Pyridinyl polythiazole class peptide antibiotic micrococcin P₁, secreted by foodborne Staphylococcus equorum WS2733, is biosynthesized nonribosomally

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Recently, foodborne Staphylococcus equorum WS2733 was isolated from a French red smear cheese on account of its strong inhibitory activity against Gram-positive pathogens such as Listeria. The antagonistic substance was identified as macrocyclic peptide antibiotic micrococcin P₁, which had previously not been reported for the genus Staphylococcus. Micrococcin P₁, also a potent inhibitor of the malaria parasite Plasmodium falciparum, is structurally related to thiostrepton, thiocillins and nosiheptide. Although all of these peptide antibiotics have been known for quite a long time, their mode of biosynthesis had not been determined in detail yet. By using degenerated PCR, a gene fragment encoding a nonribosomal peptide synthetase (NRPS) could be amplified from S. equorum. The corresponding chromosomal locus was disrupted by insertional mutagenesis, and it could be shown that all mutants obtained displayed a micrococcin P₁-deficient phenotype. Sequence analysis of a coherent 2.8-kb fragment revealed extensive homology to known NRPSs, and allowed the assignment of the domain organization ‘condensation-adenylation-thiolation-condensation’; an arrangement predicted only for two loci within the presumably 14-modular, 1.6-MDa biosynthetic NRPS template. Biochemical characterization of the adenylation domain exhibited selectivity for the substrate amino-acid threonine. All of these data substantiate that the macrocyclic peptide antibiotic is biosynthesized nonribosomally, and provide the basis for the characterization of the entire biosynthetic gene cluster. The biosynthetic machinery of micrococcin will serve as a model system for structurally related, pharmacologically important pyridinyl polythiazole class peptide antibiotics. Furthermore, this knowledge will enable the manipulation of its NRPS template, which in turn may grant the targeted engineering of even more potent anti-listerial and anti-malaria drugs.

Keywords: macrocyclic peptide antibiotic; micrococcin P₁; nonribosomal peptide synthetase; Plasmodium falciparum; Staphylococcus equorum.

Foodborne Staphylococcus equorum WS2733 has been recently isolated from French Raclette cheese on account of its bacteriostatic effect on a variety of Gram-positive bacteria [1]. It was demonstrated that this activity is based on secretion of the unique and highly modified macrocyclic peptide antibiotic micrococcin P₁, which had previously not been reported for the genus Staphylococcus. Micrococcin P₁, also a potent inhibitor of the malaria parasite Plasmodium falciparum, is structurally related to thiostrepton, thiocillins and nosiheptide. Although all of these peptide antibiotics have been known for quite a long time, their mode of biosynthesis had not been determined in detail yet. By using degenerated PCR, a gene fragment encoding a nonribosomal peptide synthetase (NRPS) could be amplified from S. equorum. The corresponding chromosomal locus was disrupted by insertional mutagenesis, and it could be shown that all mutants obtained displayed a micrococcin P₁-deficient phenotype. Sequence analysis of a coherent 2.8-kb fragment revealed extensive homology to known NRPSs, and allowed the assignment of the domain organization ‘condensation-adenylation-thiolation-condensation’; an arrangement predicted only for two loci within the presumably 14-modular, 1.6-MDa biosynthetic NRPS template. Biochemical characterization of the adenylation domain exhibited selectivity for the substrate amino-acid threonine. All of these data substantiate that the macrocyclic peptide antibiotic is biosynthesized nonribosomally, and provide the basis for the characterization of the entire biosynthetic gene cluster. The biosynthetic machinery of micrococcin will serve as a model system for structurally related, pharmacologically important pyridinyl polythiazole class peptide antibiotics. Furthermore, this knowledge will enable the manipulation of its NRPS template, which in turn may grant the targeted engineering of even more potent anti-listerial and anti-malaria drugs.

Keywords: macrocyclic peptide antibiotic; micrococcin P₁; nonribosomal peptide synthetase; Plasmodium falciparum; Staphylococcus equorum.

Abbreviations: A domain, adenylation domain; C domain, condensation domain; Cy domain, heterocyclization domain; NRPS, nonribosomal peptide synthetase; Ox domain, oxidoreductase domain; Ppant, cofactor 4'-phosphopantetheine; T domain, thiolation domain; BHI, brain–heart-infusion.

Note: web pages available at http://www.chemie.uni-marburg.de/~stachel and http://www.wzw.tum.de/micbio

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noted. The first type is exemplified by the maturation of the peptide antibiotic microcin B17, where heterocyclic ring formation occurs post-translationally by modification of the gene-encoded precursor McbA [19]. An alternate mode of heterocyclization is observed in a family of so-called non-ribosomal peptide synthetases (NRPSs) that function as multimodular templates for the assembly of a large group of peptide antibiotics. The biosynthetic templates for some virulence-conferring siderophores (i.e. yersiniabactin and exochelin), DNA-binding peptides such as bleomycin, and the branched-cyclic topical antibiotic bacitracin, for example, all contain specialized catalytic heterocyclization (Cy) domains that act during elongation of the covalently tethered nascent peptidyl chain [13,20,21].

Based on analogy, Mocek et al. postulated that pyridinyl polythiazole class peptide antibiotics might be assembled by this latter, nonribosomal mode of action [9]. The current understanding of the protein template-driven synthesis is founded on the multiple carrier thiotemplate mechanism [20,22]. According to this model, the number and organization of iterated modules, which constitute a NRPS template, dictate the primary structure, size and complexity of the synthesized peptide. Each module activates its cognate amino acid in a two-step reaction via a pair of closely coupled domains. An adenylation (A) domain selects the substrate and generates the corresponding aminoacyl adenylate [23,24]. Subsequently, the aminoacyl moiety is covalently tethered to the sulfhydryl residue of a prosthetic phosphopantetheinyll (Ppant) group [22,24,25], which had been post-translationally introduced into the adjacent thiolation (T) domain (also known as peptidyl carrier protein) by a dedicated Ppant transferase [26,27]. The nascent peptidyl chain grows directionally in incremental steps of elongating acyl-S-Ppant intermediates. Peptide bond formation and chain translocation usually occurs when a peptidyl-S-Ppant donor is attacked by a monomeric aminoacyl-S-Ppant nucleophile under the catalytic control of a condensation (C) domain [28]. Alternatively, a Cy domain replaces a C domain in modules that incorporate Cys, Ser or Thr residues and mediate the formation of heterocyclic thiazoline or oxazoline rings [13,21]. This catalytic role has been demonstrated recently in vitro in the yersiniabactin system [29].

Initially, heterocyclization as effected by Cy domains leads to the formation of oxazoline or thiazoline rings. Examples are known though, in which the heterocyclic rings have been further reduced or oxidized to yield the corresponding oxazolidines (or thiazolidines) and oxazoles (or thiazoles), respectively. These modifications are usually crucial for the bioactivity of the synthesized peptides, and are effected by specialized oxidoreductase (Ox) domains. These domains have been observed in two different loci in NRPS modules, and can be situated either as insertion between core motifs A8 and A9 of the A domain or following the T domains [30–32]. All six cysteine residues of micrococcin P1 are
heterocyclized and oxidized to the corresponding thiazole ring (Fig. 1).

In this study, we used degenerated PCR primers directed against the coding sequence of highly conserved core motifs of known NRPSs to identify putative NRPS genes in the micrococcin P1 producer strain *S. equorum* WS2733. In order to demonstrate the involvement of the PCR amplified gene fragment in the biosynthesis of the pyridinylpolythiazole class peptide antibiotic, a gene disruption experiment was carried out using insertional mutagenesis. A coherent 2.8-kb gene fragment was sequenced and analyzed, and the A domain encoded was characterized biochemically. These studies indicated that the gene fragment identified probably encodes the third module of the micrococcin P1 synthetase complex. This is believed to be valuable information for the future characterization of the entire biosynthetic gene cluster, which may serve as a precedent for the whole family of pharmacologically important pyridinyl polythiazole class peptide antibiotics.

**Experimental Procedures**

**Bacterial strains, plasmids, media and bacteriocin assay**

*S. equorum* WS2733 was isolated from a French Raclette cheese using the hydrophobic grid membrane filter method [33], grown on slants of plate count agar (Merck) at 30 °C and stored at 4 °C. For individual experiments, cells were subcultured in brain–heart infusion broth (BHI, Merck; 27.5 g·L⁻¹ brain–heart extract, 2 g·L⁻¹ p(+)–glucose, 5 g·L⁻¹ sodium chloride, 2.5 g·L⁻¹ disodium hydrogen phosphate pH 7.4) at 30 °C. For preparation of competent cells, BHI medium was supplemented with 2% glycine. *Listeria ivanovii* WSLC3061 (Weihenstephan *Listeria* collection) was grown as described elsewhere [33]. *Escherichia coli* strains were grown in Luria–Bertani medium. Bacterial strains and plasmids used for cloning are summarized in Table 1. Antibiotic activity was determined by the critical dilution method as described elsewhere [34].

**DNA isolation and sequencing**

DNA techniques were performed using standard procedures [35]. Chromosomal DNA was isolated by the alkaline SDS method. For DNA preparations from *S. equorum*, the cell wall was digested with 23.5 U·mL⁻¹ lysozyme (Sigma) for 1 h at 37 °C. All fragments for cloning were separated by agarose gel electrophoresis and purified using the QIAquick-spin PCR purification kit and DNA gel extraction kit (Qiagen) in accordance with the manufacturer’s instructions. DNA sequencing was performed by SequiServe (Vaterstetten, Germany). A partial nucleotide sequence for the micrococcin synthetase gene has been deposited in the GenBank database under accession number AF183902.

**PCR and cloning**

For the identification of a putative NRPS gene in *S. equorum* WS2733, oligonucleotide primers were derived from highly conserved regions of known NRPSs [36,37]. Forward primer: PSF 5′-GG(AC)T(AC)GG(AC)ACT(AC)GCC(AC)ACT(AC)GGG-3′; reverse primer: PSR2 5′-GGCCA(GT)CCAT(CT)(CT)(CT)GGCA(AG)GTC(AGCT)CC(GT)GT-3′. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). PCRs were carried out in 50 μL volumes containing 5 μL 10× PCR reaction buffer, 2.0 mM MgCl₂, 100 pmol each oligonucleotide primer, 0.2 mM dNTPs and 1 U Goldstar DNA polymerase (Eurogentec, Seraing, Belgium). After an initial denaturation step (95 °C, 5 min), amplification was carried out with 30 cycles (95 °C for 15 s, 60 °C for 1 min, followed by a final extension step at 72 °C for 5 min. PCR products were purified using the QIAquick-spin PCR purification kit (Qiagen) and cloned into the vector pUCK57/T. After restriction mapping, selected plasmids were subjected to DNA sequencing. For the

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in vitro amplification of the upstream and downstream flanking regions of the 728 bp fragment identified, inverse PCR was performed as described by Silver [38]. Chromosomal DNA of *S. equorum* WS2733 was digested with the restriction endonuclease AccII (Eurogentec), self-ligated and used as template for PCR amplification with specific primers derived from the known sequence of the peptide synthetase as obtained by the first PCR (forward 32InvF2: 5′-CCAATCGGGCAAAACCACTTC-3′; reverse 32InvR1: 5′-GGTTTTAGTGCTTCTCAGTACG-3′).

Southern hybridization to a DIG-labelled probe was performed at 60 °C overnight. Subsequent washing was performed at 60 °C, using 0.2 × SSPE buffer (2.2 mM sodium dihydrogenphosphate pH 7.4, 30 mM sodium chloride, and 0.2 mM EDTA) for 25 min. For detection, the DIG kit from Boehringer Mannheim was used according to the manufacturer’s instructions.

**Transformation and electroporation**

Transformation was carried out by electroporation as described by Dover et al. [39] for *E. coli* and Lee [40] for *S. equorum*, using a Bio-Rad Gene Pulser (200 Ω, 25μFD, 2.5 kV) and sterile 2-mm electroporation cuvettes (EquiBio Ltd, Kent, UK). Transformants were selected on Luria–Bertani agar plates supplemented with ampicillin (final concentration 100 μg·mL⁻¹), erythromycin (150 μg·mL⁻¹) and tetracycline (12 μg·mL⁻¹). Colonies harbouring recombinant plasmids were selected by blue–white screening. Plasmids were isolated by standard procedures [35].

**Construction of the disruption plasmid and insertional mutagenesis**

Oligonucleotides PepSyn5′ (5′-ATCTGAATTTCCAGATGAG TCTAATCTTATGATG-3′) and PepSyn3′ (5′-ATCT TCTAGAGCTTTAATGTAAGTCTCCTCCC-3′) were used to amplify a 1223-bp DNA fragment of the putative NRPS gene (restriction endonuclease recognition sites shown in italic). The fragment was cloned into the *Eco*RI–*Xba*I sites of vector pBluescript ISK(-) (Table 1). Then, the tetracycline resistance gene *tetM* from *Enterococcus faecalis* DS16 transposon *Tn916* [40a] was amplified by PCR using oligonucleotides introducing *Eco*RV restriction sites (TetMS5: 5′-ATCT GATATCGAGCCAGAAGGACAC-3′ and TetMS3: 5′-ATCTGATATCTCAACTCAAATAATACATTGA-3′) (restriction endonuclease recognition sites shown in italic). *tetM* was cloned into the unique *Swa*I restriction site of the plasmid pMC1, disrupting the NRPS gene fragment and providing the interface for subsequent homologous recombination. The construct was designated pMC2 (Table 1).

Cells of wild-type *S. equorum* WS2733 were transformed with the disruption plasmid pMC2, as described above. This plasmid is unable to replicate in Gram-positive hosts, thus forcing its integration into the chromosome. Transformants were selected on agar plates containing tetracycline (5 μg·mL⁻¹), and successful recombination was verified by PCR. For this purpose, an additional set of primers was used: 5′-micA (see Biochemical characterization), and 3′-micAT: 5′-ATTAGATCTTTGTGGAAATCTCTGATATCT GTATGG-3′. The genomic arrangement before and after insertional mutagenesis, as well as the relative localization of the primers are depicted below.

**Anti-listerial assays**

The macrocolony and spot-on-the-lawn assays were used to check for inhibitory activity against a set of 12 *L. monocytogenes* strains [41]. First, indicator strains were grown in tryptose broth (Merck: 20 g tryptose, 1 g d(+)-glucose, 5 g sodium chloride, 5 mg thiamine/HCl, 1.25 mL 1 M potassium chloride, adjusted to 1 L dH₂O and pH 7.0) for 24 h at 30 °C. For preparation of the indicator plates, 6.5 mL tryptose broth soft agar [tryptose broth supplemented with 0.8% (w/v) agar] were mixed at 47 °C with 100 μL overnight culture, and poured into Petri dishes (10 cm diameter). Now, 10 μL of an overnight culture of *S. equorum* could be spotted onto the surface of these plates. Alternatively, for the detection of inhibitory substances secreted into the growth medium, *S. equorum* test strains were grown in BHI broth and samples were taken at various time points. After centrifugation (10 000 g, 10 min, 4 °C), supernatants were transferred to fresh tubes, neutralized, and membrane-filtered (Spin X 8160, Costar, Badhoevedrop, Netherlands). Ten microlitres of the samples were spotted onto a lawn of *Listeria* indicator cells. All indicator plates were incubated at 30 °C, and examined for the appearance of inhibition zones after 24–72 h.

**Computer analysis**

Sequence similarity searches were carried out using BLAST 2.0 provided by the National Center for Biotechnology Information. Multiple sequence alignments were performed with CLUSTAL software provided by the Baylor College of Medicine Search Launcher of the Human Genome Sequencing Center. All sequences were obtained from the publicly accessible databases.

**Biochemical characterization**

The gene fragment encoding the unknown A domain of the micrococcin synthetase complex was PCR amplified from chromosomal DNA using *Pfu* Turbo polymerase (Stratagene) in accordance with the manufacturer’s protocol. The following set of primers was used and exploited to generate the terminal restriction sites required for subsequent cloning in the expression vector pQE60 (Qiagen): 5′-micA: 5′-AT CCGGGAATTACAAAAAGGATCTTCTTC-3′, and 3′-micA: 5′-ATAGATCTTTTATATCTTCTGTTCGGCGC-3′ (NcoI and BglII restriction endonuclease recognition sites shown in italic). The integrity of the resulting construct, pMicA-His (Table 1), was confirmed by DNA sequencing on an ABI Genetic Analyser 310.

Expression of the A domain and purification of the His₆-tagged protein was performed as described previously [23]. The gene product is appended by a C-terminal ‘RSHHHHHH’ tag and could be purified, as judged by SDS/PAGE, to apparent homogeneity by single step Ni²⁺-affinity chromatography. Fractions containing the recombinant protein were pooled and dialysed against assay buffer (20 mM Mes pH 8.0, 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM EDTA). The protein could be stored at ~80 °C over several weeks with no observable loss. **
of activity. Protein concentration was determined using the calculated extinction coefficient for the absorbance at 280 nm (A_{280nm}) = 56 400 M^{-1}cm^{-1}.

The ATP-pyrophosphate exchange assay was carried out as described previously [23], using all proteinogenic amino acids and proposed constituting amino acids of micrococcin.

**RESULTS AND DISCUSSION**

**Amplification of a putative NRPS gene fragment**

Because of their highly conserved domain organization, PCR has been shown to be a suitable tool for the amplification of putative NRPS genes from various sources, simply by using primers directed against the coding sequence of their signature core motifs [20,37]. Based on this observation, we performed sequence comparison of NRPS genes encoding gramicidin S synthetase 2 and tyrocidine synthetase 2 from *B. brevis* [42,43], bacitracin synthetase 3 from *B. licheniformis* [21], fengycin synthetase A from *B. subtilis* [44], pyoverdine synthetase D from *Pseudomonas aeruginosa* [45], and AcvA synthetase from *Penicillium chrysogenum* [46]. After determination of their consensus sequence, degenerated oligonucleotides were constructed against the coding sequence of the highly conserved core motifs A3 (YTSGTTGPKG) and A7 (LxRTGD). By using these primers in PCR a DNA fragment of the expected size (≈740 bp) could be amplified from chromosomal DNA of the micrococcin P1 producer strain *S. equorum* WS2733 (data not shown). The amplified DNA fragment was subcloned in pUC57/T (see Table 1). Transformation of *E. coli* XL-1 blue yielded several transformants, from which 24 were selected for further analysis. Restriction endonuclease digests indicated that all the plasmids apparently contained the expected insert size (2.8 kb) and were digested by the restriction enzymes *HindIII* and *PstI*. Sequence analysis revealed that the predicted insert size was confirmed by DNA sequencing of three selected clones. Subsequent sequence analysis revealed a continuous open reading frame encoding all the expected core motifs A3 to A7 in their correct order (Fig. 2).

In order to verify that the PCR product indeed originated from the *S. equorum* strain, Southern blotting was carried out. Chromosomal DNA derived from the micrococcin P1 producer was digested with different restriction enzymes and probed with the DIG-labelled PCR product (Fig. 3). Because of the restriction enzymes chosen only *HindIII* has a restriction site within the 738 bp probe, the additional band consistently visible in all lanes may be the result of cross-hybridization with the corresponding gene fragments of homologous domains. Given the fact that NRPSs consist of repetitive and highly homologous modules, this observation is not unusual for NRPS gene clusters, and multiple signals have been reported in earlier studies performing analogous experiments [36]. Notwithstanding this objection, the result clearly indicates that the NRPS gene fragment identified really originated from the micrococcin producer.

**Characterization of a putative NRPS gene fragment**

To get access to the DNA sequence of the regions flanking the PCR product, chromosomal DNA of *S. equorum* was digested with the restriction enzyme *Acc* II, self-ligated, and used as template for an inverse PCR [38]. The specific oligonucleotides required were derived from the known sequence of the NRPS gene fragment acquired during the first PCR. A PCR product of ≈2.8 kb fragment was obtained, subcloned in pUC57/T (see Table 1) and sequenced by using primer walking. Sequence analysis revealed that the 2747 bp-fragment clearly represents a genuine part of a NRPS gene. The nucleotide sequence has been deposited in the GenBank database under accession number AF183902.

As outlined above, the most common organization of a NRPS elongation module is reflected in the domain arrangement CAT. Alternatively, a Cy domain replaces the C domain in modules that mediate incorporation of a Cys (or Ser) residue and subsequent formation of a heterocyclic thiazoline (or oxazole) ring. As shown in Fig. 1, this latter situation applies to six of the presumably 14 modules of the micrococcin synthetase complex. Consequently, the deduced amino-acid sequence of the determined micrococcin synthetase fragment was aligned with homologous sequences of known NRPSs with varying domain organizations, namely CAT/C as represented by TycC2/3, Gsr3B/4, and SrfAA2/3, and CyAT/C and CyAT/Cy as found in BacA2/3 and BlmIV, respectively. As shown in Fig. 2, the micrococcin synthetase fragment contains the following sequence of characteristic core motifs: C6, NQEYPLE; C7, SDLSRNPL; A1, LNYDEL; A2, LKAGAYVPIP; A3, LMYVIYTSGTTGPKGV; A4, FDVS; A5, NLYGPT; A6, GELCIAGVYTAGYL; A7, YRTGD; A8, GRDEQVKI RGYRIELGEIE; A9, LPDYMP; A10, NGKLNK; T, DNIFEMGGHL; C1, SQKRLYVL; C2, HHEILRTRF; C3, MHHIISDGLS; C4, YRDYSEW. However, neither the partially characterized N-terminal nor the C-terminal domain reveals any evidence for the appearance of a Cy domain. Thus the order of core motifs unequivocally prescribes the domain arrangement CAT/C, an organization predicted for only two loci within the putative micrococcin biosynthetic template (see below).

**Insertional inactivation of the putative peptide synthetase gene**

To validate that the gene fragment identified contributes to the synthesis of the peptide antibiotic micrococcin P1, insertional mutagenesis was performed. To interrupt the NRPS gene in the chromosomal locus of *S. equorum*, the disruption plasmid pMC2 was constructed, containing the antibiotic resistance gene *tetM* (mediating tetracycline resistance) flanked on both sides by a 600-bp fragment of the putative micrococcin synthetase gene. This plasmid is unable to replicate in Gram-positive hosts, thus its integration into the chromosome can be forced by selecting for tetracycline resistance (5 μg·mL^{-1}).

*S. equorum* WS2733 was transformed with the disruption plasmid pMC2, and transformants were selected on plates containing tetracycline. Several colonies were obtained of which six were chosen for further characterization. First, integration of the antibiotic resistance cassette via homologous recombination into the NRPS gene was verified by PCR using specific primers for the *tetM* gene (data not shown). To determine the orientation of the integrated plasmid, an additional PCR was performed using different primer combinations targeting the T3 and T7 promoter sequence of the plasmid, as well as the sequences of the flanking NRPS gene (compare Fig. 4; primer combinations used PepSyn5/T3 promoter, T7 promoter/
This analysis unequivocally revealed that the entire plasmid was integrated into the genome by a single crossover event. Furthermore, it confirmed the genomic arrangement after insertional mutagenesis as shown in Fig. 4.

The mutant strains derived were investigated for growth in BHI medium and their ability to inhibit the indicator strain *L. ivanovii* WSLC3061. By comparing growth rates in liquid culture, it was found that wild-type and mutant strains were not distinguishable based on this test (data not shown). By contrast, all mutants revealed a micrococcin P1-deficient phenotype, as inhibition of the indicator *L. ivanovii* WSLC3061 due to antibiotic production could not be observed (Fig. 4). From these data, we concluded that the NRPS system detected is responsible for the non-ribosomal biosynthesis of the peptide antibiotic micrococcin P1. Consequently, this NRPS system was designated micrococcin P1 synthetase.

To further corroborate the observed micrococcin P1-deficient phenotype of the mutant, we performed an HPLC/MS analysis. Both wild-type and mutant strains were grown in BHI medium, cells were pelleted by centrifugation, and the supernatants concentrated by ammonium sulfate precipitation. After resuspension, the yellowish solutions were applied to reverse-phase HPLC/MS and analysed as described recently [1]. The elution profile of the wild-type preparation confirmed the strong binding of micrococcin P1 to the C18 column, which had been already observed in earlier studies (data not shown). Furthermore, the NRPS system detected is responsible for the non-ribosomal biosynthesis of the peptide antibiotic micrococcin P1. Consequently, this NRPS system was designated micrococcin P1 synthetase.

![Diagram](image-url)
coupled MS analysis confirmed the purity of the eluted substance and its molecular mass of 1143 Da [1]. By contrast, neither micrococcin P_1 nor any intermediate that might be caused by a premature termination of the biosynthetic process (as one might expect for a disrupted biosynthetic template) could be observed in the sample obtained from a mutant strain. An explanation for this finding could be that any intermediate formed might not be released from the disrupted NRPS template due to the absence of the terminal thioesterase (Te) domain [20]. Alternatively, the abortion product may be unstable, not secreted or not extractable, and/or the gene disruption might have occurred at the very beginning of the biosynthetic NRPS template.

Biochemical characterization of the encoded adenylation domain

From current data, it was difficult to conclude at what position within the micrococcin synthetase gene cluster insertion of the tetracycline resistance cassette did occur. A hint towards the answer, however, could be obtained by determination of the substrate selectivity of the A domain discovered. In order to do so, two complementary strategies were applied. First, we took advantage of the selectivity-conferring code of NRPS A domains, and determined the proposed binding-pocket constituents [23]. To this end, phylogenetic studies were carried out considering only these 10 residues. This analysis revealed that the unknown A domain derived from micrococcin synthetase presumably recognizes and activates Ser or Thr (Fig. 5C). Second, the gene fragment of the A domain was amplified by PCR and cloned into the His_{6}-tag expression vector pQE60, yielding plasmid pMicA-His (see Table 1). Gene expression in *E. coli* M15(pREP4), as well as purification and biochemical characterization of gene product was performed as described earlier [23]. An ATP-pyrophosphate exchange assay was carried out using all proteinogenic amino acids, as

![Figure 3](image-url)  
**Fig. 3. Southern hybridization to genomic DNA of *S. equorum*. (A) Restriction digest of chromosomal DNA. (B) Southern hybridization of the DIG-labelled original PCR fragment to the restriction digests shown in (A).**

![Figure 4](image-url)  
**Fig. 4. Insertional inactivation of the putative micrococcin synthetase. (A) Genomic arrangements before and after insertional mutagenesis. Relative localization of the primers used for the analytic PCR are indicated: primer A, 5'-micA; B, PepSyn5; C, T7 prom; D, TetM5; E, TetM3; F, T3 prom; G, PepSyn3; H, 3'-micAT. (B) Bioassays with *S. equorum* wild-type strain WS2733, and micrococcin P_1 deficient mutants (*mic_-). Left, macro-colony assay using liquid cultures of the test strains; right, spot-on-the-lawn assay performed with sterile-filtered culture supernatants.**
As shown in Fig. 5A and B we found that the A domain preferentially activates \( l\)-Thr (not \( l\)-Ser) with a catalytic efficiency \( \frac{k_{\text{cat}}}{K_m} \) of 34 \( \text{mm}^{-1}\text{min}^{-1} \) \( (k_{\text{cat}} = 29.4 \pm 0.9 \text{ min}^{-1}, K_m = 0.86 \pm 0.1 \text{ mM}) \).

**Model for the putative domain organization of micrococcin P1 synthetase**

To date several dozen NRPS systems have been characterized by means of molecular biological and biochemical techniques, providing us with a wealth of information about the general appearance of those biosynthetic templates [20,47–49]. In this connection, a recurrencing principle is the colinearity between NRPS gene(s), enzymatic protein template and final peptide product. Applying the same rule(s) on the pyridinyl polythiazole class peptide antibiotic micrococcin P1 (Fig. 6), the following model for the putative domain organization of the micrococcin P1 synthetase complex can be proposed. The peptide antibiotic consists of 14 amino acids and thus, the NRPS template should be assembled of 14 individual modules. Modules 1 and 7 may

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**Fig. 5. Biochemical characterization of the A domain fragment discovered.** (A) The A domain was tested for recognition and activation of all proteinogenic amino acids, as well as all proposed constituting amino acids of micrococcin P1. The highest ATP-pyrophosphate exchange activity observed (\( l\)-Thr) was set to 100\%, and only data for the eight amino acids revealing the highest exchange rates are shown. (B) Michaelis–Menten plot for the activation of \( l\)-threonine. (C) Comparison of the proposed binding pocket constituents of the Mic A domain with a Ser and Thr nonribosomal codon, respectively [23].

**Fig. 6. Chemical structure of the pyridinyl polythiazole class peptide antibiotic micrococcin P1 and indication of the presumed monomeric precursor amino acids.** Locations for which a domain organization CAT/C would be predicted in the corresponding NRPS template are highlighted. Abbreviations are: Dbu, dehydrobutyrine; Dha, dehydroalanine; HPA, 2-hydroxypropylamine.
be involved in pyridine ring formation, which according to Bycroft and Gowland occurs by interaction of two dehydroalanine units [3]. Possibly, two A domains activating amino acids with uncharged polar side chains (Ser, Cys, or Thr) are to be expected at these positions, and dehydration of these precursors may yield the final dehydro residues. Alternatively, activation of nonproteinogenic amino acids is a common feature of NRPSs, and thus, even the direct activation and incorporation of two dehydroalanine units is conceivable. Additional catalytic domains (internally) or enzymes (externally) are probably required to catalyse the branching and heterocyclization of the peptide backbone, as well as the desamination of the N-terminal amino group of the starter amino acid to yield the final pyridine ring.

Six modules (positions 2, 5, 7, 9, 11 and 12) are necessary for the incorporation of the thiazole moieties. Consequently, Cys-specific A domains are required, along with T, Cy and Ox domains (putative domain organization: CyACysTOx), respectively. Modules 3, 4, 8, 13, and 14, in contrast, presumably activate and incorporate Thr residues into the nascent peptide product. While the Thr moieties at position 3 and 8 remain unmodified, they may only represent precursors for the formation of two dehydrobutyryne residues (Dbu; positions 4 and 13), as well as one hydroxypropylamine moiety (HPA; position 14). Module 6 is responsible for the activation and incorporation of Val.

The molecular biological and biochemical data presented in this study revealed that the 2.8-kb fragment identified encodes a NRPS with the proposed domain organization CyACysTOx. Taken into account the colinearity principle of NRPSs [20,22], the biosynthetic template of micrococcin P1 would feature only two locations, which are in agreement with this arrangement, namely module 3 and module 13 (Fig. 6).

**CONCLUSION**

More than a decade ago, Rieckmann pointed out that multidrug resistance of the malaria parasite *Plasmodium falciparum* is developing into a major problem [50]. Malaria already ranks among the top three global death-causing infectious diseases, making the search for alternative drugs a pressing demand. Recently, the macrocyclic antibiotics thiostrepton [51] and micrococcin [52] have been reported to inhibit *in vitro* growth of *P. falciparum*. The inhibitory potential of micrococcin proved to be about 100-fold higher than that of thiostrepton, and as good as that of the classical antimalarial drugs pyrimethamine [53] and mefloquine [54].

The discovery of the genetic locus in *S. equorum* WS2733 responsible for the nonribosomal assembly of micrococcin P1, as reported in this study, provides the prerequisite for the complete characterization of its entire biosynthetic gene cluster. This way, it will serve as a precedent for the whole family of pharmacologically important pyridyl polythiazole class peptide antibiotics. Furthermore, it provides the basis for the targeted engineering of even more potent micrococcin P1 derivatives. The knowledge can be exploited, i.e. to optimize production rate of the drug, and to manipulate its biosynthetic NRPS template by module fusion or substitution at the genetic level [55,56].

Finally, it should be also noted that *S. equorum* is traditionally used for food production [1] and therefore has been considered to be a ‘generally recognized as safe’ (GRAS) type of organism. The potential of peptide antibiotic micrococcin to develop into a potent inhibitor of malaria, however, may require reconsideration of this status for micrococcin-producing, foodborne bacteria.

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