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Reconstitution and Minimization of a Micrococcin Biosynthetic Pathway in *Bacillus subtilis*

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ABSTRACT

Thiopeptides represent one of several families of highly modified peptide antibiotics that hold great promise for natural product engineering. These macrocyclic peptides are produced by a combination of ribosomal synthesis and extensive posttranslational modification by dedicated processing enzymes. We previously identified a compact, plasmid-borne gene cluster for the biosynthesis of micrococcin P1 (MP1), an archetypal thiopeptide antibiotic. In an effort to genetically dissect this pathway, we have reconstituted it in *Bacillus subtilis*. Successful MP1 production required promoter engineering and the reassembly of essential biosynthetic genes in a modular plasmid. The resulting system allows for rapid pathway manipulation, including protein tagging and gene deletion. We find that 8 processing proteins are sufficient for the production of MP1 and that the tailoring enzyme TclS catalyzes a C-terminal reduction step that distinguishes MP1 from its sister compound micrococcin P2.

IMPORTANCE

The emergence of antibiotic resistance is one of the most urgent human health concerns of our day. A crucial component in an integrated strategy for countering antibiotic resistance is the ability to engineer pathways for the biosynthesis of natural and derivatized antimicrobial compounds. In this study, the model organism *B. subtilis* was employed to reconstitute and genetically modularize a 9-gene system for the biosynthesis of micrococcin, the founding member of a growing family of thiopeptide antibiotics.

Maintaining an arsenal of effective antibiotics is a tremendous biomedical challenge, as antibiotic discovery is outpaced by the evolution of resistance (1–3). Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are natural compounds with the appealing attributes of being derived directly from a genetic template and possessing numerous exotic chemical features that contribute to stability and antimicrobial activity (4). Thiopeptides, characterized by posttranslationally formed sulfur- and nitrogen-containing heterocycles, constitute a rapidly expanding class of RiPP antibiotics (5). Over 100 different thiopeptides have been chemically identified from numerous culturable bacterial producers, and the mining of genomic and metagenomic data promises to uncover many more chemical species that have eluded discovery by conventional means (6–8).

Thiopeptides are generally encoded by chromosomal or plasmid-localized gene clusters (example in Fig. 1A) that consist of approximately 6 to 25 protein-coding genes (7). These include a gene encoding the precursor peptide that is modified to become the thiopeptide product as well as an assemblage of posttranslational modification genes that are required for thiopeptide maturation and genes for host immunity and other functions. The precursor peptide for thiopeptides and other RiPPs consists of an N-terminal leader that acts as a required docking site for the biosynthetic machinery as well as a C-terminal core peptide that is the direct substrate for posttranslational modifications (9, 10). The leader peptide is ultimately cleaved from the modified core during posttranslational processing, releasing the active compound (example in Fig. 1B). In 2009, multiple independent researchers reported this general scheme for thiopeptide synthesis (11–15). In the years since, several additional biosynthetic principles have been elucidated (16–18). The process appears to involve three core steps: (i) cyclodehydration/oxidation of Cys residues to form thiazole heterocycles (Ser and Thr residues may also be modified to oxazoline or oxazole), (ii) dehydration of Ser and Thr residues yielding dehydroalanines and dehydrobutyrines, respectively, and (iii) cycloaddition of two of the dehydrated residues to yield the final macrocyclic thiopeptide scaffold (Fig. 1B).

Specific physical interactions between thiopeptide biosynthetic enzymes and cognate leader peptides are generally thought to license each processing enzyme to act relatively promiscuously on multiple substrate moieties within the core (19–21). This means that a single biosynthetic active site can work processively at multiple locations on the core peptide. For a typical thiopeptide gene cluster in nature, one often encounters numerous additional genes that obscure the simplicity of central thiopeptide chemistry. The recent *in vitro* reconstitution of the thiomuracin biosynthetic pathway illustrates this simplicity (17). In that study, five different protein enzymes, along with an additional RiPP precursor peptide recognition element (RRE)-containing protein, were found to be sufficient for the synthesis of a bioactive thiopeptide scaffold. This and other studies pursuing minimal thiopeptide biosynthetic

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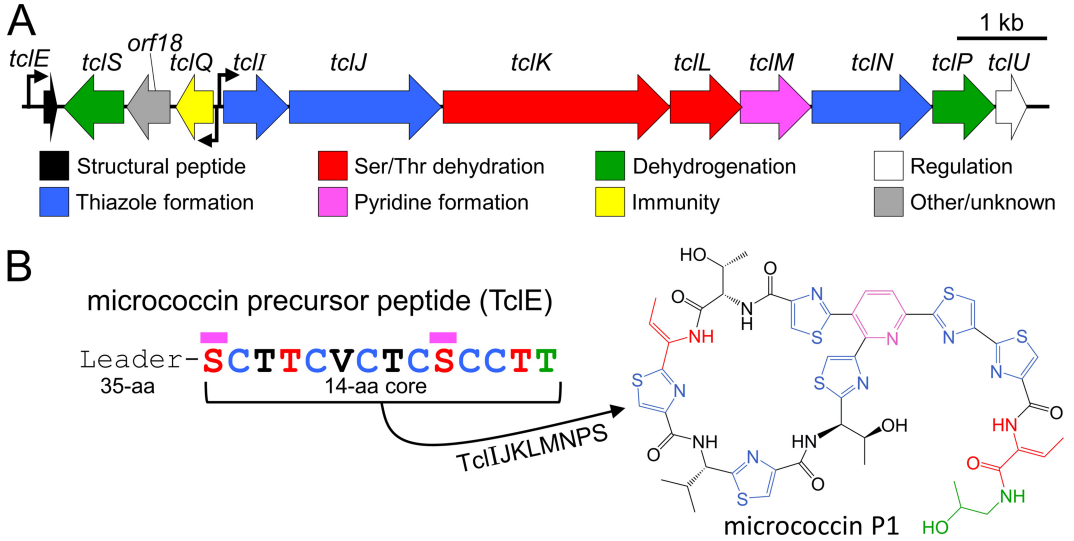

FIG 1 The *M. caseolyticus* strain 115 *tcl* gene cluster is responsible for the production of the antibiotic MP1. (A) The *tcl* gene cluster from *M. caseolyticus* (GenBank accession number KM613043) with predicted functions. Bent arrows represent putative promoters. (B) Sequence of the micrococcin precursor peptide TclE. The 14-amino-acid core peptide gives rise to the bioactive thiopeptide MP1. Magenta bars indicate residues joined to form the macrocycle.

pathways (7) inspire efforts to create easily manipulated *in vivo* platforms for the investigation of thiopeptide biology and for the derivation of novel antimicrobial compounds.

In this paper, we address the biosynthesis of micrococcin, the founding member of the thiopeptide antibiotic family (22–24). The genetic basis of its synthesis was first discovered in the producer strain *Bacillus cereus* ATCC 14579 (12). The cluster of 24 *tcl* genes in this strain controls the production of a mixture of structurally similar compounds, including several thiocillins (hence the *tcl* gene designation), micrococcin P1 (MP1), and micrococcin P2 (MP2). MP1 and MP2 share a structure and intracellular target but differ by a pair of hydrogen atoms as will be described later. A number of incisive studies have delineated key players from the 24-gene *tcl* cluster and have provided insights into how the TclI-processing machinery tolerates alterations in the core peptide sequence (25–28). More recently, we identified a second micrococcin gene cluster in a strain of *Staphylococcus epidermidis* (now classified as *Macrococcus caseolyticus*) strain 115 (29). This gene cluster is considerably smaller (12 genes) (Fig. 1) and was found to produce only a single thiopeptide product, MP1. We report here our effort to minimize and modularize the *M. caseolyticus* micrococcin gene cluster for expression in the laboratory model organism *Bacillus subtilis*, enabling rapid genetic analysis and pathway engineering.

MATERIALS AND METHODS

Basic bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are summarized in Table 1. The micrococcin-producing strain 115, previously identified as *S. epidermidis* (30, 31), was investigated by multilocus sequence typing (MLST) and reassigned as *M. caseolyticus*. MLST primers and results are summarized in Tables S1 and S2 in the supplemental material. Plasmid DNA from *M. caseolyticus* 115 was used as the template for *tcl* gene amplification. The thiopeptide expression chassis *B. subtilis* 168 was used for promoter analysis, and a micrococcin-resistant derivative (BS 168R) with a single-codon deletion in the *rplK* gene (ΔP22) was used for micrococcin expression experiments. The indicator strain used in all spot-on-lawn bioassays was *Staphylococcus aureus* ATCC 6538. All plasmids in this study were selected and maintained in *Escherichia coli* DH5α. All strains listed above were maintained in liquid or solidified lysogeny broth (LB) unless described otherwise. The antibiotics used were ampicillin (Ap, 100 μg/ml), chloramphenicol (Cm, 5 μg/ml), and spectinomycin (Sp, 80 μg/ml). All bacterial cultures were grown at 37°C.

***B. subtilis* transformation.** Transformation of *B. subtilis* was accomplished by using the previously described starvation-induced competence protocol (29). Following selection, colonies were verified for correct integration of transgenes using a modified colony PCR protocol as follows: 1 ml of a saturated overnight culture was pelleted and resuspended in 200 μl of *B. subtilis* lysis buffer (50 mM Tris with 0.5 mg/ml lysozyme). Cells were then incubated at 30°C for 20 min and vortexed briefly before boiling for 3 min to complete cell lysis. Lysates were cleared by microcentrifugation (16,100 × *g*, 5 min), and 1 μl of supernatant was used as the template for PCR with primers flanking the integration junctions.

Strain construction for *tcl* promoter analysis. To generate the reporter strains that were used in the promoter analysis experiments, a two-step integration strategy was used. First, integration of the *thrC*::P_{xylA}-*tclU* insert was accomplished using a derivative of the *thrC* integrative plasmid pBS4S (32), in which the plasmid was modified to include the xylose-responsive P_{xylA} promoter and its cognate repressor (*xylR*) upstream of the putative regulatory gene *tclU* (full sequences provided in Fig. S1 in the supplemental material). The correctly assembled construct was transformed into *B. subtilis* 168, and the resulting strain was used as the recipient strain for subsequent transformations. To generate the promoter-*lacZ* fusion plasmids pLacZ_E, pLacZ_Q, and pLacZ_I, the *amyE* integrative vector pDG1661 (33) was modified to include promoters P_{tclE}, P_{tclQ}, and P_{tclI}. These promoters were amplified from *M. caseolyticus* plasmid DNA and were ligated into pDG1661 within the polylinker upstream of the *spoVG-lacZ* reporter gene (full sequences provided in Fig. S2 in the supplemental material). The promoter inserts consisted of approximately 500 bp of sequence immediately upstream of the start codons of the corresponding genes. Once constructed, each plasmid was transformed into the *B. subtilis* strain carrying *thrC*::P_{xylA}-*tclU* described above to yield the strains used in β-galactosidase assays.

β-Galactosidase assays. *B. subtilis* strains were grown overnight in LB-Sp and were then diluted 1:100 into fresh antibiotic-free broth and cultured until late-exponential phase (optical density at 600 nm [OD_{600}] ≈ 1.0). Cultures were then placed on ice for 10 min to arrest growth before quantifying OD_{600} values. A total of 150 μl of culture was added to 750 μl

TABLE 1 Strains and plasmids used in this study

Strain or plasmid[a]	Description[b]
Strains	
M. caseolyticus 115	MP1 producer, source of all *tcl* genes used in this study
B. subtilis 168	Heterologous host for promoter activity assays
B. subtilis BS 168R	Micrococcin-resistant (*rplK*ΔP22) derivative of *B. subtilis* 168 used for heterologous expression
S. aureus ATCC 6538	Indicator strain for spot-on-lawn bioassays
Plasmids	
pDG1661	*spoVG-lacZ* reporter for *amyE* integration; Cm^r in *B. subtilis* and Ap^r or Sp^r in *E. coli*
pLacZ_E	pDG1661 with P_{tclE}-*spoVG-lacZ*
pLacZ_Q	pDG1661 with P_{tclQ}-*spoVG-lacZ*
pLacZ_I	pDG1661 with P_{tclI}-*spoVG-lacZ*
pBS4S	*B. subtilis thrC* integration vector; Sp^r in *B. subtilis* and Ap^r in *E. coli*
pPxyl_U	pBS4S derivative with P_{xylA}-*tclU*
pTclE	pBS4S derivative with P_{strong}-*tclE*; Sp^r
pTclE_His	pTclE with His_6-*tclE* (N-terminal tag)
pTclE_GST	pTclE with GST-*tclE* (N-terminal tag)
pDG1662	*amyE* integration vector; Cm^r in *B. subtilis* and Ap^r or Sp^r in *E. coli*
pLEGO_proto	pDG1662 derivative with p15A ori, P_{xylA} promoter, and new MCS; Cm^r
pLEGO	pLEGO_proto with P_{xylA}-*tclIJKLMNPS*
pLEGO_strepI	pLEGO with Strep-*tclI* (N-terminal tag)
pLEGO_strepJ	pLEGO with Strep-*tclJ* (N-terminal tag)
pLEGO_strepK	pLEGO with Strep-*tclK* (N-terminal tag)
pLEGO_Kstrep	pLEGO with *tclK*-Strep (C-terminal tag)
pLEGO_strepL	pLEGO with Strep-*tclL* (N-terminal tag)
pLEGO_Lstrep	pLEGO with *tclL*-Strep (C-terminal tag)
pLEGO_strepM	pLEGO with Strep-*tclM* (N-terminal tag)
pLEGO_Nstrep	pLEGO with *tclN*-Strep (C-terminal tag)
pLEGO_strepP	pLEGO with Strep-*tclP* (N-terminal tag)
pLEGO_Pstrep	pLEGO with *tclP*-Strep (C-terminal tag)
pLEGO_strepS	pLEGO with Strep-*tclS* (N-terminal tag)
pLEGO_Sstrep	pLEGO with *tclS*-Strep (C-terminal tag)
pLEGO_ΔS	pLEGO with *tclS* removed
pLEGO_ΔP	pLEGO with *tclP* removed

[a] Strains and plasmids not from this study are cited in the Materials and Methods.
[b] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Sp^r, spectinomycin resistance.

of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 20 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) before adding 10 μl of toluene to permeabilize cells. Suspensions were vortexed for 15 s, warmed to 30°C for 5 min, combined with 150 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution (4 mg/ml in buffer Z), and incubated at 30°C for 30 to 40 min. Reactions were stopped by adding 400 μl of stop buffer (1 M Na_2CO_3). Insoluble debris was sedimented by microcentrifugation (16,100 × *g*, 5 min) before measuring OD_{420} values. A sample processed in an identical manner but omitting cells was used as a blank. Miller units were calculated according to reference 34.

Construction of pLEGO and derivatives. To construct pLEGO, numerous alterations were made to the *amyE* integrative vector pDG1662 (33). In brief, the high-copy-number ColE1 replication origin was replaced with the lower-copy-number p15A origin from pACYC184 (35) followed by removal of the β-lactamase (*bla*) gene and destruction of many backbone restriction sites. The small polylinker within the integrating region of the plasmid was expanded to include the following restriction sites: BamHI, AvrII, SphI, KpnI, PstI, SacI, SalI, AatII, XhoI, BglII, and EcoRI. Finally, the *xylR*-P_{xylA} promoter cassette from pHCMC04 (36) was introduced into the polylinker between BamHI and AvrII sites. We disrupted XhoI, EcoRI, and SalI sites within *xylR* in the process. This construct, termed pLEGO_proto, served as the base upon which pLEGO was constructed. To complete the assembly of pLEGO, the eight selected *tcl* processing genes (*tclIJKLMNPS*) were amplified from *M. caseolyticus* strain 115 and introduced between appropriate restriction sites in the pLEGO_proto polylinker. In several cases, mutagenic PCR was employed to disrupt restriction sites within *tcl* genes without altering the amino acid sequence of gene products. The complete sequence of pLEGO is provided in Fig. S3A in the supplemental material. Epitope-tagged derivatives of pLEGO-encoded enzymes were made by reamplifying *tcl* genes from pLEGO with primers designed to append the 8-amino-acid (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) Strep tag (sequences given in Fig. S3B). For pLEGO derivatives lacking *tclP* or *tclS*, the genes were removed by digesting pLEGO at the appropriate flanking restriction sites followed by treatment with Klenow polymerase and religation.

Construction of pTclE and derivatives. The plasmid pTclE was engineered as a derivative of the *thrC* integrative vector pBS4S (32). The strong constitutive *B. subtilis* promoter P_{strong} (iGEM biological parts registry BBa_K780003) was introduced between the NotI and XbaI sites of pBS4S using hybridizing oligonucleotides, and the *tclE* gene was cloned downstream between the XbaI and PstI sites to generate the final construct, pTclE. The complete sequence of pTclE is provided in Fig. S4A in the supplemental material. Plasmid derivatives for appending hexahistidine (His_6) or glutathione *S*-transferase (GST) tags to the N terminus of TclE were generated in a similar manner by incorporating the tag in the primer (His_6) or using overlap extension PCR (GST). The GST tag was amplified from pGEX-4T-1 (GE Life Sciences, Marlborough, MA). Detailed sequences are provided in Fig. S4B.

Micrococcin susceptibility bioassays. Methanolic extracts from *B. subtilis* strains expressing *tcl* genes were obtained as follows. After overnight growth in antibiotic-supplemented LB, cells were diluted 1:60 into 6 ml of LB supplemented with xylose (1%, wt/vol). After an additional 8 h of growth, 4.8 ml of each culture was pelleted using iterative microcentrifugation (16,100 × *g*, 2 min for each run) and pellets were stored at −20°C prior to extraction. Extractions were accomplished by suspending thawed cell pellets in 1 ml of high-pressure liquid chromatography (HPLC)-grade methanol and vortexing vigorously for 3 min. Suspensions were left to incubate at room temperature for 5 min, followed by vortexing for an additional 3 min. Insoluble material was sedimented by microcentrifugation (16,100 × *g*, 5 min), and supernatants were transferred to fresh microcentrifuge tubes. These extracts were concentrated at room temperature to 80-μl volumes using a DNA120 SpeedVac (Savant Instruments Inc., Holbrook, NY) and stored at 4°C until assayed. The bioactivity of each extract was evaluated using a spot-on-lawn bioassay as described previously (29). The spot-on-lawn indicator strain *S. aureus* ATCC 6538 was grown overnight and diluted 1:20 into fresh LB before 200 μl was spread onto LB agar and allowed to dry. Once dry, 5-μl spots of concentrated extracts were placed upon bacterial lawns, allowed to dry, and then incubated at 37°C. After 8 h, plates were assessed for zones of inhibition and imaged.

Extraction and electrospray ionization mass spectrometric analysis of MP1 and MP2. For each MP1- or MP2-producing strain, a single colony was grown overnight in LB-Sp and used to inoculate LB-Sp supplemented with 1% xylose (1 liter, 37°C, 200 rpm, 16 h). The cells were harvested by centrifugation (7,878 × *g*, 15 min), resuspended in 25 ml of methanol, vortexed for 30 s, and gently agitated on a benchtop shaker for 20 min at room temperature. Sodium sulfate (10 g) was added to the suspended cells before centrifugation (16,420 × *g*, 5 min). The methanol fraction was evaporated to <1 ml, and 30 ml of ethyl acetate was added. The mixture was transferred to a separatory funnel, washed with 30 ml of water, and subsequently washed with 30 ml of saturated sodium chloride. The combined aqueous washes were back extracted with ethyl acetate. The ethyl acetate layers were combined, dried with sodium sulfate, and fil-

tered, and the volatiles were removed under reduced pressure. The resulting oily residue was redissolved in 50 μl of methanol, and 5 μl was transferred to a 96-well sample plate and evaporated under a stream of air. The residue was taken up in 20 μl of dimethyl sulfoxide (DMSO), injected (10 μl) into a Waters XBridge C_{18} column (3.5 μm, 4.6 by 50 mm), and separated using a Waters Alliance 2795 HPLC system (Waters, Milford, MA). The following linear gradient was used: 5% to 100% B over 8 min (A = water/0.1% formic acid, B = acetonitrile/0.1% formic acid, 1 ml/min). The eluent was analyzed by UV absorbance (Waters 2996 diode array UV detector) and electrospray ionization (ESI) positive-mode mass analysis (Waters ZQ-4000 quadrupole mass spectrometer). MP1 and MP2 products were eluted between 7.3 and 7.6 min.

RESULTS

Identification of the essential elements of an engineered micrococcin expression system. To aid in the initial design of a minimized *B. subtilis* micrococcin expression system, we combined bioinformatic analyses (29), structural modeling, and a detailed literature survey to identify essential elements within the *M. caseolyticus tcl* gene cluster (shown in Fig. 1A). Homology arguments (12, 17, 29) suggest that TclI, TclJ, and TclN are involved in converting each of the core peptide Cys residues to thiazoles (Fig. 1B, blue), with TclI as the Ocin-ThiF-like RRE-containing protein, TclJ performing ATP-dependent heterocyclization (to thiazoline), and TclN oxidizing the thiazoline groups to thiazoles. TclK and TclL are likely responsible for the four Ser/Thr dehydration reactions (Fig. 1B, red), with TclM catalyzing peptide macrocyclization via the two dehydrated serines (magenta). TclP and TclS have homology with short-chain dehydrogenases, and we hypothesized that these enzymes are involved in decarboxylation of the C-terminal Thr residue (Fig. 1B, green) as has also been proposed for the homologous proteins in the *B. cereus tcl* cluster (12). TclQ is a ribosomal L11 homologue that we have shown to be involved in immunity (29), TclU is a putative transcriptional regulator (discussed below), and the function of the protein potentially encoded by *orf18* is unclear.

Analysis of *tcl* promoter activity in *B. subtilis*. The *tcl* gene cluster is organized into three apparent transcriptional units that are controlled by promoters upstream of *tclE*, *tclQ*, and *tclI* (Fig. 1A). The *tclE* unit is monocistronic, encoding only the TclE precursor peptide; the *tclQ* unit is a three-gene operon encoding TclQ, Orf18, and TclS; and the *tclI* transcriptional unit encodes eight proteins, most of which are predicted to participate in post-translational processing of the TclE core peptide. The final gene in the *tclI* operon (*tclU*) encodes a putative MerR-type transcriptional regulator. Sequence alignments and structural modeling of TclU (not shown) indicate the presence of a conserved helix-turn-helix DNA-binding domain; however, the effector domain typically found in MerR regulators appears to be truncated or absent in TclU, making it difficult to predict its regulatory role with respect to *tcl* gene expression.

In transferring a functional *tcl* gene cluster into *B. subtilis*, transcriptional compatibility was a critical consideration. We sought to test whether the three *tcl* promoters described above are active in *B. subtilis* and whether TclU influences their activity. As an initial step, we employed a *lacZ* reporter assay to monitor the activity of the three predicted *tcl* promoters in *B. subtilis*. The 500-bp regions immediately upstream of the *tclE*, *tclQ*, and *tclI* start codons were fused to the *spoVG-lacZ* reporter, and these fusions were integrated into the *B. subtilis* chromosome. In each of these reporter strains, *tclU* (controlled by the xylose-inducible promoter P_{xylA}) was integrated at a second chromosomal locus (Fig. 2A). Promoter strength was quantified on the basis of β-galactosidase activity in the presence or absence of TclU induction (Fig. 2B). From these results, we conclude that P_{tclE} and P_{tclQ} exhibit modest to strong activity, whereas P_{tclI} is inactive. TclU appears to play only a minor role, if any, in the regulation of these promoters in *B. subtilis*, justifying its exclusion from the heterologous expression system.

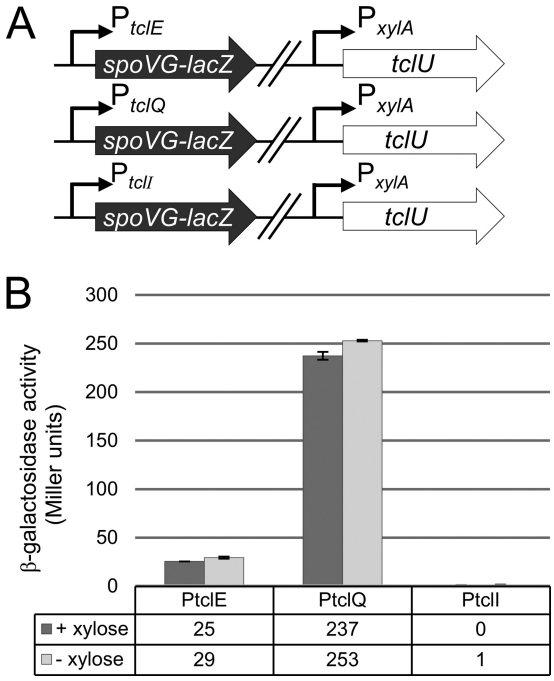

FIG 2 Analysis of *M. caseolyticus tcl* promoter activity in *B. subtilis* strain 168. (A) The promoter regions upstream of *tclE*, *tclQ*, and *tclI* were fused to a *lacZ* reporter and integrated into the *B. subtilis* chromosome. P_{xylA}-*tclU* was similarly integrated at a separate locus for inducible expression of *tclU*. (B) β-Galactosidase activity was measured to quantify *tcl* promoter strength in the presence (+ xylose) or absence (− xylose) of *tclU* expression. Error bars represent the standard deviation from the mean using three biological replicates per condition.

Genetic refactoring of the *tcl* gene cluster supports production of MP1 in *B. subtilis*. We next turned to the construction of a functional *tcl* gene cluster in the laboratory model organism *B. subtilis* strain 168. We sought to accomplish three primary objectives: (i) minimization of the ensemble of *tcl* genes required for micrococcin production, (ii) modularization of these genes to permit rapid manipulation of the pathway, and (iii) transcriptional optimization of the precursor peptide-encoding gene *tclE* and the *tcl* biosynthetic operon.

We decided on a two-plasmid system, allowing independent manipulation of different components of the pathway. In a preliminary expression system, the naturally cotranscriptionally arranged genes *tclIJKLMNP* were introduced downstream of P_{xylA} in a vector designed for transgene integration at the *B. subtilis amyE* locus. The *tclU* gene was omitted in this construct. The remaining four genes (*tclE*, *tclS*, *orf18*, and *tclQ*) were introduced into a second vector that was designed for integration at the *thrC* locus. These genes were maintained in their original configuration (Fig. 1A) under the control of their native promoters (P_{tclE} and P_{tclQ}). Integration of these two preliminary plasmids into *B. subtilis*, fol-

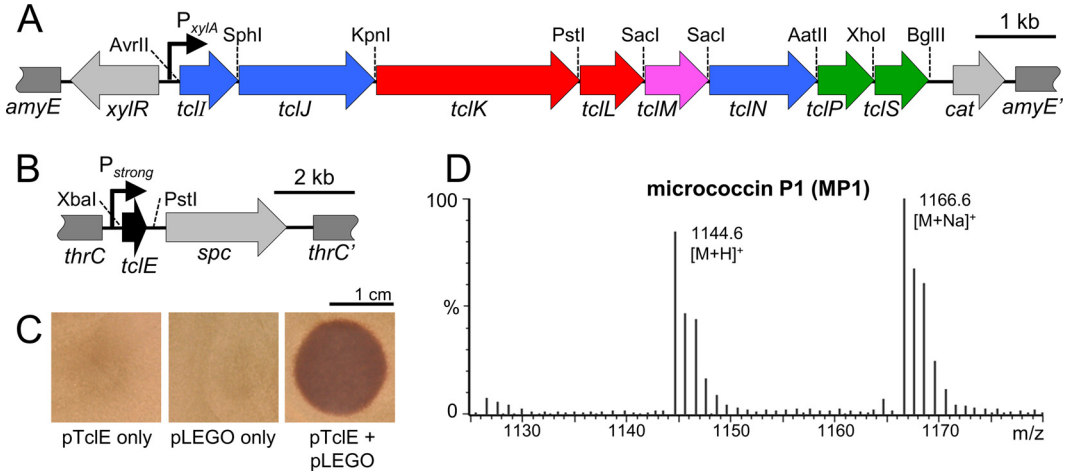

FIG 3 Minimization and modularization of the *tcl* biosynthetic pathway in *B. subtilis*. (A) Gene map of the *tcl* region of pLEGO showing the xylose-inducible promoter (bent arrow), relevant processing genes (colored arrows), unique restriction sites, and homology regions (dark gray) for recombination into the *B. subtilis* chromosome. Light gray arrows indicate the xylose-responsive regulatory gene (*xylR*) and the chloramphenicol resistance determinant (*cat*). (B) Gene map of the TclE-encoding portion of pTclE showing the constitutive promoter (bent arrow), *tclE*, homology regions for integration into the *B. subtilis* chromosome (dark gray), and the spectinomycin resistance determinant (*spc*). (C) Spot-on-lawn bioassays with methanolic extracts from strains transformed with pTclE only, pLEGO only, or both. (D) ESI-MS analysis of the bioactive extract from the pTclE + pLEGO strain. MP1: observed 1,144.6 $[M+H]^+$, calculated 1,144.2 $[M+H]^+$; observed 1,166.6 $[M+Na]^+$, calculated 1,166.2 $[M+Na]^+$.

lowed by growth in the presence of xylose, led to the production of a methanol-extractable product with bioactivity against wild-type *B. subtilis*, based on a qualitative spot-on-lawn assay (not shown).

With the success of the proof-of-concept experiment described above, we further reduced and modularized our two-plasmid heterologous expression system. This system is summarized in Fig. 3. For the *amyE* integration plasmid, the putative biosynthetic genes were all arranged as a single operon under the control of the xylose-inducible P_{xylA} promoter. To accomplish this, *tclS* was moved from the *thrC* integration plasmid described above, completing the eight-gene *tclIJKLMNPS* biosynthetic operon. This operon was punctuated by unique intergenic restriction sites, multiple overlapping regions between *tcl* open reading frames were resolved, and artificial ribosome binding sites were installed. As such, each biosynthetic gene constitutes a translationally independent unit that can be altered or removed while minimally affecting the expression of neighboring genes. This final plasmid (pLEGO) is represented in Fig. 3A, and its full sequence is provided in Fig. S3 in the supplemental material. The *thrC* integration plasmid was dedicated to *tclE* expression. This required the removal of *tclS* (moved to pLEGO), *orf18* (a gene of unknown function), and the immunity gene *tclQ*. We alleviated the requirement for *tclQ* by selecting a spontaneous micrococcin-resistant *B. subtilis* mutant, which was used for all subsequent micrococcin expression experiments. This strain (BS 168R) was found to possess a single-codon deletion in *rplK*, which encodes the known ribosomal protein target of micrococcin (37). Given the relatively weak expression level of the native *tclE* promoter in *B. subtilis* (Fig. 2B), we replaced it with a strong constitutive promoter (P_{strong}). The resulting P_{strong}-*tclE* expression plasmid (termed pTclE) is represented in Fig. 3B. Its complete sequence is given in Fig. S4 in the supplemental material.

The pLEGO and pTclE plasmids were transformed into BS 168R, and transformants were evaluated for micrococcin production (Fig. 3C and D). Methanolic extracts from strains transformed with pTclE only, pLEGO only, or pTclE + pLEGO were tested for bioactivity against the *S. aureus* reference strain ATCC 6538. Only the strain with both transgenic components supported the production of a bioactive compound (Fig. 3C). Mass analysis of this methanol-extractable product was consistent with its identity as MP1 (expected *m/z* 1,144.2 $[M+H]^+$; observed *m/z* 1,144.6 $[M+H]^+$), which is in agreement with earlier work characterizing the product of this pathway (29). In addition, methanolic extracts from strains that were missing either TclI or TclP lacked bioactivity and were deficient in compounds detectable by mass spectrometry (MS) within the expected mass range for MP1 and MP2 (not shown). Taken together, these results support the identity of the antibacterial compound as MP1 and demonstrate the success of this highly engineered system in reconstituting the *M. caseolyticus* micrococcin pathway in *B. subtilis*.

Testing the utility of the heterologous, modularized genetic system. The successful reconstitution of this pathway in *B. subtilis* opens the door for detailed mechanistic studies of this pathway and its constituents. Such studies will inevitably require purification of substrate, enzymes, or both and would be benefitted by a better understanding of how Tcl proteins in this pathway tolerate N- or C-terminal affinity tags. To address this, we tagged each pathway component at one or both termini. As illustrated in Fig. 1B, the TclE precursor peptide consists of a 35-amino-acid N-terminal leader (which presumably recruits the biosynthetic machinery) and the C-terminal 14-amino-acid core peptide. Because this core peptide undergoes C-terminal decarboxylation as part of its maturation process, we did not attempt to fuse tags to this end of TclE. We were gratified to observe that fusions of glutathione *S*-transferase (GST) and hexahistidine (His_6) to the N terminus of TclE result in the production of a bioactive compound (Fig. 4A), which suggests that such tags do not interfere with posttranslational processing and would be suitable for purifying TclE intermediates at various stages of maturation. We also tested each of the biosynthetic Tcl proteins for their ability to tolerate the 8-ami-

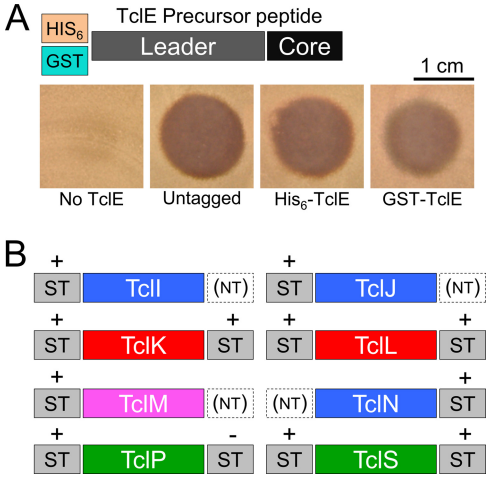

FIG 4 Each component of the micrococcin biosynthetic pathway is amenable to affinity tagging. (A) Graphical representation of the affinity tags fused to the N terminus of the precursor peptide TclE. Spot-on-lawn assays were used to test the bioactivity resulting from tagged TclE. (B) Representations of the various locations in which Strep tags (ST) were fused to Tcl-processing proteins. The effect of these tags on bioactivity is also indicated; +, the fusion protein was functional; −, the fusion abolished bioactivity; NT, the tag was not tested.

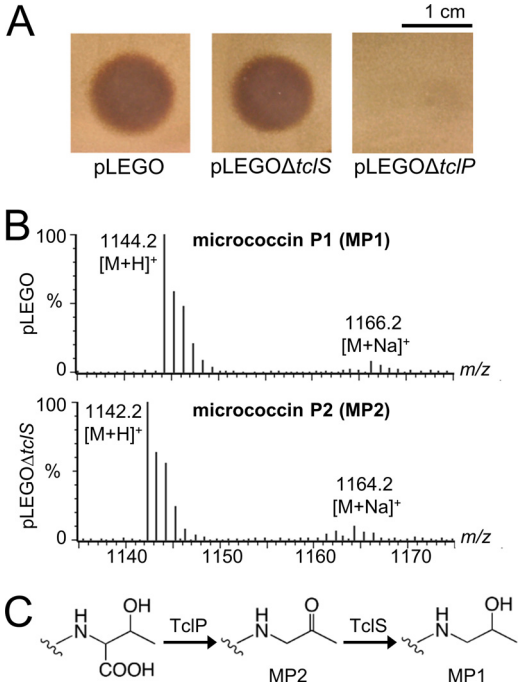

FIG 5 Analysis of the role of TclS in micrococcin C-terminal processing. (A) Spot-on-lawn assays with methanolic extracts from *B. subtilis* strains transformed with pLEGO and pLEGO derivatives lacking *tclS* and *tclP*. In these experiments, the pTclE plasmid had been modified to express the GST-TclE fusion. (B) ESI-MS analysis of purified extracts from pLEGO and pLEGOΔ*tclS* strains. MP1: observed 1,144.2 $[M+H]^+$, calculated 1,144.2 $[M+H]^+$. MP2: observed 1,142.2 $[M+H]^+$, calculated 1,142.2 $[M+H]^+$. (C) Proposed roles of TclP and TclS in C-terminal processing of MP1 and MP2.

no-acid Strep tag at their N or C termini. Based on the ability to produce biologically active micrococcin, all eight proteins are compatible with this tag in at least one orientation (Fig. 4B). Of the 12 Strep-tagged variants tested, only one (TclP-Strep) failed to support micrococcin biosynthesis. Taken together, these results demonstrate that every protein component in this pathway can be modified in a manner that would allow for affinity purification and detection with commercial reagents. These results also demonstrate the relative ease with which alterations can be made to individual components in this expression system without perturbing the rest of the pathway.

Accumulation of MP1 versus MP2 is determined by the activity of the putative C-terminal dehydrogenase TclS. The modularity of this micrococcin production system also allows one to rapidly perform specific *tcl* gene removal experiments. Of the eight processing proteins in this system, six have predictable roles in thiazole installation (TclI/TclJ/TclN), Ser/Thr dehydration (TclK), and macrocyclization (TclM). We preserved the two putative short-chain dehydrogenases (TclP and TclS) in our expression system with the assumption that they are somehow involved in the decarboxylation of the core peptide C-terminal Thr residue; however, defined roles for these enzymes have remained unclear. To address this, either *tclP* or *tclS* was removed from pLEGO by simple restriction digestion and religation. The resulting plasmids were integrated into a *B. subtilis* strain background harboring the GST-*tclE* cassette. Methanol extracts from these strains were then tested for bioactivity by spot-on-lawn assay (Fig. 5A) and the presence of thiopeptide compounds by electrospray ionization mass spectrometry (ESI-MS). Interestingly, the extract from the *tclP* deletion strain showed no bioactivity and no detectable thiopeptide product; however, the *tclS* deletion mutant produced a bioactive compound with a mass consistent with MP2 rather than MP1 (Fig. 5B). MP2 has a methyl ketone group at the C terminus, whereas MP1 is reduced to the alcohol at the same position. Therefore, we conclude that TclS is responsible for the conversion of MP2 to MP1 (Fig. 5C). TclP appears to play an essential role in micrococcin production, acting at some point prior to TclS in the biosynthetic pathway. Its presumed function as a short-chain dehydrogenase points to a possible role for TclP in the initial oxidative decarboxylation of the C-terminal Thr as illustrated in Fig. 5C.

DISCUSSION

Heterologous thiopeptide expression serves several purposes. (i) It allows confirmation of the sufficiency of a given gene cluster to facilitate the production of a specific thiopeptide product. (ii) With the gene cluster cloned on a plasmid, it allows the researcher to probe the biosynthetic logic of the pathway with greater facility. (iii) It opens the door to the rational design and large-scale production of new thiopeptide analogs with improved pharmacological or bioactivity properties. Attempts to heterologously express diverse thiopeptide gene clusters have been met with mixed success (7, 15, 38–40). In most cases, *Streptomyces* species, such as *Streptomyces lividans* and *Streptomyces coelicolor*, have been employed. These expression hosts can be limiting due to low growth rate and potential incompatibility with genes derived from distantly related organisms, such as the low-GC firmicutes. It is remarkable that *B. subtilis* has rarely (if ever) been utilized for thiopeptide production. The *B. subtilis* strain used in this study is fast-growing and straightforward to genetically manipulate, with its natural competence and high recombinogenicity as well as the

wealth of genetic tools developed by a large community of researchers. *B. subtilis* is particularly attractive for the production of thiopeptides, whose native producers (such as *Bacillus*, *Staphylococcus*, and *Macrococcus* spp.) share close evolutionary relatedness.

Several levels of potential incompatibility should be considered with respect to heterologous thiopeptide production. These include codon bias, transcriptional efficiency, self-intoxication by the bioactive product, and cofactor compatibility. Worthy of special mention is the $tRNA^{Glu}$ glutamate donor that is employed by the RiPP Ser/Thr dehydratase systems (TclK and TclL in the work presented here). Initially demonstrated for the two-domain NisB lantibiotic dehydratase (41), $tRNA^{Glu}$ is presumably the source of the glutamyl group used to activate Ser/Thr residues for subsequent dehydration in many RiPP pathways. In the *in vitro* reconstituted thiomuracin biosynthetic system (17), all thiopeptide biosynthetic components can be produced and purified from *E. coli*; however, the *E. coli* $tRNA^{Glu}$ was not a suitable substrate for Ser/Thr dehydration, so a hybrid system that utilized the *E. coli* aminoacyl $tRNA^{Glu}$ synthetase and *Thermobispora bispora* $tRNA^{Glu}$ was required. $tRNA^{Glu}$ is therefore a crucial consideration in transferring thiopeptide biosynthetic systems across bacterial taxa. In moving the micrococcin pathway from *M. caseolyticus* to *B. subtilis*, we were satisfied with the similar codon preferences of the two organisms, and we were able to override transcriptional incompatibilities by using promoters with predictable behavior in the producer strain. We were encouraged by the close similarity in the $tRNA^{Glu}$ sequence (a 1-bp difference located in the acceptor stem) (see Fig. S5 in the supplemental material), and successful micrococcin production in *B. subtilis* indicates that there was not a problem with $tRNA^{Glu}$ incompatibility.

Our functional genetic reconstruction of the *M. caseolyticus* micrococcin pathway in *B. subtilis* defines a minimally sufficient gene set for micrococcin biosynthesis. By preselecting a micrococcin-resistant variant of the producer strain, we could omit immunity functions from the engineered gene cluster. We also excluded genes for micrococcin export; thus, it is not surprising that micrococcin can be successfully extracted from *B. subtilis* cell pellets. It is not known whether our strains secrete residual micrococcin into the medium either passively or via endogenous efflux systems. Our data show that the minimal genetic requirement for conversion of the TclE precursor peptide to micrococcin (MP2) is the seven-gene set *tclIJKLMNP*, with *tclS* responsible for the reduction of MP2 to MP1. TclI, which we predict to be an Ocin-ThiF-like RRE-containing protein, is required for micrococcin production (not shown), TclJ and TclN constitute a putative thiazole installation module, TclK and TclL constitute a Ser/Thr dehydration system, TclM is predicted to catalyze the [4 + 2] cycloaddition required for macrocycle formation, and TclP is likely involved in oxidative decarboxylation of the core peptide C terminus.

The genetic refactoring described here enables unprecedented control over the properties of this system as demonstrated by our affinity tagging of every protein component and our ability to easily remove the processing enzymes TclP and TclS. These experiments constitute the beginning of an ongoing series of tests that are allowing us to dissect the entire micrococcin biosynthetic pathway in great detail. These experiments capitalize on the ability to genetically block the pathway and rapidly purify the affinity-tagged precursor peptide TclE at various stages of maturation. This approach may be employed to assess the biosynthetic consequences of numerous *tcl* gene deletions and point mutations, all of which are easily incorporated using standard molecular cloning techniques. We hope to be able to leverage this system to study physical interactions between pathway components, to explore how well the biosynthetic proteins tolerate core peptide sequence variation, and to create a system for combinatorial biosynthesis of never-seen-by-nature bioactive compounds.

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