# Reconstituted Biosynthesis of the Nonribosomal Macrolactone Antibiotic Valinomycin in *Escherichia coli*

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S *Supporting Information*

ABSTRACT: The structural complexity of nonribosomal peptides (NRPs) impeding economic chemical synthesis and poor cultivability of source organisms limits the development of bioprocesses for novel bioactive compounds. Since nonribosomal peptide synthetases (NRPSs) assemble NRPs from simple amino acid building blocks, heterologous expression of NRPSs in a robust and easy to manipulate expression host is an attractive strategy to make pharmaceutically relevant NRPs more accessible and is also a basis for engineering of these enzymes to generate novel synthetic bioactive compounds. Here we show a systematic approach for the heterologous expression of the 654 kDa heterodimeric valinomycin synthetase (VlmSyn) from *Streptomyces tsusimaensis* in a soluble and active form in *Escherichia coli*. VlmSyn activity and precursor requirements were determined *in vitro* and provided evidence for a previously proposed model of valinomycin biosynthesis. *In vivo* production of recombinant valinomycin, a macrolactone antibiotic with reported antifungal, antibacterial, and antiviral activities, was achieved using an engineered *E. coli* strain growing in inexpensive media and independent of the supplementation with precursors and further optimization of the cultivation conditions. Tailoring of VlmSyn in *E. coli* paves the way to the production of novel valinomycin analogues in the future.

KEYWORDS: valinomycin biosynthesis, nonribosomal peptide synthetase, heterologous expression, cyclodepsipeptide antibiotic, secondary metabolite production, synthetic biology
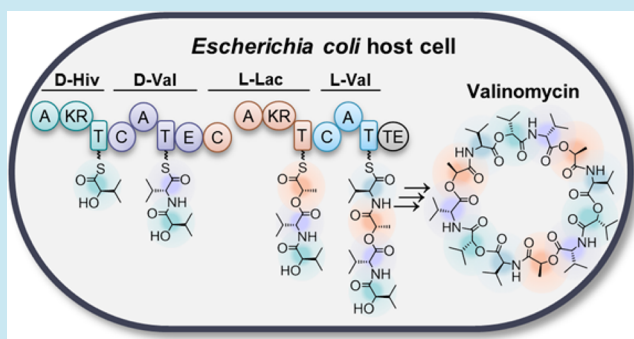
Nonribosomal peptides (NRPs) belong to a class of microbial secondary metabolites that has been a prolific source of bioactive compounds with a wide spectrum of medicinal applications, including, but not limited to, antibiotics (*e.g.*, vancomycin), immunosuppressants (*e.g.*, cyclosporine A), and antineoplastics (*e.g.*, bleomycin A2). This structurally diverse group is assembled from amino acid building blocks by a common thiotemplate-directed multistep reaction mechanism catalyzed by large modular enzymes termed nonribosomal peptide synthetases (NRPSs).[1,2] Each module comprises domains for the incorporation of a single building block, typically involving an adenylation (A) domain for substrate recognition and activation, a thiolation (T) domain for covalent substrate tethering, and a condensation (C) domain for peptide bond formation. The building blocks of NRPs are not limited to the 20 proteinogenic amino acids, but can include modified versions of amino acids (D-forms, methylated, and hydroxylated) as well as α-hydroxy and other carboxylic acids.[3]

Unlike structurally simpler, synthetic small molecules, NRP discovery and industrial exploitation can be limited because of finite sustainable sources and impractical chemical synthesis. The supply is especially restricted when source organisms are rare or cannot be cultured readily in the laboratory, as is the case for an estimated 99% of all bacteria.[4] Assembly and

tailoring of the NRP scaffold can directly be linked to the genetically encoded domain architecture of NRPSs, and modern synthetic biology approaches often involve the expression and manipulation of the necessary biosynthetic pathway constituents in a surrogate host that combines robust growth with the potential for genetic engineering.[5] The qualities of *Escherichia coli* as a versatile host with numerous available genetic tools and favorable growth characteristics are undisputed. *E. coli* has been used successfully to produce several polyketides, which are assembled by a closely related thiotemplate-mechanism, as well as hybrid polyketide-nonribosomal peptides.[6,7] However, relatively few examples of bioactive NRPs produced in *E. coli* as an autonomous cell factory have been reported to date.[8−11] However the ability of recombinant NRPS production will enable the formation of novel synthetic bioactive compounds as shown by the comprehensive work on echinomycin.[12]

Valinomycin is a cyclooligomer depsipeptide (COD) produced by several *Streptomyces* strains. It displays a diverse spectrum of biological activities that range from antifungal[13] and antibacterial,[14] insecticidal-nematocidal,[15] antiparasitic[16]
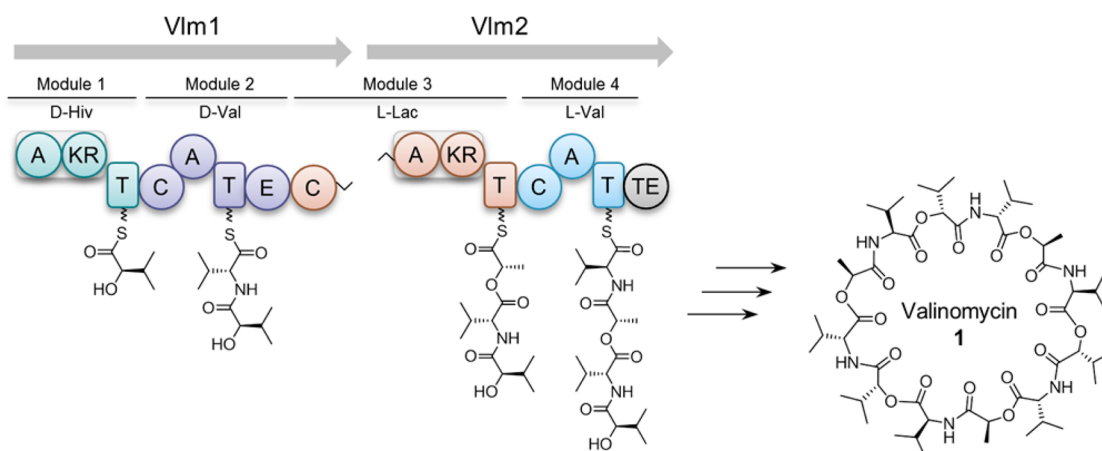
Figure 1. Proposed model for valinomycin biosynthesis. The iterative, tetramodular valinomycin synthetase includes domains with adenylation (A), ketoreductase (KR), thiolation (T), epimerase (E), and thioesterase (TE) functions divided into two distinct NRPSs Vlm1 and Vlm2. Valinomycin is formed by macrolactonization of three tetradepsipeptide monomers. The NRPS domain organization is homologous to the structurally closely related cyclodepsipeptide cereulide.
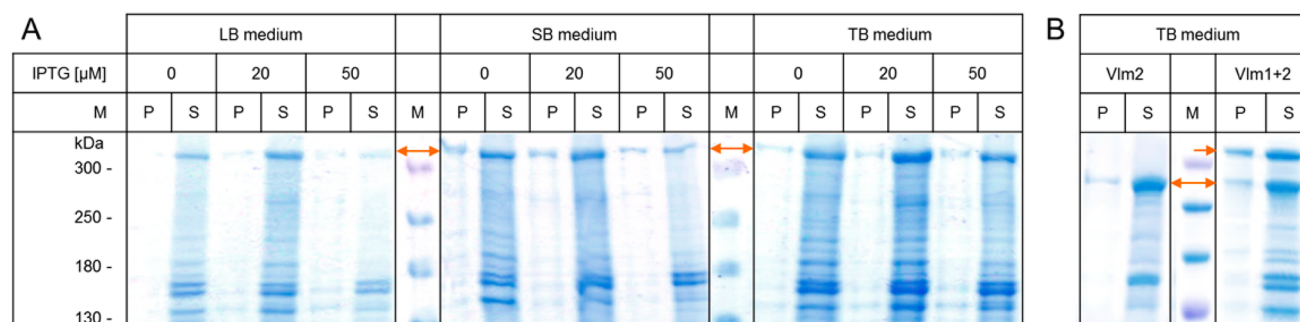
Figure 2. SDS-PAGE (5%) analysis of valinomycin synthetase expression screening. (A) Expression of Vlm1 with *E. coli* BL21 Gold induced with various IPTG concentrations in different media at 30 °C. (B) Expression of Vlm2 and coexpression of Vlm1 + 2 with *E. coli* BL21 Gold induced with 20 μM IPTG in TB medium at 30 °C. Applied pellet [P] and soluble [S] protein fractions were normalized to OD_{600}.

and antiviral[17] to antitumor[18] efficacy. This dodecadepsipeptide with a 3-fold rotational symmetry consists of a D-α-hydroxyisovaleric acid-D-valine-L-lactate-L-valine monomer with alternating amide and ester bonds. The biosynthetic gene cluster of valinomycin has previously been characterized, and a model for the biosynthesis of valinomycin has been proposed (Figure 1).[19,20] Valinomycin is synthesized by the tetramodular valinomycin synthetase (VlmSyn), which is coded by two distinct NRPS genes with the domain organization A_1-KR_1-T_1-C_2-A_2-T_2-E_2-C_3 for Vlm1 and A_3-KR_3-T_3-C_4-A_4-T_4-TE for Vlm2. The iterative type B VlmSyn deviates from the colinearity rule[21] and reuses its four modules to assemble three tetradepsipeptide monomers, which are subsequently oligomerized and macrolactonized. The NRPSs that synthesize the structurally closely related emetic toxin cereulide from *Bacillus cereus* were shown to activate α-keto acid precursors that are subsequently stereospecifically reduced to α-hydroxy acids by dedicated ketoreductase (KR) domains. A similar mechanism was proposed for the valinomycin biosynthesis.[20] This is contrasted by the assembly of the fungal CODs enniatin from *Fusarium equiseti* and beauvericin from *Beauveria bassiana* where α-hydroxy acids provided by an independently acting ketoreductase are directly activated and incorporated.[22]

In this study, we have screened conditions for the coexpression of Vlm1 and Vlm2 in *E. coli* as a heterologous host and determined enzyme activities and precursor requirements in a subsequent *in vitro* assay. Autonomous formation of

valinomycin without the necessity of precursor substrate feeding was achieved in an engineered *E. coli* strain. The recombinant production of the 36-membered macrolactone antibiotic valinomycin broadens the scope of bioactive natural products synthesized in *E. coli* and will be the basis to engineer the valinomycin biosynthetic assembly line and to generate structural analogues.

The two valinomycin NRPS genes *vlm1* (10,287 bp) and *vlm2* (7,968 bp) were PCR amplified from *Streptomyces tsusimaensis* genomic DNA. Silent mutation single-cutter restriction sites were introduced into linker regions between modules to facilitate a directed exchange of modules (Supporting Information, Figure S1). *Vlm1* was inserted into plasmid pCTUT7_His for expression from the IPTG-inducible P_{lac_CTU} lac promoter derivative with an N-terminal His$_6$-tag.[23] The *vlm2* gene was inserted into plasmid pKS01, a derivative of vector pCTUT7_His with complementary ampicillin resistance marker and the plasmid stabilizing *parB* locus[24] for stable coexpression of Vlm1 and Vlm2. The resulting plasmids pVlm1 and pVlm2 were introduced into *E. coli* strain BL21 Gold, and relevant cultivation parameters (inducer concentration, media composition, temperature) were tested for expression.

Key to soluble expression of Vlm1 (370 kDa) was a low induction of protein expression with IPTG inducer concentrations below 50 μM (Figure 2A). Even uninduced leaky expression led to substantial amounts of Vlm1, while under stronger induction conditions (>50 μM) expression of Vlm1

was almost completely abolished. Since large proteins are prone to misfolding and aggregation, weaker promoters under low induction conditions may be generally applicable for NRPS expression in *E. coli*. A richer medium (TB > SB > LB) proved to be beneficial for both specific production of Vlm1 (Figure 2A) and volumetric production due to enhanced *E. coli* cell growth (data not shown). Favorable conditions for the soluble expression of Vlm1 were found to allow soluble expression of Vlm2 (284 kDa) and coexpression of Vlm1 + 2 from two individual plasmids (Figure 2B) as well. While expression at 20 °C increased the amount of soluble protein produced per OD_{600} compared to 30 °C, overall cell growth was almost decreased by half (Supporting Information, Figure S2). We used mass spectrometric analysis of tryptic digest fragments to verify the identity of recombinant Vlm1 and Vlm2 (data not shown).

Early precursor feeding experiments with *Streptomyces* valinomycin producer strains identified L-Val as precursor for both the L- and D-form of valine in valinomycin. However, in these experiments the direct precursors of the two valinomycin hydroxy acid constituents, D-α-hydroxyisovaleric acid (D-Hiv) and L-lactate (L-Lac), could not be unambiguously identified.[25] Furthermore, alignment of signature binding pocket residues of the corresponding A domains did not result in predicted substrate binding specificities. The putative biosynthesis of the α-hydroxy acids from α-amino acids occurs *via* deamination to the corresponding keto acids and subsequent reduction (Figure 3A).
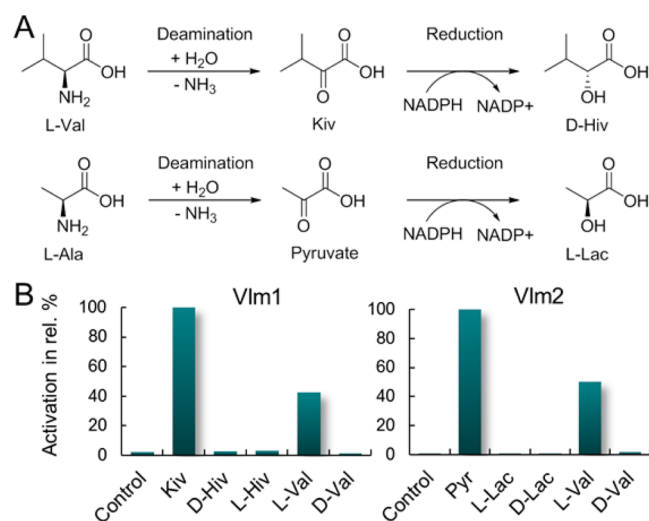
Figure 3. Precursor requirements of valinomycin biosynthesis. (A) Proposed route for the biosynthesis of valinomycin hydroxy acid constituents D-α-hydroxyisovaleric acid (D-Hiv) and L-lactate (L-Lac) from the corresponding amino acid precursors *via* α-ketoisovaleric acid (Kiv) and pyruvate (Pyr). (B) Substrate specificities of Vlm1 and Vlm2 adenylation domains were determined by ATP-PPi exchange assay. In the negative control amino acid/hydroxy acid was omitted. Activities are shown in relation to the substrate with the strongest activity.

We performed an *in vitro* ATP-PPi exchange assay with purified Vlm1 and Vlm2 to assess the activity of the recombinant enzymes and to determine substrate specificities of the A domains (Figure 3B). Vlm1 and Vlm2 were purified from *E. coli* BL21 Gold cell lysates in a two-step purification with His-tag Ni^{2+}-affinity chromatography and gel filtration. As

predicted both NRPSs Vlm1 and Vlm2 activated L-Val. Furthermore, the keto acids α-ketoisovaleric acid (Kiv) and pyruvate were activated selectively by Vlm1 and Vlm2, respectively. The hydroxy acids D-Hiv (or L-Hiv) and L-Lac (or D-Lac) were not activated. This confirms the proposed model of valinomycin biosynthesis in analogy to the cereulide biosynthesis in *Bacillus cereus*.[22] Since Kiv is a direct precursor in the branched chain amino acid metabolic pathway of the L-Val anabolism, *E. coli* should be able to provide all necessary precursors for autonomous valinomycin biosynthesis. D-Hiv itself is not known to be an *E. coli* metabolite even though recent work suggests otherwise.[26]

The subsequent aim of our study was the *in vivo* heterologous production of valinomycin in the *E. coli* host. In order to achieve this goal, one has to consider that nonribosomal peptide biosynthesis requires the posttranslational modification of NRPS thiolation domains with a 4′-phosphopantetheinyl (Ppant) prosthetic group. This modification for covalent substrate tethering is carried out by a dedicated phosphopantetheinyl transferase (PPTase). Within the borders of the two characterized valinomycin gene clusters from *S. tsusimaensis* and *Streptomyces levoris* A9 no PPTase gene could be identified.[19,20] Therefore, we decided to use the promiscuous PPTase Sfp from *Bacillus subtilis*, which has been frequently applied for the phosphopantetheinylation of heterologous PKS and NRPS substrates.[27]

Hence, we integrated *sfp* under the control of the constitutive tryptophanase promoter into the *xylA* locus of the *E. coli* BL21 Gold genome *via* λ Red recombineering to generate *E. coli* strain BJJ01 (Supporting Information, Figure S3). Upon overexpression of Vlm1 and Vlm2 from the two-plasmid system pVlm1 and pVlm2 in BJJ01, valinomycin formation could be detected and identified in culture extracts by LC−MS (Figure 4B) and LC−MS/MS (Figure 4C), respectively. The data was consistent with the chemical structure of valinomycin and in comparison to a commercial standard (Supporting Information, Figure S4). Interestingly, valinomycin production could also be detected upon coexpression of Vlm1 and Vlm2 in the unmodified *sfp⁻* *E. coli* strain BL21 Gold (Figure 4A). Valinomycin produced from *E. coli* BL21 Gold corresponded to ~50% of the amount produced by the engineered *sfp⁺* strain BJJ01. Obviously, Vlm1 and Vlm2 are accepted to a limited extent as substrates by any of the native *E. coli* PPTases AcpS, EntD and AcpT (YhhU).[28]

We monitored valinomycin production with *E. coli* BJJ01 over a time-course of 36 h in a batch cultivation with TB medium. Valinomycin was quantitated by multiple reaction monitoring (MRM) mass analysis (Figure 5). Most valinomycin accumulated within the first 8 h of fermentation to a titer of 0.25 ± 0.02 mg L^{-1}. After the culture entered the stationary phase, valinomycin only further accumulated up to a final concentration of 0.3 ± 0.01 mg L^{-1} after 36 h. Valinomycin formation correlated with the growth of the *E. coli* host cells, probably because the availability of valinomycin building blocks (pyruvate, Kiv, L-Val) within the cell depends on the fluxes through the primary metabolism. Upon depletion of nutrients in the batch medium, *E. coli* growth and therefore the supply of valinomycin precursors would become limiting. The valinomycin in the culture remained stable, even after the cell number declined after 24 h.

Switching from a batch to a system with a continuous glucose delivery (EnPresso, BioSilta Oy, Finland) to prolong cell growth greatly benefits the heterologous production of
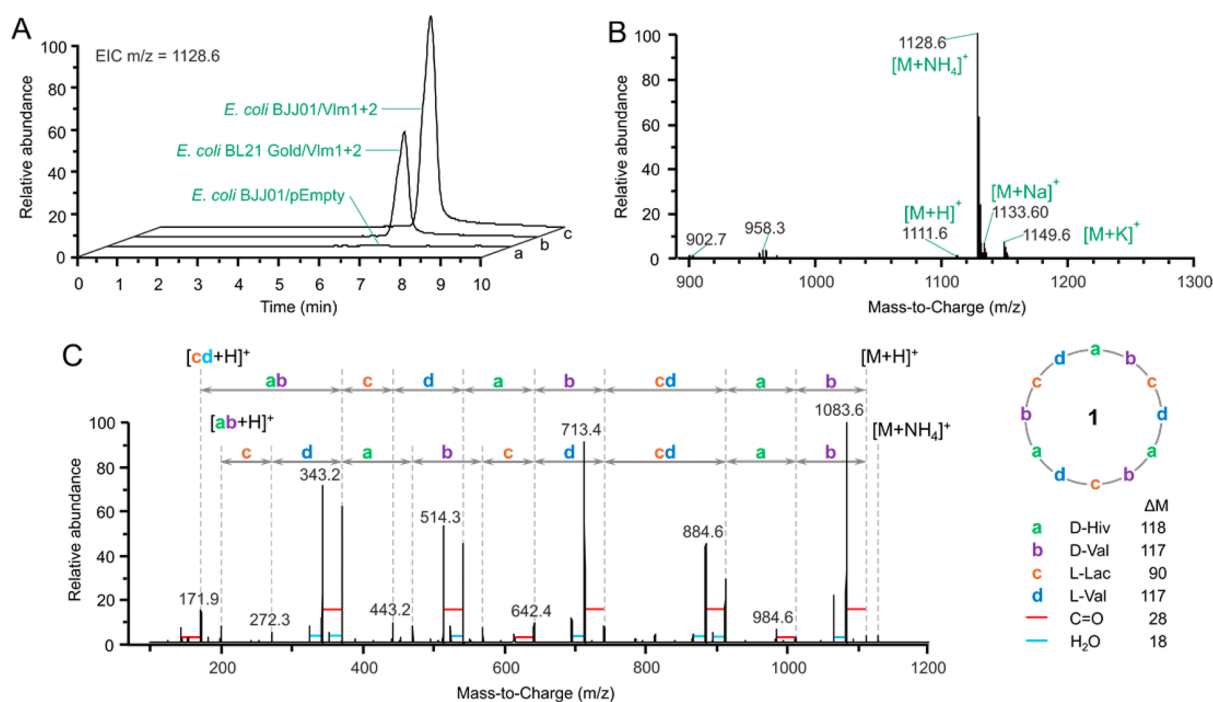
Figure 4. LC−MS analysis and characterization of recombinant valinomycin produced in *E. coli*. (A) Extracted ion chromatograms of valinomycin $[M + NH_4]^+$ *m/z* 1128.6 from culture extracts of engineered *E. coli* strain BJJ01 with an empty control plasmid (a), strain BL21 Gold expressing Vlm1 and Vlm2 (b), and BJJ01 expressing Vlm1 and Vlm2 (c). (B) MS spectrum of valinomycin peak. (C) MS/MS spectrum of valinomycin. The fragment ions represent the sequential loss of amino and hydroxy acid constituents and carbonyl groups from the cyclooligodepsipeptide **1**.
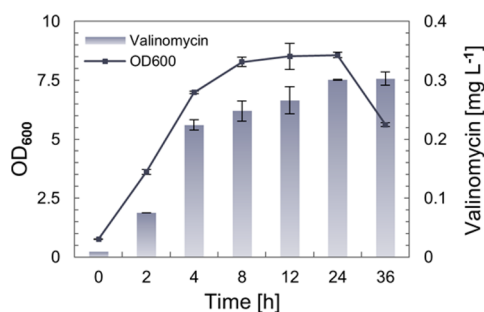
Figure 5. Time-course of valinomycin formation and cell growth (OD_{600}). Valinomycin titers produced by BJJ01 expressing Vlm1 and Vlm2 in TB medium at 30 °C after induction with 20 μM IPTG were monitored with multiple reaction monitoring (MRM).

valinomycin in *E. coli*. We have optimized the valinomycin production through small-scale high cell density EnPresso B cultivations, and the final titer was improved up to approximately 10 mg L^{-1}, which represents a 33-fold increase compared to the initial TB batch cultivations.[29] In addition, coexpression of the type II thioesterase (TEII, ORF15), a discrete protein that serves as a repair enzyme to hydrolyze the misacylated thiol groups of 4′-phosphopantetheine moieties of the T domains restoring active holo-NRPS,[30] further improved the valinomycin titer to approximately 13 mg L^{-1}, which is 43-fold higher than the initial TB batch cultivations (unpublished data). Our results indicate that *E. coli* is a robust host for heterologous production of valinomycin with competitive titers that are comparable to the productivity of several native *Streptomyces* producers.[31]

Toxicity is a major concern if a bioactive compound is to be produced in a heterologous host. *E. coli* was shown to be insensitive to externally applied valinomycin because of its outer membrane, which acts as a selective barrier in Gram-negative bacteria.[32] To assess the toxicity of valinomycin on *E. coli*, we performed a disk diffusion assay with the *E. coli* mutant strain BAS3023, which carries an outer membrane permeability defect and shows sensitivity toward antibiotics that are usually not active against Gram-negative bacteria.[33] Between 10 and 500 μg of valinomycin per paper disc were tested and did not result in growth inhibition (Supporting Information, Figure S5). Likewise, no difference in growth could be detected between *E. coli* strain BL21 Gold/pVlm1/pVlm2 and the engineered BJJ01/pVlm1/pVlm2 *sfp*$^+$ strain (data not shown). This shows that valinomycin has no toxic effect on *E. coli*, at least under the conditions applied in this study. However, valinomycin was mainly detected in *E. coli* cell extracts and only to a minor degree in the culture supernatant (data not shown), indicating that valinomycin accumulated within the cell and was not efficiently transported into the cell environment. Self-resistance determinants and/or specific exporters are commonly associated with natural product biosynthetic gene clusters. A gene coding for a putative transporter, *vlmC*, was identified in the valinomycin cluster of *S. levoris* A9,[20] but it is doubtful if the transport system of a Gram-positive organism like *Streptomyces* would work in a Gram-negative host due to the additional outer membrane barrier. An *E. coli* strain with engineered overexpression of its native transport systems associated with multidrug efflux, like the AcrAB-TolC pump, has been suggested as a general measure to address the export and toxicity of recombinant natural products.[10,33] This strategy may also be applied to support valinomycin efflux and increase final product titers.

The reconstituted biosynthesis of valinomycin adds another example for the versatility and adaptability of *E. coli* as a heterologous natural product host. Two GC-rich (~70% GC) genes of *Streptomyces* origin could be coexpressed to produce

the large valinomycin NRPSs Vlm1 and Vlm2 in a soluble and active form without the necessity of codon adaption. While *E. coli* was able to provide the necessary NRPS posttranslational phosphopantetheinylation to a limited extend, coexpression of *sfp* from a genomic locus significantly increased *in vivo* valinomycin production. Valinomycin formation was independent of the supplementation of precursors and solely relied on growth in inexpensive media. While initial valinomycin yields of approximately 0.3 mg L^{-1} from unoptimized batch cultivations are still modest, the valinomycin titer in small scale cultivations could be further increased to more than 10 mg L^{-1} by controlled glucose feeding and coexpression of the type II thioesterase form the valinomycin cluster (unpublished results). Further improvement in *E. coli* will permit straightforward optimization by metabolic (*e.g.*, overexpression of an efflux pump) and bioprocess engineering (*e.g.*, fed-batch cultivation and scale-up). Finally, the ease of genetic manipulations in *E. coli* will facilitate the development of valinomycin analogues by combinatorial biosynthesis.

■ METHODS

Strains, Plasmids, and Culture Conditions. Strains used in this study are listed in Table S1 (Supporting Information). For the isolation of genomic DNA containing the valinomycin biosynthetic gene cluster (GenBank accession no. DQ174261) *Streptomyces tsusimaensis* ATCC 15141 was cultivated in Seed medium[34] with 0.5% glycine at 30 °C for 24 h. *E. coli* BL21 Gold was used as host strain for Vlm1 and Vlm2 cloning and overexpression. Plasmids used in this study are listed in Table S2 (Supporting Information). Vlm1 and Vlm2 expression screenings were carried out in 24-well round-bottom plates with 3 mL of Luria−Bertani broth (LB), Superbroth (SB), or Terrific Broth (TB) culture medium[35] supplemented with appropriate antibiotics (34 μg mL^{-1} of chloramphenicol, 100 μg mL^{-1} of ampicillin) at either 30 or 20 °C in an incubation shaker at 250 rpm. Cultures were inoculated from a fresh overnight culture to a starting OD_{600} of 0.1, and expression was induced at OD_{600} 0.6−0.8 with varying IPTG inducer concentrations (0, 20, 50, 100, 500 μM, and 1 mM). Cultures were harvested 5 h after induction and subjected to SDS-PAGE analysis. *In vivo* valinomycin formation was induced with 20 μM IPTG in *E. coli* BJJ01 in baffled shake flasks (20% filling volume) with TB supplemented with 17 μg mL^{-1} of chloramphenicol and 50 μg mL^{-1} of ampicillin at 30 °C in an incubation shaker at 200 rpm. Culture samples were taken at certain time points for valinomycin extraction and LC−MS analysis. The outer membrane permeability mutant *E. coli* BAS3023 and its wild-type precursor strain MC4100 were used for the valinomycin disc diffusion assay.

PCR Amplification and Cloning of NRPS Expression Plasmids. *S. tsusimaensis* ATCC 15141 genomic DNA was isolated with the Invisorb Genomic DNA Kit III (Stratec Molecular, Berlin, Germany). All oligonucleotides used in this study were purchased from TIB MOLBIOL (Berlin, Germany) and are listed in Table S3 (Supporting Information). Vlm1 and Vlm2 gene coding sequences were amplified in three subfragments each with flanking *att*B sites for Gateway recombinational cloning and additional single-cutter restriction sites (Supporting Information, Figure S2). PCR amplifications were carried out with the KAPA HiFi DNA Polymerase and HiFi GC buffer (Peqlab Biotechnology, Erlangen, Germany) according to the manufacturer's protocol. Gateway recombinational cloning reactions were performed with the LR and BP

clonase enzyme mixes (Invitrogen Life Technologies, Darmstadt, Germany). The *vlm1* and *vlm2* subfragments were introduced into the pDONR201 plasmid *via* Gateway BP reaction and subsequently reassembled *via* restriction digest and ligation to yield plasmids with the complete *vlm1* and *vlm2* gene sequences (pDONR201/Vlm1 and pDONR201/Vlm2). DNA sequencing by LGC Genomics (Berlin, Germany) confirmed the sequence integrity of *vlm1* and *vlm2*. The *vlm1* gene was transferred into pCTUT7 and *vlm2* into pKS01 *via* Gateway LR reaction to obtain the plasmids pVlm1 and pVlm2.

Construction of an *E. coli* Strain with Genomically Expressed *sfp*. The *B. subtilis* PPTase *sfp* gene[27] under the control of the weak constitutive tryptophanase promoter was introduced into the *E. coli* BL21 Gold locus of the nonessential *xylA* gene by λ Red Recombineering.[36] An overview of the cloning and integration strategy is depicted in Figure S3 (Supporting Information). *sfp* amplified from plasmid pAP16 and a chloramphenicol resistance (cm^R) cassette flanked by *FRT* (FLP recombination target) sites amplified from plasmid pKD3 were linked by overlap PCR. The *sfp*-cm^R cassette was cloned into the pTNAmod plasmid between the constitutive tryptophanase P_{TNA} promoter and a λ t_0 terminator *via* restriction enzymes *Mlu*I and *Not*I. The p_{TNA}-*sfp*-cm^R-λ t_0 cassette was amplified with the primers XylA-Sfp_F and XylA-Sfp_R to generate a PCR product that contained ~50 base pairs at its ends with homology to the target sequence up- and downstream of the *xylA* gene in the *E. coli* chromosome. This linear substrate was transformed by electroporation into *E. coli* BL21 Gold, in which the λ Red functions *gam*, *beta*, and *exo* had been preinduced from plasmid pSIM6 according to the method described by Sharan et al.[36] Successful recombinants unable to ferment D-xylose were selected on MacConkey indicator agar (Difco, Heidelberg, Germany) supplemented with 1% D-xylose and 10 μg mL^{-1} of chloramphenicol.[37] The cm^R cassette was excised from the chromosome by FLP recombinase from plasmid pCP20. The BL21 Gold strain with the *xylA*::*sfp* allele was termed strain BJJ01.

LC−MS Analysis and Quantitation of Valinomycin. *E. coli* culture samples were pelleted by centrifugation. The supernatant was extracted with an equal volume of ethyl acetate. The pellet was resuspended in the same volume of methanol and extracted after lysis by sonication. Cell debris was removed by centrifugation. The solvent was evaporated under a vacuum and the residue was taken up in one-fifth of original sample volume methanol for LC−MS analysis. LC−MS analysis of extracts was performed with the TripleQuad LC−MS 6460 mass spectrometer (Agilent) in combination with the Agilent 1290 Infinity HPLC system. For HPLC separation prior to MS analysis the Zorbax Eclipse Plus C18 RRHD column (2.1 × 50 mm, 1.8 μm) from Agilent was used with water +0.1% HCOOH (v/v) as eluent A and acetonitrile +0.1% HCOOH (v/v) as eluent B. Samples were separated at a flow rate of 0.3 mL min^{-1} with a linear gradient from 5 to 100% B over 2.5 min, an isocratic elution at 100% B for 5.5 min and a linear gradient from 100 to 5% B over 4 min. For the MS/MS analysis and valinomycin quantitation by multiple reaction monitoring (MRM) the $[M + NH_4]^+$ adduct (m/z 1128.6) was used as precursor ion. For MRM the characteristic mass transitions m/z 1128.6 → 1083.6, m/z 1128.6 → 713.4, and m/z 1128.6 → 343.3 were used to quantify valinomycin in culture extracts in comparison to a calibration curve prepared with commercial valinomycin as standard.

ATP-PPi Exchange Assay. *E. coli* BL21 Gold expressing His$_6$-tagged Vlm1 or Vlm2 was pelleted and lysed by sonication in His-buffer [50 mM NaH$_2$PO$_4$·H$_2$O (pH 7.4), 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM DTT, 1 mM PMSF]. The cleared cell lysate was loaded onto a 5 mL Ni-NTA Superflow cartridge (Qiagen), washed, and eluted with His-buffer containing 300 mM imidazole. Fractions containing Vlm1 or Vlm2 were pooled, loaded onto the HiLoad 16/60 Superdex 200 column (GE Healthcare), and eluted with gel filtration buffer [50 mM Tris-HCl (pH 8), 100 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF]. Protein was quantitated by absorbance at 280 nm.

100 μL of ATP-PPi reaction mix [5 mM ATP, 5 mM MgCl$_2$, 0.1 mM Na$_4$P$_2$O$_7$, 0.09 μCi [^{32}P]-Na$_4$P$_2$O$_7$ (Perkin-Elmer)] was supplemented with 5 mM precursor substrate or water as negative control. The reaction was initiated by addition of 100 μL of purified Vlm1 or Vlm2 in gel filtration buffer, incubated for 10 min at 30 °C, and quenched by mixing with 1 mL of stop solution [168 mM Na$_4$P$_2$O$_7$, 377 mM HClO$_4$, 1.4% (w/v) activated carbon]. The carbon was retained on a glass fiber filter and washed thrice with 2 mL of ddH$_2$O. Radioactivity was measured in 4 mL of LumaSafe Plus scintillation cocktail (Perkin-Elmer) with the Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer).

Valinomycin Susceptibility Assay. *E. coli* BL21 Gold, the outer membrane permeability mutant strain BAS3023, and its wild-type precursor strain MC4100 were tested for susceptibility to valinomycin. Fresh overnight colonies of each strain were suspended in LB medium and diluted to a final OD$_{600}$ between 0.08 and 0.13 (corresponds to ~1–2 × 10^8 cfu mL^{-1}). 150 μL of cell suspension were streaked onto a LB agar plate and left to dry. Six-millimeter filter discs were applied onto the inoculated agar surface. Ten-microliter valinomycin solutions with different concentrations (1, 5, 10, 20, 50 μg μL^{-1} in EtOH) were pipetted onto the filter disks. As controls EtOH, a 1.7 μg mL^{-1} of chloramphenicol, and a 1 μg μL^{-1} of kanamycin solution were applied. The plates were incubated at 35 °C for 18–20 h.

ASSOCIATED CONTENT

S **Supporting Information**

Supporting tables, figures, and references. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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