycins isolated from *Streptomyces* sp. M10

Based on the HR–ESI–MS and NMR spectra and those found in references, the M10 compounds were assigned the carbon skeleton of bafilomycins, but differed in *O*-methylation at C-19 and fumarate moiety or *N*-(3-hydroxy-2-cyclopentenone-2yl)-fumarylester monoamide moiety at C-21 (Fig. 3) [7, 20, 36].

Production of multiple secondary metabolites derived from similar precursors might result in competition for these precursors, and negatively affect the titers of each compound. Although valinomycin and bafilomycins are derived from two different kinds of biosynthetic machineries, non-ribosomal peptide synthases (NRPSs) and PKSs, we wished to determine what effect bafilomycin biosynthesis might have on valinomycin titers in strain M10. For this purpose, we cloned the bafilomycin biosynthetic gene cluster and constructed a mutant strain that did not produce bafilomycin derivatives.

Cloning and disruption of bafilomycin biosynthetic genes

We constructed a genomic DNA library of *Streptomyces* sp. M10 to screen and sequence the bafilomycin biosynthetic gene cluster. The fosmid clones, 1G-80, 1E-53, 11E-1013, and 12F-1122, contained overlapping parts of the gene cluster. The sequence of the gene cluster (76,091 bp) is deposited in GenBank under accession number KM245330. The predicted functions, gene homologies, and protein domains of the bafilomycin biosynthetic genes from M10 strain are shown in Supplementary Table S3 and Supplementary Fig. S2 and S3.

Two bafilomycin PKS gene clusters were known so far. The bafilomycin PKS gene cluster in *S. lohii* is composed of five ORFs (*bafAI–bafAV*, 59 kb) [39]. Another bafilomycin PKS gene cluster (*bafSI–bafSV*, 58.5 kb) was identified in the genome of *S. griseus* DSM 2608 [18]. The five ORFs of strain M10 (*bafI–bafV*) showed 99–100 % identity with those of *S. griseus* and 91–98 % identity with those of *S. lohii*.

To investigate the effect of bafilomycin biosynthesis on valinomycin titer, a disruption mutant (M10bafm) was generated. Bafilomycins were not detected in the culture extract of the M10bafm strain, but instead a 1.2- to 1.5-fold (4.8–6.0 mg/L) higher yield of valinomycin was reproducibly observed (data not shown). Valine is a building block of valinomycin, and its deamination product (2-Kiv) produced by valine dehydrogenase is also recruited by valinomycin

NRPSs after reduction to D-Hiv by D-Hiv dehydrogenase (Fig. 1). Therefore, the precursor pool of valine is critically important in supplying the building blocks for valinomycin production. However, the valinomycin NRPSs of strain M10 possibly share the valine precursor pool with bafilomycin PKSs, because 2-Kiv can also be catalyzed by BCDH to give isobutyrate, the starter unit of bafilomycin. The blockade of the valine pool draining toward bafilomycin production may be a reason for the increased synthesis of valinomycin in the M10bafm strain. We speculate that the *Streptomyces* sp. M10 genome contains BCDH gene clusters to supply isobutyryl-CoA to the loading module-AT domains of bafilomycin PKSs and that disruption of the BCDH-catalyzed pathway possibly has a positive impact on valinomycin production.

Increased production of valinomycin in a BCDH null mutant

BCDH-catalyzed amino acid catabolism is a crucial step in providing branched-chain acyl-CoA precursors for fatty acid and polyketide biosynthesis [8, 10, 34]. The BCDH complex catalyzes oxidative decarboxylations of 2-Kiv, 2-keto-3-methylvalerate, and 2-ketoisocaproate (the deamination products of the branched-chain amino acids, valine, isoleucine, and leucine, respectively), releasing CO_2 and generating corresponding acyl-CoA and NADH [26]. *Streptomyces* spp. were reported to contain two sets of BCDH gene clusters, each of which encodes the E1α (a dehydrogenase), E1β (a decarboxylase), and E2 (an acyltransferase) subunits of the BCDH complex.

We identified two sets of a gene cluster, which most likely constitutes a BCDH complex, by PCR-based screening of the M10 strain genomic DNA library. The products of the first gene cluster (3544 bp) in fosmid clone 1E-52 showed 86–97 % identity to the *bkdA*, *bkdB*, and *bkdC* products of *S. avermitilis*, and those of the other cluster (3920 bp) found in fosmid clone 4A-292, revealed 84–95 % identity to the *bkdF, bkdG,* and *bkdH* products (Fig. 4a). Therefore, these ORFs were termed *bkdA*, *bkdB*, *bkdC*, *bkdF, bkdG,* and *bkdH*. The GenBank accession numbers of the *bkdABC* and *bkdFGH* gene clusters of strain M10 are KM216827 and KM216828, respectively.

Null mutants of *bkdA* and *bkdF* were generated by replacing the corresponding sequences with an apramycin resistance gene. The replacement was confirmed by southern blot hybridization and PCR analysis with a primer set (bkdA-F/R or bkdF-F/R) designed to amplify 100-bp upstream and downstream of the replaced region (Fig. 4). The size of the DNA fragment amplified from the wild-type genomic DNA was ~1.1 kb, but the null mutant strains, M10bkdAm (*ΔbkdA::acc(3)IV*) and M10bkdFm (*ΔbkdF::acc(3)IV*), each generated a 1.5-kb DNA fragment, reflecting the size of the disruption cassette (Fig. 4b). The *SmaI*-digested DNA fragment of the M10bkdAm (or M10bkdFm)-containing apramycin cassette was larger than that of the wild-type M10 strain in southern blotting analysis (Fig. 4c). The production rates of bafilomycin and valinomycin by the M10bkdAm strain were not significantly altered relative to those of the wild-type M10 strain (Fig. 4d). However, bafilomycin production in the *bkdF* null mutant (M10bkdFm) was completely abolished (Fig. 4e). Moreover, valinomycin production in the M10bkdFm strain was fourfold (16.0 mg/L) higher than that in the wild-type strain. The M10bkdFm culture extract also showed higher antifungal activity against *B. cinerea* (Fig. 4f, g).

The *bkdF* gene product is crucial for production of polyketide compounds having a branched-chain fatty acid as the starter unit. In avermectin biosynthesis, isobutyryl-CoA and 2-methylbutyryl-CoA are used as starter units. A deletion of the 5′ region of the *bkdFGH* gene cluster in *S. avermitilis* results in a complete loss of the ability to synthesize avermectins [10]. Elevated valinomycin yields in M10bkdFm are likely the result of redistribution of the metabolic flux from bafilomycin to valinomycin biosynthesis, because the compounds share 2-Kiv as a precursor in their biosynthetic routes. L-valine is converted into 2-Kiv by valine dehydrogenase and then D-Hiv dehydrogenase catalyzes the dehydrogenation of 2-Kiv to generate D-Hiv [21]. Disruption of the *bkdF* gene causes the loss of BCDH activity and thereby the 2-Kiv intermediate is mainly converted into D-Hiv, resulting in increased valinomycin synthesis (Fig. 1).

Compared with the increase in valinomycin yield in the M10bkdAm null mutant (1.2- to 1.5-fold), the increase in the M10bkdFm null mutant (fourfold) was markedly higher. One explanation is that disruption of bafilomycin biosynthesis in the M10bkdAm mutant did not result in the exclusive redirection of the valine and 2-Kiv flux toward valinomycin biosynthesis, probably because the M10 strain has other biosynthetic machineries that recruit branched-chain fatty acids catalyzed by the BCDH complex.

Role of the *bkdR* gene product in bafilomycin production

We investigated the ORFs surrounding the BCDH gene clusters of strain M10 and identified an ORF in fosmid clone 1E-52 with a deduced amino acid sequence homologous to that of the regulatory protein, bkdR (Fig. 4). The *bkdR* gene (594 bp) is located upstream of *bkdA*. In a neighbor-joining tree based on amino acid sequence homology, *bkdR* clustered with other *bkdR* genes from *Streptomyces* spp. The group was confirmed by its high bootstrap value (100 %) (Fig. 5a). The *bkdR* gene product

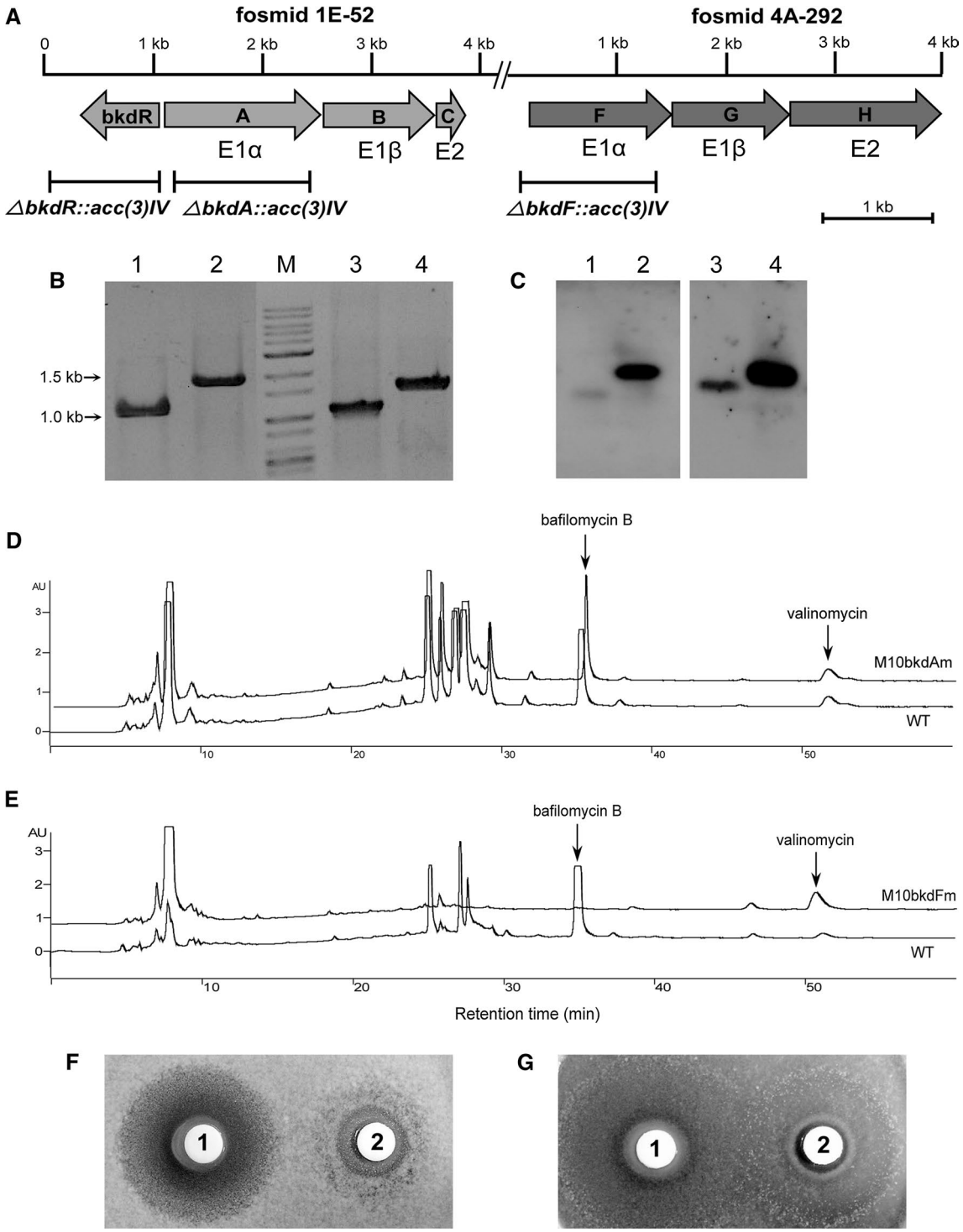


Fig. 4 **a** Organization of the *Streptomyces* sp. M10 BCDH gene clusters. The deletions in the *ΔbkdA::acc(3)IV* and *ΔbkdF::acc(3)IV* mutants are indicated by *solid lines* below the clusters. **b** PCR confirmation of the double-crossover mutation. M, 1.0-kb DNA plus ladder; *lane 1*, PCR product of *bkdA* gene in wild-type M10 strain; *lane 2*, corresponding PCR product in M10bkdAm strain; *lane 3*, PCR product of *bkdF* gene in wild-type M10 strain; *lane 4*, corresponding PCR product in M10bkdFm strain. **c** Southern blot analysis of wild-type M10 strain (*lane 1*, *3*), mutant M10bkdAm strain (*lane 2*), and mutant M10bkdFm strain (*lane 4*). **d**, **e** HPLC analysis of culture extract from wild-type M10 strain versus mutant M10bkdAm strain (**d**), and wild-type M10 strain versus mutant M10bdkFm strain (**e**). **f**, **g** Antifungal activity against *C. orbiculare* (**f**) and *B. cinerea* (**g**). 1, Culture extract from wild-type M10 strain; 2, culture extract from M10bkdFm strain

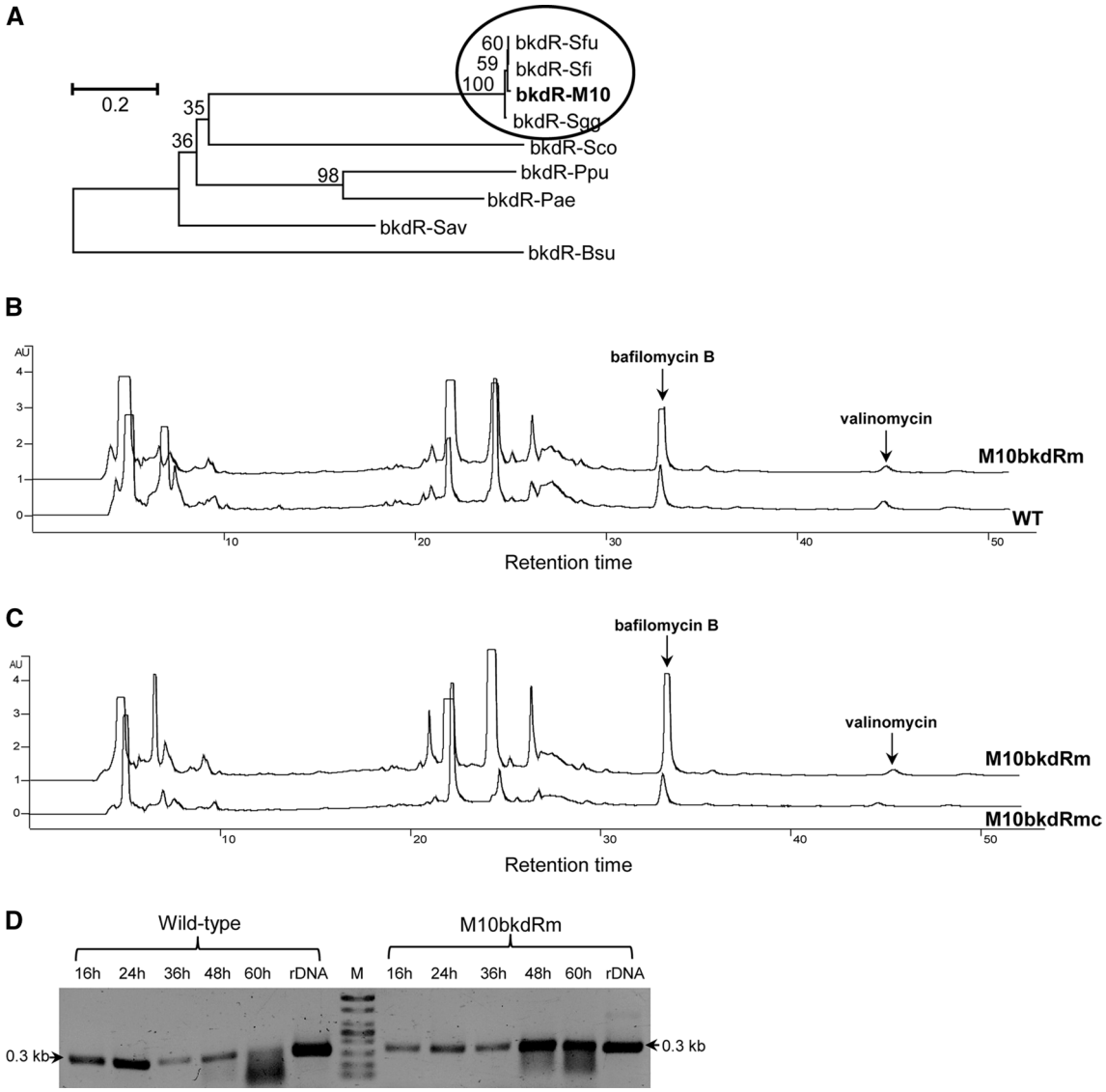


Fig. 5 **a** Neighbor-joining tree constructed from multiple alignments of proteins predicted from *bkdR* genes encoding transcriptional regulators of the branched-chain amino acid dehydrogenase complex. bkdR-Sfu, *S. fulvissimus* DSM 40593; bkdR-Sfi, *S. filamentosus*; bkdR-Sgi, *S. griseus* XylebKG-1; bkdR-Sgg, *S. griseus* subsp. *griseus* NBRC 13350; bkdR-Sco, *S. coelicolor* A3(2); bkdR-Sav, *S. avermitilis*; bkdR-M10, *Streptomyces* sp. M10; bkdR-Ppu, *P. putida*; bkdR-Pae, *Pseudomonas aeruginosa*; bkdR-Bsu, *B. subtilis*. **b** HPLC analysis of culture extracts of wild-type M10 and mutant M10bkdRm strains. **c** HPLC analysis of culture extracts of mutant M10bkdRm and M10bkdRmc strains. **d** RT-PCR gene expression analysis of *bkdF* transcripts in wild-type M10 and mutant M10bkdRm strains, harvested after 16, 24, 36, 48, and 60 h of growth in SGGP broth medium. Transcription of the 16S *rRNA* gene was used as an internal control

showed high similarity to the leucine-responsive regulatory protein (Lrp) class of transcription factors. Most Lrp homologues are specific regulators of amino acid metabolism-related genes [3]. To investigate whether the *bkdR* product affects the metabolism of branched-chain amino acids involved in valinomycin and bafilomycin production, we constructed a *bkdR* gene disruption mutant (M10bkdRm, *ΔbkdR::acc(3)IV*) (Fig. 4a), and compared its capacity to generate antibiotics with that of the wild-type M10 strain. The M10bkdRm strain produced 2.4-fold more bafilomycin B than the wild-type strain (Fig. 5b). To exclude the possibility that the increased production of bafilomycin B was due to a polar effect of *bkdR* gene disruption, a plasmid (pSET152::*bkdR*), constructed with the wild-type *bkdR* gene under the control of the *erm*E* promoter, was introduced into the M10bkdRm strain. HPLC analysis showed that the amount of bafilomycin B produced by the complemented strain (M10bkdRmc) was similar to that produced by the parental strain (Fig. 5c), indicating that *bkdR* gene disruption did not result in a polar effect.

RT-PCR was employed to confirm that the *bkdR* gene product affected the precursor supply catalyzed by the BCDH complex through transcriptional regulation of the BCDH gene cluster. Total RNA was isolated from wild-type and M10bkdRm cells harvested after 16, 24, 36, 48, and 60 h of culture in SGGP broth. Primer sets for RT-PCR were designed to produce ~300-bp cDNAs of *bkdF*. The cDNA amplified from the 16S rRNA gene was used as an internal control. As shown in Fig. 5d, a transcript amplified from *bkdF* in wild-type cells was abundant during the early growth stage (16–24 h). This transcript apparently decreased as growth proceeded, nearly disappearing at 60 h after inoculation. However, the transcript level detected in the M10bkdRm cells was dramatically increased during growth at 48–60 h after inoculation. These results indicate that the *bkdR* gene product negatively regulates the transcription of *bkdF* and the disruption of *bkdR* gene results in the increased production of bafilomycin B.

We speculated that the increased production of bafilomycin B would cause depletion of 2-ketoisovaleric acid (2-Kiv) and valine and then result in decrease of valinomycin yield. However, the valinomycin yield was not significantly affected by *bkdR* gene disruption. A possible explanation for the result will be replenishment of valine through linking central carbon metabolism to the pathway flux toward valine. Since valine is an amino acid essential for maintaining metabolism, the strain would recruit metabolic pathways to replenish valine pool. Through *de novo* synthesis of valine, pyruvate flux can be channeled to L-valine via 2-Kiv [11]. As far as the pool of 2-Kiv is maintained, valinomycin yields may not be affected by increased production of bafilomycins.

In previous reports on *Pseudomonas putida* and *Bacillus subtilis*, the *bkdR* gene was reported to act as a transcriptional activator. In *P. putida*, transcription of the *bkd* gene cluster was activated by binding of branched-chain amino acids to the bkdR protein [25]. Isoleucine and valine utilization were overturned in *bkdR* disruption mutants of *B. subtilis*, and the expression of the *bkd* operon was induced by adding isoleucine or valine to the growth medium [9]. However, the *bkdR* gene in *S. coelicolor* apparently encodes a transcriptional repressor of the *bkdFGH* gene cluster and affects morphogenesis and antibiotic production [33]. Given the increase in bafilomycin production and the lack of transcriptional repression of *bkdF* in the M10bkdRm strain, we conclude that the *bkdR* gene product also acts as a transcriptional repressor of a BCDH gene cluster and in this manner directs the 2-Kiv flux toward bafilomycin biosynthesis.

Antibiotic production from precursor feeding

Three kinds of branched-chain amino acids (valine, isoleucine, and leucine) are converted into α-keto acids by valine dehydrogenase in the pathways of branched-chain amino acid catabolism in *Streptomyces* species. The valine-, isoleucine-, and leucine-derived α-keto acids are then transformed into isobutyryl-CoA, α-methylbutyryl-CoA, and isovaleryl-CoA, respectively, through oxidative decarboxylation catalyzed by BCDH [34]. The *bkdF* disruption mutant showed that the BCDH-catalyzed reaction is an essential step in providing the precursor for bafilomycin. To examine the effect of diverse precursors catalyzed by BCDH on valinomycin and bafilomycin synthesis, three branched-chain amino acids (L-valine, L-leucine, and L-isoleucine) and their deamination intermediates (isobutyric acid, isovaleric acid, and α-methyl-butyric acid) were, respectively, added to the culture medium of the wild-type M10 and mutant M10bkdFm strains.

As shown in Fig. 6, antibiotic production in the wild-type strain was increased by ~2.0-fold by the addition of 40–80 mM valine for bafilomycin and 10–20 mM valine for valinomycin, while the biomass of the wild-type strain was not significantly changed by the addition of the amino acid (Fig. 6a). We found that L-valine could be used as a direct precursor source to improve bafilomycin and valinomycin titers. Addition of isoleucine (20 mM) resulted in ~1.6-fold higher bafilomycin production in the wild-type strain (from 16.6 to 26.7 mg/L), suggesting that isoleucine catabolism provides some of the precursors for bafilomycin biosynthesis. Isobutyric acid, the product of valine dehydrogenase-/BCDH-catalyzed reaction of valine, led to a ~ 1.8-fold higher yield of bafilomycin, without any detectable effect on biomass. Similar results were obtained for the M10bkdFm strain when isobutyric acid was added to the culture medium (Fig. 6b).

The M10bkdFm strain did not produced any detectable amount of bafilomycins. Isobutyric acid supplementation, however resulted in resumption of bafilomycin production (~6.8 mg/L). The fact that bafilomycin production was recovered by precursor feeding suggests that the starter unit (isobutyryl-CoA) of bafilomycin is mainly supplied by the BCDH-catalyzed reaction. Bafilomycin production in the M10bkdFm strain was recovered by supplementation of the medium with diverse precursors. However, under all conditions tested (except for the addition of isobutyric acid), the recovery was limited, suggesting that the supplemented components are not readily transformed into bafilomycin precursors in the mutant strain with a defect in BCDH-facilitated catalysis.

The M10bkdFm strain produced 11.7–22.1 mg/L valinomycin when grown in SGGP media supplemented with valine (Fig. 6b), with ~40 % more valinomycin generated by the addition of 10 or 100 mM valine. No significant increase in valinomycin yield was observed at other valine concentrations. As noted above, the BCDH null mutation resulted in fourfold higher valinomycin production than

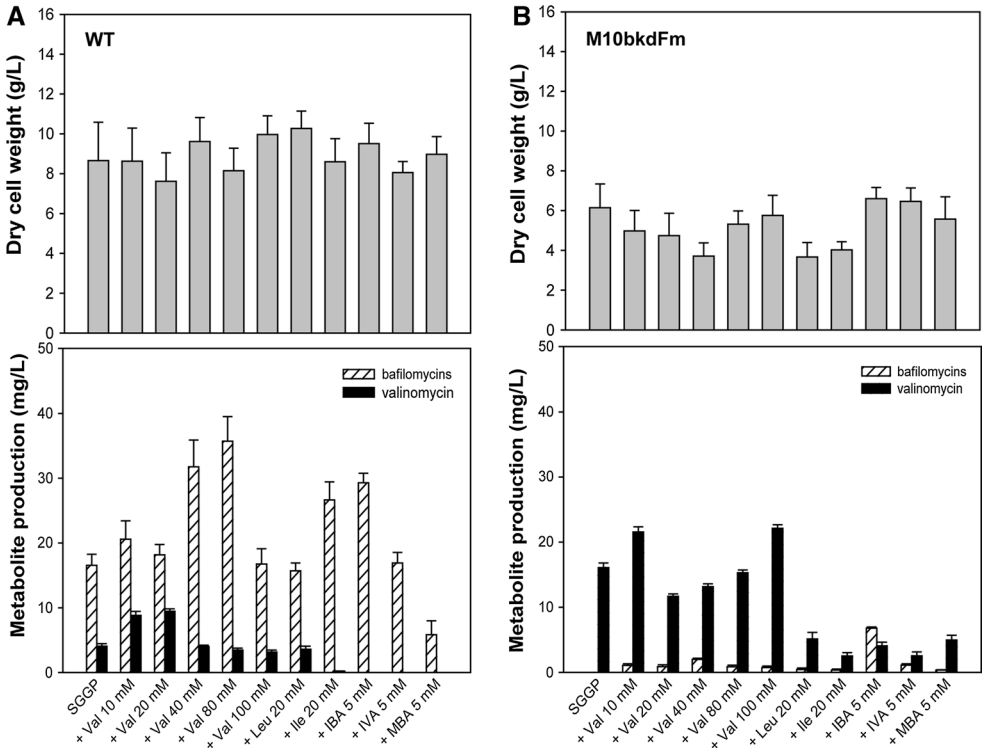


Fig. 6 Bafilomycin and valinomycin production from culture medium supplemented with branched-chain amino acids and their deaminated intermediates in the wild-type M10 strain and the *bkdF* gene disruption mutant (M10bkdFm) in SGGP medium. **a** Dry cell weight and metabolite production in wild-type M10 strain. **b** Dry cell weight and metabolite production in mutant M10bkdFm strain. *WT* wild-type, *Val* L-valine, *Leu* L-leucine, *Ile* L-isoleucine, *IBA* isobutyric acid, *IVA* isovaleric acid, *MBA* α-methylbutyric acid. Values are presented as averages ± standard deviations from three independent measurements

the wild-type strain without valine feeding. The relatively small increase in valine feeding in the mutant shows that valine feeding cannot exert a marked stimulatory effect on valinomycin yields in the mutant strains lacking BCDH activity. Therefore, we speculate that attenuating precursor draining away from bafilomycin biosynthesis relieves the limit in the supply of precursors toward valinomycin, and such situation, precursor concentration may not be a major limiting factor anymore in the valinomycin production.

Conclusions

Streptomyces is a promising resource for biofungicides to control plant diseases. However, strain improvement to increase the titers of active ingredients is an indispensable procedure during commercialization. Although multiple-fold increases in the titers of active ingredients might not result in a drastic increase in biofungicide efficiency, two-fold or threefold increases in active ingredient yield might result in appreciable reductions in production costs by up to one-half or one-third. Genomic research on assorted *Streptomyces* spp. has revealed multiple biosynthetic gene clusters of secondary metabolites and the production of multiple metabolites derived from similar precursors in the same cell might be competitive and negatively affect the titers of each generated compound. Although the impact of precursor limitation and the regulatory relationships between the metabolites are still unclear, directing the precursor flux toward active ingredient biosynthesis could be used as a tool to increase the yield of active ingredients of biofungicides.

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