The ADEP Biosynthetic Gene Cluster in *Streptomyces hawaiiensis* NRRL 15010 Reveals an Accessory *clpP* Gene as a Novel Antibiotic Resistance Factor

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ABSTRACT The increasing threat posed by multiresistant bacterial pathogens necessitates the discovery of novel antibacterials with unprecedented modes of action. ADEP1, a natural compound produced by *Streptomyces hawaiiensis* NRRL 15010, is the prototype for a new class of acyldepsipeptide (ADEP) antibiotics. ADEP antibiotics deregulate the proteolytic core ClpP of the bacterial caseinolytic protease, thereby exhibiting potent antibacterial activity against Gram-positive bacteria, including multiresistant pathogens. ADEP1 and derivatives, here collectively called ADEP, have been previously investigated for their antibiotic potency against different species, structure-activity relationship, and mechanism of action; however, knowledge on the biosynthesis of the natural compound and producer self-resistance have remained elusive. In this study, we identified and analyzed the ADEP biosynthetic gene cluster in *S. hawaiiensis* NRRL 15010, which comprises two NRPSs, genes necessary for the biosynthesis of (4*S*,2*R*)-4-methylproline, and a type II polyketide synthase (PKS) for the assembly of highly reduced polyenes. While no resistance factor could be identified within the gene cluster itself, we discovered an additional *clpP* homologous gene (named *clpP*_{ADEP}) located further downstream of the biosynthetic genes, separated from the biosynthetic gene cluster by several transposable elements. Heterologous expression of ClpP_{ADEP} in three ADEP-sensitive *Streptomyces* species proved its role in conferring ADEP resistance, thereby revealing a novel type of antibiotic resistance determinant.

IMPORTANCE Antibiotic acyldepsipeptides (ADEPs) represent a promising new class of potent antibiotics and, at the same time, are valuable tools to study the molecular functioning of their target, ClpP, the proteolytic core of the bacterial caseinolytic protease. Here, we present a straightforward purification procedure for ADEP1 that yields substantial amounts of the pure compound in a time- and cost-efficient manner, which is a prerequisite to conveniently study the antimicrobial effects of ADEP and the operating mode of bacterial ClpP machineries in diverse bacteria. Identification and characterization of the ADEP biosynthetic gene cluster in *Streptomyces hawaiiensis* NRRL 15010 enables future bioinformatics screenings for similar gene clusters and/or subclusters to find novel natural compounds with specific substructures. Most strikingly, we identified a cluster-associated *clpP* homolog (named *clpP*_{ADEP}) as an ADEP resistance gene. ClpP_{ADEP} constitutes a novel bacterial resistance factor that alone is necessary and sufficient to confer high-level ADEP resistance to *Streptomyces* across species.

KEYWORDS 4-methylproline, acyldepsipeptides, antibiotics, caseinolytic protease, natural products, nonribosomal peptide synthetase, polyketide synthase, resistance, streptomycetes

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The overuse of antibiotics has led to a worldwide increase in multidrug-resistant bacterial pathogens, which are now challenging our health care systems by severely complicating the treatment of serious and life-threatening bacterial infections, even making some bacterial infections untreatable (1, 2). Thus, there is an urgent need to discover and develop novel antibiotics with resistance-breaking properties. In this regard, antibiotic acyldepsipeptides (ADEP), a new natural product-derived class of antibiotics, showed promising antibacterial activity against various Gram-positive bacterial pathogens, including vancomycin-resistant enterococci (VRE), penicillin-resistant streptococci (PRSP), methicillin-resistant staphylococci (MRSA), and mycobacteria and Gram-negative *Neisseria* and *Wolbachia* endobacteria (3–8). ADEP was shown to be effective in treating MRSA infections in rodent models, even outcompeting the gold standard antibiotic linezolid (3) and, in combination with rifampin, successfully eradicated *Staphylococcus aureus* persister cells *in vitro* and in deep-seated biofilm infections in mice (9).

ADEP acts by deregulating the bacterial ATP-dependent caseinolytic protease (Clp) (3). Clp plays a crucial role in protein homeostasis and protein quality control, as well as in the proteolytic regulation of a variety of differentiation and developmental processes (10–12). The target of ADEP is ClpP, the proteolytic core of the protease, which is only catalytically active in the form of a barrel-shaped tetradecamer. Two heptameric rings of ClpP monomers stack to form a central proteolytic chamber where 14 catalytic triads are shielded from the environment. Due to the limited diameter of two entrance pores gating access to the proteolytic chamber, ClpP alone can degrade only small peptides, but it becomes an active protease when one of its cognate regulatory Clp-ATPases translocates unfolded protein strands through the pores (12, 13). ADEP binds to ClpP and in this process displaces the Clp-ATPases from their binding sites, which prevents natural substrate degradation. In addition, ADEP stabilizes the ClpP tetradecamer, stimulates catalysis allosterically, and widens the entrance pores to the proteolytic chamber (14, 16). As a consequence, nonnative polypeptide and protein substrates, e.g., the central cell division protein FtsZ (15), are now allowed to enter the proteolytic chamber of ClpP, resulting in their untimely degradation and bacterial death (3, 16, 17).

ADEP1 (acyldepsipeptide 1, “factor A”), the ADEP prototype and progenitor of synthetic ADEP derivatives, is a natural product produced by *Streptomyces hawaiiensis* NRRL 15010 as part of the A54556 antibiotic complex, which comprises several closely related congeners (Fig. 1) (18). The ADEP macrolactone core is composed of five amino acids cyclized by a depsipeptide moiety (18, 19). Attached to the ring structure is an *N*-acylated L-phenylalanine (Phe), which bears a polyene side chain (18, 20). A noncanonical (2*S*,4*R*)-4-methylproline (MePro) residue present in the ADEP1 macrocycle is replaced by L-proline (Pro) in “factor B”; factor B and ADEP1 are the most abundant ADEP congeners produced by *S. hawaiiensis* NRRL 15010. ADEP1 exhibits, depending on the organism tested, a 4- to 8-fold higher antibiotic activity than factor B, which highlights MePro as an important feature for antibacterial activity (20, 21).

During the last few years, ADEP antibiotics received considerable attention in the antibiotic field and the Clp community, and a number of studies have explored their antibacterial potency, structure-activity relationship, and mechanism of action. However, the biosynthetic pathway responsible for the production of ADEP in *S. hawaiiensis* NRRL 15010, as well as the mechanism of self-immunity in the producer, remained elusive. In the current study, we report on a fast, small-scale purification procedure for ADEP1 and describe the acyldepsipeptide (*ade*) biosynthetic gene cluster (BGC) in *S. hawaiiensis* NRRL 15010, thereby revealing a new and unprecedented antibiotic resistance factor.

RESULTS

Constitutive production of ADEP by *S. hawaiiensis* NRRL 15010 enables robust small-scale purification. To characterize ADEP production, we tested culture supernatants of *S. hawaiiensis* NRRL 15010 in *Bacillus subtilis*-based bioassays. In all *Firmicutes*

FIG 1 Structures of congeners of the A54556 antibiotic complex produced by *S. hawaiiensis* NRRL 15010 (18).

investigated to date, ClpP is not essential for viability under moderate growth conditions, and a deletion of the *clpP* gene leads to high-level resistance against ADEP. Thus, wild-type *Bacillus subtilis* 168 and a corresponding $\Delta clpP$ mutant were instrumental as ADEP indicator strains to rule out growth inhibition by compounds other than ADEP.

S. hawaiiensis NRRL 15010 showed stable ADEP production under all medium conditions tested, and no further antibacterial agent was detected in the strain (see Fig. S1 in the supplemental material). Quantification of growth and ADEP production in yeast malt (YM) medium revealed detectable amounts of ADEP already at the beginning of the exponential growth phase, increasing in parallel with the biomass (Fig. 2A). Under given conditions, peak production of ADEP1 occurred after 56 h of growth (9 to 10 mg/liter), as determined by bioassay and high-performance liquid chromatography (HPLC) analyses using pure ADEP1 as a standard. We next developed a fast and robust purification protocol to purify ADEP1, the most active of the two main components of the A54556 complex, from the supernatant. Two chromatographic steps efficiently separated the compound from medium components and the other congeners present, in particular the highly similar factor B, and obtained about 3 mg/liter of pure ADEP1 (Fig. S3). The antibiotic was tested in bioassays for its potency against the ADEP-sensitive relative *Streptomyces lividans* TK24 in comparison to previously described synthetic derivatives ADEP2, ADEP4, and ADEP7 (3, 14) (Fig. 2B and C). In previous studies, ADEP2 and ADEP4 had proven by far superior to the natural product ADEP1 with regard to their *in vitro* and *in vivo* efficacy against *S. aureus*. However, against streptomycetes, the natural product ADEP1 was more potent than any of those synthetic congeners (Fig. 2B).

Structure-guided analysis facilitated identification of the *ade* BGC. The genome of *S. hawaiiensis* NRRL 15010 was sequenced using PacBio RSII and Illumina next-generation sequencing technologies and subsequently analyzed by antiSMASH 3.0 (22) and BLAST software tools (23). Based on the ADEP1 and factor B primary structures, we expected to find a BGC consisting of three subclusters (24) for biosynthesis of the peptide macrocycle, the noncanonical amino acid MePro, and the polyene side chain, respectively, which led to the identification of a candidate *ade* BGC consisting of 12 open reading frames (ORFs) (Table S1).

A vast number of peptidic natural products emanate from biosynthesis by nonribosomal peptide synthetases (NRPSs). Following the collinearity rule for incorporation of amino acids in thiotemplate-based natural product biosynthesis, NRPSs are composed of a certain number of modules (M) corresponding to the number of incorporated amino acid residues. Each module minimally comprises three functional domains: an adenylation domain (A) for selection and activation of an amino acid building block, a peptidyl carrier protein (PCP), which tethers the growing peptide chain to the complex, and a condensation domain (C) catalyzing amide bond formation between the next amino acid monomer and the peptide chain (25). Therefore, we anticipated that the cluster contains an NRPS with six modules (M1-M6) possessing A domains (A1-A6) specific for successive incorporation of Phe, L-serine (Ser), Pro, L-alanine (Ala), Ala, and either MePro in ADEP1 or Pro in factor B. Additionally, we expected a

FIG 2 (A) Growth and ADEP production of *S. hawaiiensis* NRRL 15010 in YM medium. Shown is dry cell mass plotted against bioactivity in the culture supernatant (determined against *B. subtilis* 168 by agar diffusion; compare to Fig. S2). One representative out of three biological replicate experiments is shown. (B) Bioassay to test the antibiotic potency of the synthetically structure-optimized ADEP derivatives ADEP2, ADEP4, and ADEP7 compared to ADEP1 against *S. lividans* TK24 (left). Five microliters of a dense spore suspension of *S. lividans* TK24 was plated on MH agar. Paper disks with 20 μ g of the respective ADEP congener and a dimethyl sulfoxide (DMSO) control were applied to the plates, which were subsequently incubated for 48 h at 30°C. While the pipecolic acid moiety seems to be tolerated (compare ADEP1 to ADEP4), the cyclohexyl side chain leads to a strong reduction of antibacterial potency against *Streptomyces* (compare ADEP2 to ADEP4). The producer *S. hawaiiensis* NRRL 15010 was used as an ADEP-insensitive control (right). (C) Structures of ADEP congeners.

methyltransferase (MT) domain in M4 involved in *N*-methylation of the incorporated Ala as well as a thioesterase (TE) domain in M6 for release and simultaneous cyclization of the peptide chain. The putative *ade* BCG comprises genes encoding two NRPSs (*adeG* and *adeH*) consisting of four and two modules, respectively (Fig. 3). As the *in silico* predicted A domain substrate specificities by antiSMASH 3.0 (22) and the structure-based prediction model of Challis et al. (26) were consistent with the ADEP depsipeptide backbone structure, we propose that *adeG* and *adeH* are responsible for the incorporation of the six respective amino acids (Fig. 3B). Downstream of *adeH*, *adel* adjoins the NRPS genes, encoding an Mbth-like protein (Fig. 3A). This protein family is often found with NRPS-containing gene clusters, binding to NRPS proteins and stimulating adenylation reactions, thereby supporting optimal secondary metabolite production in a chaperone-like manner (27, 28).

4-Methylproline is a nonproteinogenic amino acid which has been reported to be incorporated into only a limited set of natural products (see reference 29 and further

FIG 3 (A) Sequential arrangement of ORFs responsible for ADEP biosynthesis in *S. hawaiiensis* NRRL 15010. (B) The ADEP NRPS assembly line with modules M1 to M6 and specific substrates. Pro and MePro, which are both incorporated by M6, are marked in orange. Domains are given the following labels: C, condensation domain; A, adenylation domain; PCP, peptidyl carrier protein; MT, methyltransferase; TE, thioesterase.

references therein). Biosynthetic routes to the (2*S*,2*R*)- and the (2*S*,4*R*)-diastereomers have been described (29–31), and we expected to find homologs for (2*S*,4*R*)-4-methylproline biosynthesis. Two genes encoding a putative leucine hydroxylase (*adeA*) and a putative alcohol dehydrogenase (*adeB*) represent likely candidates for MePro supply in the putative *ade* BGC (Fig. 3A). Furthermore, we expected a polyketide synthase (PKS) to be present within the cluster, comprising ketoreductase (KR) and dehydratase (DH) domains for the generation of the diene or triene side chain of the ADEP congeners (Fig. 1). Most common in bacteria are iterative or noniterative PKSs of type I with large multimodular enzymes (32) and of the iterative type II comprised of discrete, monofunctional enzymes that perform multiple catalytic cycles (33). Type II PKSs minimally possess a characteristic heterodimeric ketosynthase (KS) assembled from α - and β -subunits for Claisen-type condensation reactions and an acyl carrier domain (ACP) to which the growing chain is bound during biosynthesis (34). The putative *ade* BGC contains four genes (*adeC* to *adeF*) encoding a putative ACP, KS α - and β -subunits, and a KR, which together represent a putative type II PKS (Fig. 3A).

Two further genes (*orf1* and *orf2*) located upstream of the potential biosynthesis genes represent putative regulatory components located in a bicistronic operon, while *orf3* shows highest similarity to transcriptional regulators of the xenobiotic response element (XRE) family (Fig. 3A).

Heterologous expression confirms identity of the *ade* BGC. In order to confirm that the putative *ade* BGC in *S. hawaiiensis* NRRL 15010 is in fact responsible for ADEP production and that it contains all required functionalities, we aimed for expression of the region of interest in the heterologous host *Streptomyces coelicolor* M1146 (35). In addition to the biosynthetic genes, BGCs for the production of antimicrobially active substances usually contain genes encoding resistance factors to ensure survival of the producers during biosynthesis (36, 37). For antibiotics acting on an intracellular target, BGCs often encode transporters to export the compounds out of the cells; however, we

FIG 4 Heterologous expression of the *ade* BGC in *S. coelicolor* M1146. (A) Design of the *pCAP03-adeP* shuttle vector for expression of the *ade* BGC. Elements used for propagation in yeast (red), *E. coli* (green), and *Streptomyces* (blue) are shown. The captured region of interest spans from *adeA* to *clpP_{ADEP}* (magenta). *orfA-F* encode transposable elements and hypothetical proteins (gray). (B) Activity of *S. hawaiiensis* NRRL 15010 and *S. coelicolor* M1146 extracts against *B. subtilis* 168 wild type and the ADEP-resistant $\Delta clpP$ mutant. (C) LC-MS analysis of extracts demonstrating the presence of ADEP1 and factor B in the heterologous M1146/*pCAP-adeP* strain. Chromatograms show merged extracted ion chromatograms for ADEP1 (m/z 719.37) and factor B (m/z 705.36). cps, counts per second.

did not find genes encoding putative transporters within or close to the assumed *ade* BGC borders. The biosynthesis genes are flanked by insertion sequence (IS) element fragments, which are common sites of introduction of natural product BGCs due to horizontal gene transfer (38, 39). When analyzing the region downstream of *adel*, we identified a putative *clpP* homolog, which we named *clpP_{ADEP}*, that is separated from the biosynthesis genes by several transposable elements (Fig. 4A). Due to the ClpP-modulating mode of action of ADEP and the proximity of *clpP_{ADEP}* to the biosynthesis genes, we assumed a putative role for this gene in conferring self-immunity against ADEP and, thus, included this gene in the heterologous expression experiments. To clone the region spanning from the leucine hydroxylase *adeA* to the cluster-associated *clpP_{ADEP}* into a shuttle vector, we employed transformation-associated recombination (TAR) cloning. This technique makes use of endogenous homologous recombination activity of *Saccharomyces cerevisiae* to stitch genomic DNA of interest into a shuttle vector, *pCAP03* (40). Using this approach, we successfully captured the region of interest of 33.6 kb in *pCAP03-adeP* (Fig. 4A). The *pCAP03* backbone is a yeast/*Esche-*

FIG 5 Relative substrate specificities of adenylation domains determined in a hydroxamate formation assay (41). A3 (A) and A6 (B) specificities were determined by probing a range of amino acids. Normalized values of absorbance for the assay reaction minus the absorbance for a control reaction with boiled protein are shown for one representative out of three biological replicate experiments (based on three independent protein purifications) with two technical replicates each. Error bars represent the maximal deviations from the respective mean values.

richia coli/actinobacterial shuttle vector that allowed the transfer of the cluster into *S. coelicolor* M1146 for chromosomal integration at the ϕ C31 phage *attB* site. Fermentation extracts of *S. coelicolor* M1146/pCAP03-*adep* were active against *B. subtilis* 168 but not the *B. subtilis* 168 Δ *clpP* mutant, indicating the successful heterologous production of ADEP (Fig. 4B). Furthermore, HPLC (Fig. S4) and liquid chromatography-mass spectrometry (LC-MS) (Fig. 4C and Fig. S5) analyses of *S. coelicolor* M1146/pCAP03-*adep* fermentation broth revealed two new peaks not present in the empty-vector control. These were in accordance with the retention times of ADEP1 and factor B compared to those of the *S. hawaiiensis* NRRL 15010 production culture spectra. The masses of these peaks corresponded to ADEP1 (retention time, 7.22 min; expected $[M+H]^+$, 719.376; observed $[M+H]^+$, 719.3797; error, 5.1433 ppm) and factor B (retention time, 6.89 min; expected $[M+H]^+$, 705.3606; observed $[M+H]^+$, 705.3620; error, 1.9848 ppm) (Fig. S5), which confirmed the identified cluster as responsible and sufficient for ADEP production in *S. hawaiiensis* NRRL15010.

A multispecific A domain is responsible for Pro and MePro incorporation. The *in silico*-predicted A domain substrate specificities were largely consistent with the ADEP depsipeptide backbone structure. The only exception was the A6 domain, which was predicted to incorporate Pro and L-pipecolic acid (Pip) (Fig. S6). Thus, we employed a discontinuous hydroxamate-based assay to investigate A6 substrate specificity, utilizing A3 as a positive control (41). A3 was specific for Pro (Fig. 5A), consistent with the fact that all six elucidated congener structures of the A54556 complex contain Pro in position 3. In contrast, A6 activated both Pro and MePro, reflecting their incorporation at position 6 in ADEP1 and factor B, respectively. A6 was also found to activate Ser to a moderate extent, displaying a more relaxed substrate specificity *in vitro* relative to that of A3 (Fig. 5B). However, ADEP congeners comprising Ser at position 6 have not been reported as products of the native producer.

MePro is supplied by a minimal subcluster. The *ade* BGC contains two genes encoding AdeA and AdeB, which exhibit high amino acid sequence identities/similarities to the recently described leucine hydroxylase GriE (57%/73%) and the alcohol dehydrogenase GriF (68%/80%) of the griselimycin biosynthetic gene cluster of *Streptomyces* DSM 40835, respectively (Fig. S7 to S10) (29, 42). GriE was shown to participate in (2S,4R)-4-methylproline formation in whole-cell and *in vitro* experiments, most likely catalyzing the hydroxylation of L-leucine to (2S,4R)-5-hydroxyleucine. GriF was suggested to be involved in the further oxidation, immediately followed by a spontaneous,

FIG 6 Proposed pathway for MePro biosynthesis to be incorporated into the ADEP peptide backbone.

nonenzymatic cyclization, yielding (3R,5S)-3-methyl-Δ1-pyrroline-5-carboxylic acid (29). Phylogenetic analyses of AdeA and GriE (Fig. S8) as well as AdeB and GriF (Fig. S10) revealed a close relationship between each pair and suggest their descent from a common ancestor. Thus, AdeA and AdeB most probably represent the leucine hydroxylase and alcohol dehydrogenase, respectively, involved in MePro supply during ADEP biosynthesis. A final reduction step is necessary to yield MePro, which was suggested to be performed in *Streptomyces* DSM 40835 by either GriH, an F420-dependent oxidoreductase expressed in the BGC, or ProC, a pyrroline-5-carboxylate reductase from proline synthesis. In the *ade* BGC, no enzyme with a putative reducing function is present, so that during MePro generation also a pyrroline-5-carboxylate reductase from primary metabolism might contribute (Fig. 6 and Fig. S11).

A minimal type II PKS is responsible for biosynthesis of the triene side chain.

Recently, the actinobacterial BGCs for skyllamycin, simocyclinones, and ishigamide (43–46) were reported to contain minimal type II PKS subclusters, which produce highly reduced polyene chains instead of canonical aromatic structures, the most common products by type II PKSs (45, 46). The *S. hawaiiensis* NRRL 15010 PKS genes display a similar organization and high amino acid sequence similarities to the PKS enzymes in the aforementioned clusters (Fig. S12 to S15). Phylogenetic analysis showed a close relation of AdeD with polyene producing ketosynthase α -subunits, containing a characteristic active-site motif (Cys-His-His). In AdeE, this motif is changed to Gln-Ser-Asp, and it is found in a clade in the phylogenetic tree with polyene-producing ketosynthase β -subunits, also called chain length factor (CLF) (Fig. 7A). By analogy with published systems, we propose that during generation of the ADEP polyene side chain, polyketide elongation takes place on the ACP domain AdeC, starting from acetyl coenzyme A (acetyl-CoA) and incorporating malonyl coenzyme A (malonyl-CoA) as extender units. The KS heterodimer, composed of AdeD and AdeE, performs Claisen-type condensation reactions (AdeD), yielding a tetraketide, with the chain length defined by the CLF (AdeE) (47). For maturation of the triene side chain, ketoreduction and dehydration functions are also necessary (Fig. 7B). While a putative ketoreductase gene, *adeF*, is clustered with *adeC-E*, a gene encoding a putative dehydratase is not present within the *ade* BGC. tblastn analysis of the *S. hawaiiensis* NRRL 15010 genome sequence for dehydratases revealed putative candidates for catalysis of this reaction (Fig. S16).

The *ade* BGC reveals an accessory *clpP* gene as resistance factor. Streptomycetes commonly possess multiple *clpP* homologs, e.g., three homologs in *Streptomyces*

A

B

FIG 7 Biosynthesis of the ADEP1 triene side chain by a type II PKS. (A) Phylogenetic analysis of AdeD and AdeE revealed high similarities to KS subunits of type II PKSs producing highly reduced polyenes. PUFA, polyunsaturated fatty acid; FAS, fatty acid synthase. (B) Proposed pathway for biosynthesis of the triene side chain.

griseus and five in *Streptomyces lividans*. BLAST analysis of the genome of *S. hawaiiensis* NRRL 15010 revealed a total of six *clpP* genes, with four of them being colocalized in two bicistronic operons (*clpP1P2* and *clpP3P4*) and a monocistronic *clpP5* gene, as it is common in streptomycetes (48). Unusual, however, is the presence of a sixth *clpP* gene, the monocistronic *clpP*_{ADP} associated with the *ade* BGC.

Phylogenetic analyses of ClpP proteins from various *Streptomyces* species, including *S. hawaiiensis* NRRL 15010, confirmed an organization in two homolog pairs (ClpP1 and

FIG 8 ADEP resistance in *ClpP*_{ADEP}-expressing mutant strains. (A) Spores of *S. hawaiiensis* NRRL 15010 were streaked on NE agar and incubated for 3 days. Subsequently, indicator strains were applied perpendicularly to test them against the ADEP natural product complex secreted by *S. hawaiiensis* NRRL 15010 (48). wt, wild type; vc, pSETermE* (empty vector control); *ClpP*_{ADEP}, pSETclpP_{ADEP}. (B) *S. lividans* mutant strains were grown on MH agar containing increasing concentrations of the inducer thioestrepton (as stated in the figure) to gradually increase *ClpP*_{ADEP} expression. A constant amount of 10 µg of ADEP1 was applied onto all paper discs. (Upper) *S. lividans* pIJ6902clpP_{ADEP}; (lower) *S. lividans* pIJ6902 (empty vector control).

*ClpP*2 are homologous to *ClpP*3 and *ClpP*4, respectively) (Fig. S17), as was described before for *S. lividans* (48, 49). *S. hawaiiensis* NRRL 15010 *ClpP*1-5 show high similarities to the respective *ClpP* homologs of other streptomycetes and appear in the phylogenetic tree in the respective monophyletic groups (*Streptomyces* *ClpP*1, *Streptomyces* *ClpP*2, etc.), while *ClpP*_{ADEP} does not clade within any of these subgroups (Fig. S17). However, it shares a common stem with *ClpP*1; thus, both proteins most likely descend from a common ancestor, which suggests the gene cluster-associated *ClpP*_{ADEP} fulfills a similar function.

To investigate whether *ClpP*_{ADEP} can act as a resistance factor in *Streptomyces*, we constructed a plasmid for heterologous expression of *ClpP*_{ADEP} under the control of a constitutive *ermE** promoter (pSETclpP_{ADEP}) and introduced it into representatives of three different ADEP-sensitive *Streptomyces* species: *Streptomyces lividans* TK24 (*S. lividans* pSETclpP_{ADEP}), *Streptomyces coelicolor* A3(2) (*S. coelicolor* pSETclpP_{ADEP}), and *Streptomyces griseus* Waksman (*S. griseus* pSETclpP_{ADEP}). We next streaked the mutants containing *ClpP*_{ADEP} perpendicularly to the native ADEP producer to test their sensitivity to the produced A54556 natural product complex. Indeed, while we observed growth inhibition for the corresponding wild-type strains as well as strains that had received only the empty vector, expression of *ClpP*_{ADEP} led to profound ADEP resistance and unhampered growth in all species tested (Fig. 8A). Furthermore, we introduced a construct for the regulatable expression of *clpP*_{ADEP} under the control of a thioestrepton-inducible *tipA* promoter (pIJ6902clpP_{ADEP}) into *S. lividans* TK24, generating *S. lividans* pIJ6902clpP_{ADEP}. In bioassays using a constant amount of ADEP1 and by varying inducer concentrations, we observed a clear dependence of ADEP resistance on *clpP*_{ADEP} expression levels, independently proving that the expression of *ClpP*_{ADEP} confers resistance to the A54556 natural product complex including ADEP1 (Fig. 8B).

DISCUSSION

The unusual mode of action and therapeutic potential of ADEP have already been the subject of several studies. However, the BGC for production of the ADEP natural

product complex in *S. hawaiiensis* NRRL 15010, in addition to the mode of producer self-protection, have not been investigated so far. In this study, we identified the *ade* BGC sufficient for ADEP biosynthesis in *S. hawaiiensis* NRRL 15010 and the heterologous host *S. coelicolor* M1146. Furthermore, an additional, cluster-associated *clpP* homolog from *S. hawaiiensis* NRRL 15010, designated *clpP*_{ADEP}, could be shown to act as a novel resistance determinant against ADEP in different *Streptomyces* species.

S. hawaiiensis NRRL 15010 is a stable ADEP producer under laboratory conditions, and we detected secretion of the antibiotic into any liquid (YM medium, lysogeny broth [LB], Mueller-Hinton broth [MH], *Streptomyces* antibiotic medium [SAM], and tryptic soy broth [TSB]) (Fig. 2; see also Fig. S1 in the supplemental material) or solid medium (YM medium, LB, MH, nutrient extract [NE], and mannitol soy flour agar [MS]) (Fig. 8) tested.

In liquid culture, the ADEP concentration in the supernatant increased proportionally to the biomass, whereas secondary metabolite production in the majority of characterized natural product-producing streptomycetes usually starts during the stationary growth phase (36). The apparent constitutive expression of the *ade* BGC under culturing conditions tested is unusual compared to that of BGCs from other streptomycetes, whose expression is commonly linked to the developmental life cycle or is even transcriptionally silent under laboratory conditions (50, 51). Constant ADEP production may have proven evolutionarily advantageous in the natural environment, although high production levels are costly and were shown to benefit not only the producer itself but also resistant nonproducers (52). Notwithstanding this, however, as we have not studied the regulation of ADEP production further in the current project, we cannot exclude that the constitutive expression of the *ade* BGC is due to a regulatory defect. Comparison with other producer strains is currently not possible, as *S. hawaiiensis* NRRL 15010 is the only ADEP producer described to date. While cultivation and ADEP production of *S. hawaiiensis* NRRL 15010 were unproblematic and consistent, in our hands, the producer strain was not genetically tractable for gene knockout or knockdown studies. In order to confirm the identity of the putative *ade* BGC, we expressed the region of interest heterologously in *S. coelicolor* M1146. Although the amount of ADEP produced by the heterologous host was low, the presence of intact and fully modified ADEP1 indicates the cluster borders and proves cluster integrity.

As *in silico* analysis of the cluster was, to a large extent, consistent with the ADEP primary structure and the literature concerning related enzymes, we concentrated our biochemical and phylogenetic studies on the outstanding questions regarding particular biosynthetic steps. For the multispecific A6 domain of AdeH, the *in silico*-predicted activated amino acids Pro and Pip differed from the amino acids actually present in ADEP1. To determine the substrate specificity of A6, we performed a discontinuous hydroxamate formation assay. While A3 of AdeG activated Pro only, A6 was capable of activating both Pro and MePro, which reflects the variation in the primary structures of ADEP1 (MePro) and factor B (Pro). A6 exhibited a further relaxed substrate specificity, as Ser also was activated to a moderate extent in the *in vitro* assay, which, however, has not been identified in compounds of the natural product complex A54556. The extended substrate spectrum observed *in vitro* and the increased *in vivo* specificity for MePro and Pro show the limitation of *in vitro* assays with isolated domains. In a recent study, the importance of C domains for specific incorporation of substrates by multispecific A domains was shown for the microcystin BGC (53). It provided evidence for an extended gatekeeping function of C domains to control substrate activation by A domains, suggesting that C6 of AdeH plays a crucial role for specific incorporation of Pro and MePro.

The *ade* BGC contains a gene set for the supply of MePro and a type II PKS for the biosynthesis of the triene side chain, which both showed high homologies in phylogenetic analyses to recently discovered enzymes with similar functions (29, 43–46). However, both subclusters lack one gene necessary to yield the final product. A dehydratase is missing from the *ade* type II PKS, suggesting an involvement of a dehydratase from fatty acid biosynthesis (44). Furthermore, the *ade* BGC lacks an

enzyme for the final reduction step in MePro biosynthesis. *In vitro* experiments with GriH, an oxidoreductase of the griselimycin BGC, and ProC, a pyrroline-5-carboxylate reductase from proline biosynthesis in *E. coli*, showed that both of these enzymes can catalyze the reduction of the carbon-nitrogen double bond in (3*R*,5*S*)-3-methyl- Δ^1 -pyrroline-5-carboxylic acid to yield MePro. Heterologous expression of the MePro-synthesizing enzyme set GriE-H in *S. lividans* yielded MePro, even when GriH was knocked out, suggesting that its function is complemented by ProC (29). Griselimycin and methyl-griselimycin contain two and three MePro moieties, respectively, which could necessitate the presence of a clustered enzyme for sufficient MePro supply. In contrast, for ADEP1 with only a single MePro, sufficient amounts apparently can be generated by exploiting a pyrroline-5-carboxylate reductase like ProC from primary metabolism.

In the search of a self-resistance factor, which enables *S. hawaiiensis* NRRL 15010 to withstand ADEP production unscathed, we identified an additional *clpP* homolog, *clpP*_{ADEP}, located downstream of the core ADEP biosynthesis genes. Heterologous expression in three different *Streptomyces* species demonstrated that *clpP*_{ADEP} can act as a sole resistance determinant to allow growth of otherwise sensitive strains in the immediate vicinity of the producer strain (Fig. 8A). In contrast to ClpP in *Firmicutes*, the Clp system in *Actinobacteria* is clearly more complicated and usually involves more than one ClpP homolog. Mycobacteria, for example, encode two *clpP* homologs in a bicistronic operon (*clpP1P2*), and the corresponding ClpP complex is assembled as a heterotetradecamer of two different ClpP homoheptameric rings composed of ClpP1 or ClpP2. We previously reported that ADEP kills mycobacteria by inhibiting the natural functions of Clp, as in mycobacteria, in contrast to *Bacillus*, ClpP is essential for viability (5, 54, 55). Streptomycetes, which are closely related to mycobacteria, possess an even more elaborate ClpP machinery, with three to five different *clpP* homologs. Several studies on ClpPs in *Streptomyces* were published (48, 49, 56–60), but neither the composition of the ClpP complex nor the mode of action of ADEP in these species have been understood so far. In *S. lividans*, the best-studied *Streptomyces* species with regard to the Clp system so far, four *clpP* homologs are tightly regulated in two bicistronic operons (*clpP1P2* and *clpP3P4*), of which the expression of at least one operon is essential for survival, while the fifth homolog, *clpP5*, is expressed constitutively at a very low level and seems to be nonessential (48). In a wild-type strain, the expression of *clpP1* and *clpP2* is sufficient to fulfill all requirements essential for survival while also indirectly repressing the transcription of *clpP3P4* via degradation of the transcriptional activator, PopR (for *clpP3* operon regulator). In a *clpP1* knockout strain, PopR activates transcription of *clpP3P4*, which compensates for the loss of the essential functions of ClpP1 (49, 57). This mutant also exhibits reduced sensitivity to ADEP despite all other ClpP proteins being expressed, suggesting that ClpP1 is the only ADEP-sensitive homolog in *S. lividans* (48). *S. griseus* encodes only three *clpP* homologs, *clpP1*, *clpP2*, and *clpP5*. Since we successfully induced ADEP resistance by the expression of *clpP*_{ADEP} in this species, it may be assumed that even though *Streptomyces lividans* ClpP3 and ClpP4 are ADEP insensitive, they do not seem to be involved in the immunity mechanism conferred by *clpP*_{ADEP}. Further studies are ongoing in our laboratory to investigate the role of *clpP*_{ADEP} in the ADEP producer, its mechanism of detoxifying ADEP, and the deregulated ClpP complex involving the ADEP-sensitive ClpP1. Although the explicit molecular mechanism remains to be determined, it can already be stated that a single protein, the novel antibiotic resistance factor *clpP*_{ADEP}, is sufficient for both, first to prevent ADEP from activating the Clp system of the producer strain toward destructive proteolysis and second to allow the Clp system to execute all essential natural functions. The multifaceted mode of action of ADEP might be met by a similarly multifarious, novel resistance mechanism.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All strains and plasmids used in this study are listed and referenced in Tables 1 and 2. *S. hawaiiensis* NRRL 15010 is publicly available from the Agricultural

TABLE 1 Strains used in this study

Strain	Relevant genotype	Source or reference(s)
<i>B. subtilis</i> 168	<i>trpC2</i>	67
<i>B. subtilis</i> 168 $\Delta clpP$	<i>trpC2</i> , $\Delta clpP::spc$	68
<i>E. coli</i> ET12567(pUB307)	<i>dam dcm hsdM hsdS hsdR cat tet</i> ; RP4	69–71
<i>E. coli</i> ET12567(pUZ8002) (<i>neo::bla</i>)	<i>dam dcm hsdM hsdS hsdR cat tet</i> ; pUZ8002; <i>neo::bla</i> , RP4	69, 72
<i>E. coli</i> JM109	<i>endA1 recA1 gyrA96 thi hsdR17</i> (r_K^- , m_K^+) <i>relA1 supE44</i> $\Delta(lac-proAB)$, [<i>F'</i> <i>traD36 proAB laqI^qZAM15</i>]	73
<i>E. coli</i> NiCo21(DE3)	<i>can::CBD fluA2 [lon] ompT gal</i> (λ DE3) [<i>dcm</i>] <i>arnA::CBD slyD::CBD glmS6Ala</i> $\Delta hsdS$ λ DE3 = λ sBamHlo $\Delta EcoRI-B$ <i>int::(lacI::PlacUV5::T7 gene1) i21 $\Delta nin5$</i>	New England Biolabs
<i>E. coli</i> TOP 10	<i>F⁻ mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi 80d lacZ\Delta M15$ $\Delta lacX74$ <i>recA1 araD139</i> $\Delta(araleu)7697 galU galK rpsL$ (StrR) <i>endA1 nupG</i>	Invitrogen
<i>Saccharomyces cerevisiae</i> VL6-48N	<i>MATα his3-D200 trp1-$\Delta 1$ ura3-$\Delta 1$ lys2 ade2-101 met14 psi⁺ cirO</i>	74
<i>S. coelicolor</i> A3(2)		75
<i>S. coelicolor</i> M1146	SCP1 ⁻ SCP2 ⁻ Δact Δred Δcpk Δcda	35
<i>S. griseus</i> Waksman		Günter Muth, Tübingen, Germany
<i>S. hawaiiensis</i> NRRL 15010	Native ADEP producer strain	ARS Culture Collection (NRRL)
<i>S. lividans</i> TK24	<i>str-6</i> ; SLP2 ⁻ SLP3 ⁻	76

Research Service Culture Collection (NRRL). *S. lividans* TK24, *S. coelicolor* A3(2), and *S. griseus* Waksman were kindly provided by Günther Muth (University of Tübingen, Tübingen, Germany). Wild-type strains and *clpP*_{ADEP} mutants were grown at 30°C on MS-MgCl₂ agar (2% soy flour, 2% mannitol, 2% agar, 10 mM mM MgCl₂), in tryptic soy broth (TSB; BD Biosciences), in Mueller-Hinton medium (MH; BD Biosciences), in lysogeny broth (LB; 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.3), or in yeast malt medium (YM medium; 0.4% yeast extract, 1% malt extract, 0.4% glucose, pH 7.3) with apramycin (50 μ g/ml) as appropriate. *Streptomyces coelicolor* M1146 (SCP1⁻ SCP2⁻ Δact Δred Δcpk Δcda [35]) was grown at 30°C on MS-MgCl₂ agar, in TSB, or in *Streptomyces* antibiotic medium (SAM; 1.5% soytone, 1.5% glucose, 0.5% NaCl, 0.1% CaCO₃, 2.5% glycerol, pH 7.0) with kanamycin (50 μ g/ml) and nalidixic acid (25 μ g/ml) as appropriate.

The hypertransformable *Saccharomyces cerevisiae* strain VL6-48N was a gift from Vladimir Larionov (National Cancer Institute, Bethesda, MD, USA). Yeast were propagated at 30°C in YPD medium (2% D-glucose, 1% yeast extract, 2% peptone, 100 mg/liter adenine) prior to transformation-associated recombination (TAR). After transformation, yeast cells were maintained on synthetic tryptophan dropout medium (SD-trp; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 100 mg/liter adenine, 76 mg/liter inositol, uracil, and amino acids except trp, 8 mg/liter *p*-aminobenzoic acid).

E. coli JM109, *E. coli* TOP10, and *E. coli* ET12567 (carrying pUB307 or pUZ8002 *neo::bla*) were propagated in LB medium with ampicillin (100 μ g/ml), apramycin (50 μ g/ml), kanamycin (50 μ g/ml), and/or chloramphenicol (35 μ g/ml) as required.

The pSET152ermE* Δ HindIII plasmid used for heterologous expression of ClpP_{ADEP} was a gift from Till Schäberle (University of Giessen, Giessen, Germany). The pJ6902 plasmid for inducible expression of ClpP_{ADEP} was kindly provided by Marc Buttner (John Innes Center, Norwich, UK). The pCAP03 plasmid used for capture of the *ade* BGC was a gift from Bradley Moore (UC San Diego, San Diego, CA, USA).

Growth and ADEP production time course. After inoculation with roughly 5×10^7 spores, *S. hawaiiensis* NRRL 15010 was grown in 1 liter of YM medium at 30°C and 200 rpm. Over a period of 7 days, 20-ml samples were taken at various time points, the mycelium was collected on preweighed filter papers, and the filters were washed twice with water and frozen at -20°C. After the last sample was taken, filters were dried at 50°C for 2 days and weighed to determine the dry cell mass as mass difference of

TABLE 2 Plasmids used in this study

Plasmid	Description	Source or reference
pSETermE* Δ HindIII	Integrative <i>Perme</i> * expression vector; <i>ori</i> pUC18, <i>lacZα</i> , <i>oriT</i> RK2, <i>int</i> C31, <i>attP</i> , <i>aac(3)IV</i>	77
pSETclpP _{ADEP}	Constitutive overexpression plasmid, carrying the <i>clpP</i> _{ADEP} gene under the control of <i>Perme</i> *	This study
pJ6902	Integrative <i>PtipA</i> expression vector; <i>ori</i> pUC18, <i>oriT</i> RK2, <i>int</i> C31, <i>attP</i> , <i>tsr</i> , <i>aac(3)IV</i>	78
pJ6902clpP _{ADEP}	Inducible construct with the <i>clpP</i> _{ADEP} gene under the control of <i>PtipA</i>	This study
pETDuet-1	Coexpression vector for two target genes; <i>bla</i> , <i>f1 ori</i> , <i>lacI</i>	Novagen
pETDuet-MbtH_A3	Inducible overexpression plasmid, carrying the <i>adel</i> and <i>his-a3</i> gene under the control of PT7	This study
pETDuet-MbtH_A6	Inducible overexpression plasmid, carrying the <i>adel</i> and <i>his-a6</i> gene under the control of PT7	This study
pCAP03	Yeast/ <i>E. coli</i> /actinobacterial shuttle vector, <i>int</i> C31, <i>attP</i> , <i>oriT</i> RK2, <i>ori</i> pUC, <i>pADH1</i> , <i>URA3</i> , <i>aph(3)II</i>	40
pCAP03- <i>adeP</i>	Heterologous expression of the <i>ade</i> BGC	This study

TABLE 3 Primers used in this study

Name	Use	Sequence
ADEP_hooks ^a	TAR cloning homology arms	CATGGTATAAATAGTGGC GGGCTCGAGAAGGGGCGACCAACGTGAACTCGCC CTGTGCTGAGGTGAGA gttttaaac TCTGACGCCTACTGACTGAGCCCTTTCCTA CCTCAGGCTGACGTGGCCAA TATGTGCGAAAGTACATA
Adep-D-F1	TAR diagnostic PCR set 1	CCGCTCTTCAGTACGTTGGTTTCG
Adep-D-R1	TAR diagnostic PCR set 1	GAGATGTTTCGGTCTGATCCATGC
Adep-D-F2	TAR diagnostic PCR set 2	ATGCTGGGTCAAGAGGTCGATG
Adep-D-R2	TAR diagnostic PCR set 2	CCGATATTGCCAGGAACGGTAGC
pSET-Hind	Cloning of pSETclpP _{ADEP} forward primer	TTTAAGCTTGGTAAGGAGTTACAGTGAAGG
pSET-Bam	Cloning of pSETclpP _{ADEP} and pIJ6902clpP _{ADEP} reverse primer	AAAGGATCCCTACTTCGCTGCCCGATATTG
pIJ-A-Nde	Cloning of pIJ6902clpP _{ADEP} forward primer	AAACATATGAAGGACATTAAGGAAGTACG
MbtH-for-NcoI	Cloning of pETDuet-MbtH forward primer	CATGCCATGGTGACTATCGTGTCATCC
MbtH-rev-Hind	Cloning of pETDuet-MbtH forward primer	CCCAAGCTTCTACTGAGTTGCCGTCGTGGC
His-A_for	Cloning of pETDuet-MbtH_A3 and pETDuet-MbtH_A6 forward primer	GGAATTCATATGCATCATCATCATCATGATCCGGGTGTGCGGGTGC
A3_rev	Cloning of pETDuet-MbtH_A3 reverse primer	CGCGGATCCTCAATCGACCCTGGAACTCCCAGG
A6_rev	Cloning of pETDuet-MbtH_A6 reverse primer	CGCGGATCCTCAATCGACCCGGGACATCCCCAAG

^aBoldface, left and right 50-bp hooks; lowercase, PmeI (MssI) RE site; uppercase, overlap with pCAP03.

the filter mass with and without mycelium. For detection of ADEP production, bioassays were performed with 70 μ l of supernatant taken at the same time points and tested against *Bacillus subtilis* 168 and the *B. subtilis* 168 Δ clpP mutant on MH soft agar (0.75% agar).

ADEP purification. *S. hawaiiensis* NRRL 15010 was grown in YM medium (prepared with tap water), inoculated either with spores or a 2-day-old preculture in YM medium at 30°C, 200 rpm, for 60 to 70 h. The mycelium was separated from the supernatant by filtration, and the supernatant was fractionated by column chromatography on Amberlite XAD-2. After three wash steps with water, 20%, and 50% methanol (MeOH), elution was performed with 100% MeOH. This fraction was dried by solvent extraction, redissolved in the required volume of MeOH, and mixed with water at a ratio of 1:1 to be further purified by semipreparative HPLC on a Reprosil-Pur basic C₁₈ column (10 μ m, 20 by 250 mm) (Dr. Maisch GmbH). As solvents, we used 0.1% formic acid in water (A) and MeOH (B) and ran a gradient of 70 to 100% B over 20 min with a flow rate of 24 ml min⁻¹. Absorbance was recorded at 298 nm with a P314 2-channel UV-visible detector (VWR). ADEP1 eluted at 87% B. Identity and purity were determined by LC-MS analysis (with electrospray ionization mass spectrometry) using an HP1100 Agilent Finnigan LCQ Deca XP Thermoquest. The pure fraction was dried in aliquots with a Uni Vapo 100H vacuum concentrator (UniEquip GmbH), and aliquots were stored at -80°C.

TAR of the ade BGC. TAR cloning was carried out as previously described (61). To construct the pCAP03 capture vector, 50-bp homology arms flanking a 33.6-kb region surrounding the *ade* BGC (*adeA* to *clpP*_{ADEP} in Fig. 4A) were concatenated, sandwiching an MssI restriction site (ADEP_hooks). The fragment was synthesized (Integrated DNA Technologies) and cloned between *pADH* and *URA3* in pCAP03 via Gibson assembly. Homology arms were released via MssI digestion prior to TAR. High-molecular-weight *S. hawaiiensis* NRRL 15010 genomic DNA was prepared by phenol-chloroform extraction and cotransformed into *S. cerevisiae* VL648N spheroplasts along with the linearized capture vector. Transformants were plated on SD-trp medium supplemented with 0.5% 5-fluoroorotic acid, and clones containing the *ade* BGC were identified using colony PCR. Total yeast DNA was electroporated into *E. coli* TOP10 cells in order to recover the resulting plasmid, pCAP03-*ade*. Integrity of the construct was further confirmed by restriction mapping. Table 3 lists primer and homology arm sequences.

Heterologous expression of the ade BGC. For production of ADEP in a heterologous *Streptomyces* species, pCAP03-*ade* was transformed into *E. coli* ET12567(pUZ8002) (*neo::bla*) and then conjugated into *S. coelicolor* M1146. Exconjugants were selected using nalidixic acid and kanamycin and confirmed by PCR, yielding the strain *S. coelicolor* M1146/pCAP03-*ade*. As a negative control, the pCAP03 plasmid was conjugated into *S. coelicolor* M1146 in the same way, creating *S. coelicolor* M1146/pCAP03. These strains were grown in 3 ml TSB with kanamycin for 3 days at 30°C, 250 rpm, and then diluted 1:100 in 50 ml SAM production medium and grown for 4 days at 30°C, 250 rpm. Spent medium was bound with 5% (wt/vol) HP-20 resin (Sigma) and subsequently washed with 20%, 65%, and 100% MeOH. The 100% MeOH fraction was dried and redissolved in 100 μ l of MeOH. For comparison, extracts of *S. hawaiiensis* NRRL 15010 were also cultivated and prepared in this way.

For the detection of ADEPs, extracts were analyzed by reverse-phase HPLC using an Xselect CSH C₁₈ column (5 μ m, 10 by 100 mm) and water (A)/acetonitrile (B), both with 0.05% trifluoroacetic acid, with a flow rate of 2 ml min⁻¹ with the following steps: 0 to 0.5 min, 5% B; 0.5 to 2 min, 5 to 25% B; 2 to 27 min, 25 to 100% B; 27 to 30 min, 100% B. Absorbance was recorded at 298 nm. LC-MS analysis was performed with an Agilent UHPLC 1290, Q-tof 6550 system, using an Eclipse XDB C₁₈ column (3.5 μ m, 2.1 by 100 mm) in positive mode. UHPLC conditions with water (A)/acetonitrile (B), both with 0.01% trifluoroacetic acid, and a flow rate of 0.4 ml min⁻¹, with the following steps: 0 to 0.5 min, 5% B; 0.5 to 9.5 min, 5 to 95% B; 9.5 to 10.5 min, 95% B. Disk diffusion assays were performed with 10 μ l of each extract on cation-adjusted MH (BD Biosciences) against *B. subtilis* 168 wild type and a *B. subtilis* 168 Δ clpP mutant.

Cloning, expression, and purification of His₆-tagged A3 and A6 domains. To counteract formation of inclusion bodies, A domains were coexpressed with the *ade* BGC MbtH-like protein. The DNA fragment coding for MbtH was amplified using Q5 high-fidelity DNA polymerase (NEB) and the primer pair MbtH-for-NcoI/MbtH-rev-Hind and cloned into pETDuet-1 (Novagen) between NcoI and HindIII sites, yielding pETDuet-MbtH. Primers for amplification of DNA fragments (His-A₃-for/A3_{rev} or A6_{rev}) coding for the A3 and A6 domains were designed to introduce an N-terminal His₆ tag, and fragments were cloned into pETDuet-MbtH via NdeI and XhoI sites. Constructs (pETDuet-MbtH_A3 and pETDuet-MbtH_A6) were verified by Sanger sequencing (LGC Genomics). Table 3 lists primer sequences. Both constructs were transformed into *E. coli* NiCo21(DE3) (NEB) for protein expression. Strains were cultivated in LB medium supplemented with 100 µg/ml ampicillin at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.6 to 0.8 was reached. After induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), expression was performed overnight at 18°C. Cells were pelleted by centrifugation (4,800 × *g*, 30 min, 4°C) and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.8, 200 mM NaCl, and 10% [vol/vol] glycerol). Following disruption with the Precellys Evolution homogenizer (Bertin Instruments), cell debris was removed by two centrifugation steps (4,800 × *g*, 20 min, 4°C, and 17,000 × *g*, 60 min, 4°C). The supernatant was incubated with chitin resin (NEB) for 1 h at 4°C to remove endogenous *E. coli* metal-binding proteins. The lysate was eluted by gravity flow using disposable 5-ml polypropylene columns (Thermo Fisher Scientific) and filtered through a sterile filter. After overnight incubation with nickel-nitrilotriacetic acid (Ni-NTA) agarose (Thermo Fisher Scientific) with gentle mixing, immobilized metal ion chromatography (IMAC) was performed using disposable 5-ml polypropylene columns and gravity flow. After two wash steps (20 mM Tris-HCl, pH 7.8, 200 mM NaCl, 10% [vol/vol] glycerol, and first 10 and then 20 mM imidazole), elution of the protein was performed with elution buffer (20 mM Tris-HCl, pH 7.8, 200 mM NaCl, 10% [vol/vol] glycerol, and 500 mM imidazole), yielding 10 fractions of about 300 µl. Fractions containing the protein of interest were identified by their molecular weight (His₆-A3, 62.25 kDa; His₆-A6, 61.71 kDa) via SDS-PAGE and combined and concentrated using Amicon Ultra-4 centrifugal filters with a 30-kDa cutoff (Merck) to approximately 200 µl, while simultaneously a buffer exchange to storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% [vol/vol] glycerol) was performed. Proteins were flash-frozen in liquid nitrogen and stored at -80°C.

His₆-A3/A6 substrate specificity assay. Purified proteins from different purifications were pooled, and the protein concentration was determined by the Bradford method using bovine serum albumin as a standard (62). For the hydroxylamine-trapping assay (41), reaction mixtures containing 40 µM His₆-A3 or His₆-A6, 25 mM Tris-HCl, pH 8, 15 mM MgCl₂, 2.25 mM ATP, 150 mM hydroxylamine, and 5 mM amino acid substrate [glycine, L-alanine, L-serine, L-proline, L-pipecolic acid (Sigma), L-phenylalanine (Roth), or (2S,4R)-4-methyl-proline (Key Organics)] in a final volume of 50 µl were incubated at 30°C for 20 h, and the reaction was terminated by the addition of 50 µl of stopping solution (10% FeCl₃ and 3.3% trichloroacetic acid in 0.7 M HCl). After centrifugation to remove precipitated proteins, samples were transferred to 96-well, flat-bottom microtest plates (Sarstedt), and the absorption at 540 nm was determined using an infinite M200Pro microplate reader (Tecan Group Ltd.). Reactions with boiled enzyme were used for normalization, and samples without substrate served as negative controls.

Phylogenetic analysis. Phylogenetic trees are maximum likelihood trees using the Dayhoff model for amino acid substitution and were created with MEGA6 (63). Bootstrap values were calculated over 500 bootstrap repetitions. Alignments were generated with MAFFT 7.222 (64), implemented in Geneious 9.1.6 (<https://www.geneious.com>) using the default settings, and were manually curated.

Cloning and conjugation of pSETclpP_{ADEP} and pIJ6902clpP_{ADEP}. The DNA fragment coding for *clpP*_{ADEP} was amplified from genomic DNA of *S. hawaiiensis* NRRL 15010 using Q5 high-fidelity DNA polymerase and the primer pair pSET-Hind/pSET-Bam or pIJ-A-Nde/pSET-Bam. The PCR product was cloned into pSETermE*ΔHindIII via HindIII and BamHI sites, yielding pSETclpP_{ADEP}, and into pIJ6902 via NdeI and BamHI, yielding pIJ6902clpP_{ADEP}. The constructs were verified by Sanger sequencing (LGC Genomics). The plasmids pSETclpP_{ADEP}, pSETermE*ΔHindIII, pIJ6902clpP_{ADEP}, and pIJ6902 were transformed into *E. coli* ET12567(pUB307) and then conjugated into *Streptomyces* species as described previously (65). Table 3 lists primer sequences.

ADEP sensitivity assay. Spores of *S. hawaiiensis* NRRL 15010 were streaked on nutrient extract (NE) agar (1% glucose, 0.2% yeast extract, 0.2% Casamino Acids, 0.1% Lab-Lemco powder, pH 7.0) (66) in a bar of approximately 1-cm width, and plates were incubated at 30°C for 3 days. Spore suspensions of test strains were streaked perpendicularly to the producer strain, and growth was observed and documented for 3 days.

Nucleotide sequence determination of the *ade* BGC. The *ade* BGC of *S. hawaiiensis* NRRL 15010 was identified by genome sequencing using Pacific Biosciences (PacBio) RSII and Illumina next-generation sequencing technologies (Macrogen), followed by assembly with Falcon (v0.2.1) (PacBio) and SOAPdenovo2 (Illumina) software.

Accession number(s). Gene sequences of *S. hawaiiensis* NRRL 15010 are available under the following GenBank accession numbers: *ade* BGC, [MK047367](#); putative dehydratase, [MK047368](#); putative MaoC family dehydratase, [MK047369](#); putative pyrroline-5-carboxylate reductase, [MK047370](#); *clpP1 clpP2* operon, [MK047371](#); *clpP3P4* operon, [MK047372](#); and *clpP5*, [MK047373](#).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01292-19>.

SUPPLEMENTAL FILE 1, PDF file, 3 MB.

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