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Identification and Characterization of the Lysobactin Biosynthetic Gene Cluster Reveals Mechanistic Insights into an Unusual Termination Module Architecture

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SUMMARY

Lysobactin (katanosin B) is a macrocyclic depsipeptide, displaying high antibacterial activity against human pathogens. In this work, we have identified and characterized the entire biosynthetic gene cluster responsible for lysobactin assembly. Sequential analysis of the *Lysobacter* sp. ATCC 53042 genome revealed the lysobactin gene cluster to encode two multimodular nonribosomal peptide synthetases. As the number of modules found within the synthetases LybA and LybB directly correlates with the primary sequence of lysobactin, a linear logic of lysobactin biosynthesis is proposed. Investigation of adenylation domain specificities in vitro confirmed the direct association between the synthetases and lysobactin biosynthesis. Furthermore, an unusual tandem thioesterase architecture of the LybB termination module was identified. Biochemical characterization of the individual thioesterases in vitro provides evidence that solely penultimate thioesterase domain mediates the cyclization and simultaneous release of lysobactin.

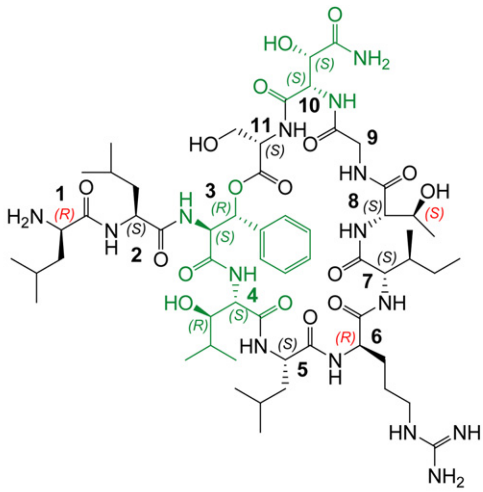
INTRODUCTION

Antibiotic resistance caused by misuse or overprescription of antibiotics has become a serious threat to public health. The rise of multiresistant pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) emphasizes the urgent requirement for the development of new antibiotics to ensure therapeutic efficiency against multiresistant pathogens in the future. Efforts to address the prevalence of pathogens include the discovery of new bactericidal compounds or the modification of known antibiotics by means of chemical, chemoenzymatic or mutasynthetic approaches (Debono et al., 1988; Mahlert et al., 2005; Nguyen et al., 2010; Weist et al., 2004).

One of the recently discovered potential antibiotics of the future is lysobactin, a depsipeptide produced by *Lysobacter sp.* ATCC 53042, which was first isolated at the Squibb Institute of Medical Research (Figure 1) (Bonner et al., 1988; O'Sullivan et al., 1988). Lysobactin displays a very strong activity against Gram-positive bacteria such as MRSA and VRE, with minimum inhibitory concentrations (MIC) of 0.39 and 0.78 μg/mL respectively, which are obviously lower (2- to 50-fold for different strains) than the MIC of vancomycin (Maki et al., 2001). In vitro studies showed that lysobactin inhibits the formation of lipid intermediates during bacterial cell wall biosynthesis, which is not inhibited by vancomycin. As this observation indicates that lysobactin differs in the mode of action from vancomycin it could potentially evade resistance mechanisms developed by vancomycin resistant strains (Maki et al., 2001). Lysobactin is therefore considered a high potential agent for the treatment of bacterial infections caused by resistant pathogens (Guzman-Martinez et al., 2007).

To obtain access to lysobactin, various synthetic pathways have been elaborated offering the opportunity to generate lysobactin derivatives with an altered or improved pharmaceutical spectrum (Campagne, 2007; Guzman-Martinez et al., 2007; von Nussbaum et al., 2007). However, the discovery of the lysobactin biosynthetic gene cluster could enable combinatorial biosynthesis of lysobactin analogs via the targeted reprogramming of genes encoding the enzymatic machinery involved in lysobactin assembly in vivo.

Lysobactin itself consists of 11 amino acids and features a 9-membered macrolactone ring with two *N*-terminal exocyclic residues (von Nussbaum et al., 2007). The peptide core is composed of a set of nonproteinogenic amino acids including two D-configured amino acids (D-Leu_1 and D-Arg_6), three β-hydroxylated amino acids (hyPhe_3, hyLeu_4 and hyAsn_{10}), and allo-Thr_8 (Figure 1). The macrolactone ring is constituted of an ester bond between the β-hydroxy group of hyPhe_3 and the carboxyl-group of Ser_{11}. As the macrocyclic structure and the nonproteinogenic residues are common features of nonribosomally assembled peptides (NRPs), it was suggested that lysobactin is synthesized by nonribosomal peptide synthetases (NRPSs). NRPSs are modularly-organized megaenzymes, which can be subdivided into individual modules with each module being responsible for the activation, covalent tethering and incorporation of a building block into the growing oligopeptide chain (Marahiel and Essen, 2009; Schwarzer et al., 2003; Walsh and Fischbach, 2010). These modules can be furthermore dissected into individual, catalytically-active domains. The adenylation (A) domains govern the recognition and activation of the cognate substrate as an aminoacyl-adenylate, which is subsequently transferred and covalently tethered onto the phosphopantetheinylated peptidyl-carrier-protein (PCP). Condensation of the immobilized substrates and peptidyl-intermediates is mediated by condensation (C) domains, leading to the translocalization of the elongated peptidyl chain toward the



D-Leu$_1$-Leu$_2$-hyPhe$_3$-hyLeu$_4$-Leu$_5$-D-Arg$_6$-Ile$_7$-aThr$_8$-Gly$_9$-hyAsn$_{10}$-Ser$_{11}$

Figure 1. The Chemical Structure and Primary Sequence of the Antibiotic Lysobactin

Lysobactin itself consists of 11 amino acids, featuring a macrolactone ring constituted of 9 amino acids and an ester bond between hyPhe$_3$ and Ser$_{11}$. Hydroxylated amino acids are accentuated in green, whereas inverted stereocenters are given in red. See also Figure S1.

C terminus of the synthetase. The release of the readily assembled enzyme-bound oligopeptide via hydrolysis or macrocyclization is generally governed by thioesterase (TE) domains, located C-terminally in the assembly line (Kopp and Marahiel, 2007a; Trauger et al., 2000).

In 1996, the involvement of a nonribosomal peptide synthetase in lysobactin biosynthesis was confirmed via hybridization of genomic libraries of *Lysobacter sp.* ATCC53042 with oligonucleotides derived from core-motifs of ACV synthetases and the gramicidin S synthetase (*Bacillus brevis*) (Bernhard et al., 1996). Marker-exchange mutagenesis, employing a 4.6-kbp NRPS-encoding DNA-fragment, gave rise to lysobactin nonproducing mutants, proving the identified DNA fragment to encode a region of the lysobactin synthetase. Bioinformatic analysis of the fragment revealed it to code for a truncated tetradomain NRPS with a C-A-PCP-C organization. Comparison of the active-site residues, determining the adenylation domain (A-domain) specificities with known A-domains, suggested this A-domain to activate asparagine. As an asparagine residue is found within lysobactin at position 10 it was assumed that the module identified was the penultimate module. Although lysobactin has first been described in 1996 (Bernhard et al., 1996), the entire sequence of the corresponding gene cluster remains elusive for more than a decade.

In this study, we report the identification and sequential analysis of the gene cluster responsible for lysobactin biosynthesis in *Lysobacter sp.* ATCC 53042. The synthetases LybA and LybB were confirmed to be involved in the assembly of the antibiotic via biochemical characterization of several adenylation domains in vitro. Annotation and bioinformatic analysis of the modular organization of LybB revealed the synthetase to harbor an unusual tandem TE architecture within the termination module. The individual TE1/TE2 domains were heterologously produced and biochemically characterized in vitro, using thioester-activated peptidyl substrates. It was shown that TE1 was exclusively responsible for the regio- and stereoselective macrocyclization of the undecapeptidyl substrate, whereas TE2 solely catalyzed hydrolysis of the peptidyl-thioester. In total, the results presented herein provide first information on the molecular architecture of the lysobactin synthetase and give insights into the catalytic function of a tandem thioesterase architecture. It also provides the opportunity for the generation of lysobactin analogs via combinatorial biosynthesis in vivo or via chemoenzymatic synthesis in vitro.

RESULTS AND DISCUSSION

Identification and Sequential Analysis of the Lysobactin Biosynthetic Gene Cluster

To identify the gene cluster responsible for lysobactin assembly, the genomic DNA of *Lysobacter sp.* ATCC 53042 was isolated and sequenced using pyrosequencing methods on a GS FLX instrument (see Supplemental Experimental Procedures available online). Lysobactin production by the strain employed was confirmed before sequencing via LC-MS analysis of culture extractions to ensure a functional biosynthetic assembly line. Lysobactin showed a retention time of t_R = 19.5 (m/z = 1276.8 $[M + H]^+$ observed, m/z = 1276.7 $[M + H]^+$ calculated) under the conditions applied (Figure S1). Genomic sequencing was carried out by GATC-biotech, providing 418 contigs ranging from 553 to 150,063 bp. One of the contigs was identified using the reported 4.6 kbp gene fragment (Bernhard et al., 1996). This contig, covering a region of 150,063 bp with an average GC-content of 70.7% was further analyzed using GeneMark2.4 annotation and has been submitted to GenBank (accession number JF412274) (Borodovsky et al., 2003). A detailed overview of the annotated genes, the deduced protein functions and the similarities to homologs is found in Table S1. The proposed *lyb* gene cluster encodes the two NRPSs LybA and LybB, three proteins conferring resistance to antibiotics (ORF78, ORF80, and ORF82) and one ABC-transporter permease/ATP-binding component (ORF79), putatively involved in the secretion of lysobactin (Figure 2).

The synthetase LybA displays 50% identity and 64% similarity (E-value = 0.0) to an NRPS from *Pseudomonas syringae pv. syringae* B728a, whereas LybB shows 39% identity and 59% similarity (E-value = 0.0) to a multimodular NRPS from *Herpetosiphon aurantiacus* ATCC 23779. Bioinformatic analysis of LybA and LybB, using the NRPS-PKS prediction software, revealed a multimodular organization (Figure 3) (Ansari et al., 2004). LybA comprises four modules with a total of 11 domains, whereas LybB harbors 7 modules and 24 domains. As the number of A-domains found within LybA and LybB directly correlates with the primary sequence of lysobactin, a linear logic of lysobactin assembly is suggested. The 4.6-kbp gene fragment was found to code for the tetradomain-region (C-A-PCP)$_{10}$-C$_{11}$, as assumed previously (Bernhard et al., 1996). Analysis of A-domain specificities was carried out with NRPSpredictor and compared the extracted active-site residues determining the A-domain specificities with known A-domains (Table S2) (Rausch et al., 2005; Stachelhaus et al., 1999) The predicted specificities of LybA and LybB nicely correlate with the primary

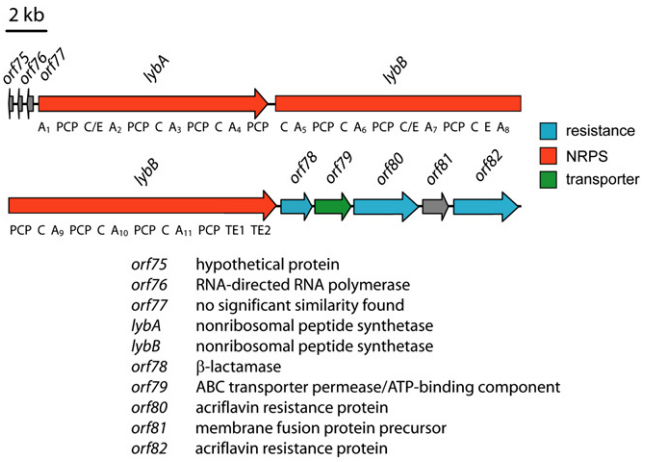


Figure 2. Schematic Overview of the Lysobactin Biosynthetic Gene Cluster and the Corresponding Upstream and Downstream Regions
Functions of the proteins encoded within this region are based on BLAST-analysis and are given in the figure. Apart from the core components for the nonribosomal assembly of lysobactin by the synthetases LybA and LybB, genes coding for transporters and resistance-conferring proteins are found. See also Table S1.

structure of lysobactin. In addition, the extracted active-site residues display a high degree of sequential identity to known active-site residue patterns with the weakest identity being 70% for LybB-A_6.

LybA A_3 is predicted to activate L-phenylalanine but shows only 80% identity of the residues determining the specificity compared to BacC A_2, suggesting the activation of a structurally analogous building block (Konz et al., 1997). This variation in the specificity conferring motif might indicate the direct incorporation of hyPhe located at position 3 in lysobactin instead of the activation of L-Phe followed by hydroxylation in *trans*. This model would require the hydroxylation of the free amino acid substrate, which has already been reported for C_β-hydroxylation of L-Arg during viomycin biosynthesis (Helmetag et al., 2009). In contrast, the extracted active-site residues of LybA A_4 are identical to those found in the L-Leu activating A-domain of SrfA-A-A_3, involved in surfactin biosynthesis (Cosmina et al., 1993). Furthermore, the specificity conferring code of LybB-A_{10} exactly resembles the code found in NosC-A_3, responsible for the recognition and activation of L-Asn. Based on these results it is suggested that LybA-A_4 and LybB-A_{10} activate the nonhydroxylated building blocks L-Leu and L-Asn, which are tethered on the adjacent PCPs. Subsequently, hydroxylation is carried out by in *trans*-operating hydroxylases, recognizing the PCP-bound substrates. This in *trans* tailoring mechanism has already been reported for the biosynthesis of kutznerides and involves nonheme Fe(II)/α-KG-dependent monooxygenase, mediating the hydroxylation of the immobilized substrates (Strieker et al., 2009). Alternatively, LybA A_4 and LybB A_{10} could activate the hydroxylated building blocks due to relaxed substrate specificities, as observed for the SalB A-domain during the biosynthesis of salinosporamide, which was exploited for the engineered biosynthesis of antiprotealide and salinosporamide derivatives (McGlinchey et al., 2008).

Examination of the synthetases revealed additional unusual features of the biosynthetic assembly line. Interestingly, modules 1 and 6, incorporating D-Leu_1 and D-Arg_6 are not equipped with an optional epimerization domain (E-domain), usually required for the stereoinversion of the α-stereocenter. Instead, the condensation domains (C-domain) located in the downstream neighboring modules (2 and 7) carry characteristic N-terminal sequences with the additional core motif HHI/LXXXXGD. This core motif has been found in dual C/E domains that catalyze the epimerization of the C_α-atom of the PCP-bound amino acid before condensation as observed during arthrofactin biosynthesis (Balibar et al., 2005). These dual C/E domains in module 2 of LybA and module 7 of LybB are therefore considered to be responsible for the C_α-epimerization of Leu_1 and Arg_6.

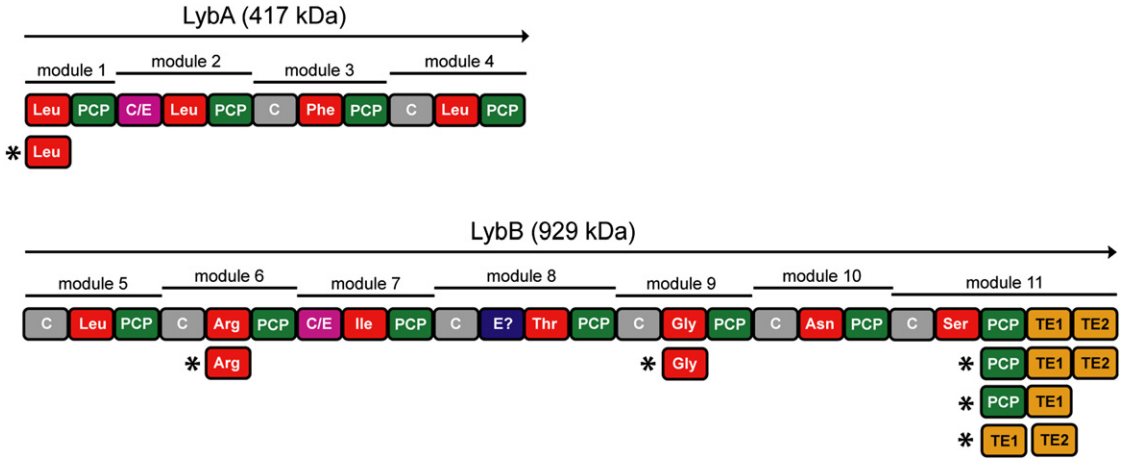


Figure 3. Modular Organization of the Lysobactin Synthetases LybA and LybB
LybA consists of 4 modules and 11 domains, respectively. LybB is constituted of 7 modules and 24 individual domains. As the number of adenylation domains found within the synthetases directly correlates with the primary sequence of lysobactin, its assembly follows a linear NRPS logic. The termination module contains two distinct thioesterase domains, responsible for release of the PCP-bound intermediate via macrolactonization. Domains that were subjected to biochemical characterization in vitro are marked with an asterisk. See also Figure S2 and Table S2.

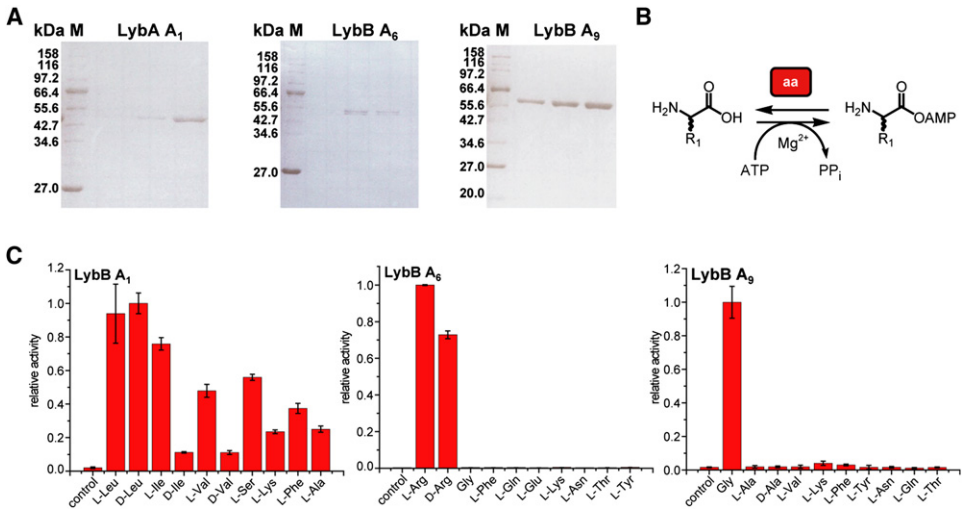


Figure 4. Analysis of Recombinant Adenylation Domains

(A) SDS-PAGE analysis of purified recombinant adenylation domains LybA A_1 (56.9 kDa), LybB A_6 (60.3 kDa), and LybB A_9 (58.2 kDa). Protein marker (M) was broad range protein marker P7702 (NEB).

(B) The reaction catalyzed by NRPS A-domains in vitro. The radioactive ATP/PP_i-exchange assay is based on the reversibility of aminoacyl-adenylate formation.

(C) Relative activities of the investigated A-domains toward various amino acid substrates as observed during pyrophosphate exchange assays.

Module 8, which incorporates allo-threonine into the final product, contains an additional domain inserted between the C_8- and the A_8-domain (Figure 3). This domain was classified as a C-domain by NRPS-PKS and the NRPS prediction blast server (Ansari et al., 2004; Challis et al., 2000). Analysis of the A-domain specificity in vitro demonstrated A_8 to display a higher affinity for L-Thr over allo-Thr (5-fold, data not shown). Based on these results it is proposed that the inserted domain acts as a side-chain epimerization domain, which inverts the stereochemistry of the C_β-atom of PCP-bound L-Thr. Further studies will be conducted to prove that LybB is an exclusive example employing an in *cis* epimerization mechanism to supply an allo-amino acid. In contrast, investigation of the biosynthetic pathway for the antimetabolite γ,γ-dichloroaminobutyrate revealed an alternative mechanism of allo-amino acid incorporation. In vitro analysis of the lone-standing A-domain CytC1 confirmed the enzyme to preferentially activate L-allo-Ile, which is directly incorporated into the natural product (Ueki et al., 2006).

The termination module of LybB harbors an unusual tandem thioesterase domain architecture similar to that of the arthrofactin synthetase ArfC (Roongsawang et al., 2003). Both TE domains, LybB TE1 and LybB TE2, share sequence homology to α/β hydrolase fold proteins and contain a catalytic triad consisting of Ser-His-Asp each, suggesting both TEs to be functional. In contrast to ArfC TE2, LybB TE2 does not house an additional conserved GXSXG sequence motif (Roongsawang et al., 2007).

The upstream flanking region of the *lyb* gene cluster is defined by *orf*s75–77 that encode hypothetical proteins and a RNA-directed RNA polymerase in the reverse direction. The downstream region of the *lyb* gene cluster comprises the β-lactamase-type protein ORF78, the RND-family acriflavin resistance proteins ORF80 and ORF82 and the ABC transporter permease/ATP-binding component ORF79. Acriflavin resistance proteins represent bacterial drug efflux proteins with a broad substrate specificity(Tseng et al., 1999). Although the participation of proteins encoded upstream and downstream of *lybA* and *lybB* remains to be confirmed we proposed the biosynthetic gene cluster for lysobactin assembly to be composed of *lybA*, *lybB*, and *orf*s78-82. The direction of transcription for *lybA*, *lybB*, and *orf*s78–82 is identical (Figure 2), suggesting a coordinated expression of the entire biosynthetic assembly line and an ATP-dependent efflux machinery, conferring either resistance to lysobactin or being involved in product secretion (Quigley et al., 1993).

Lysobactin contains three β-hydroxylated residues, namely $hyPhe_3$, $hyLeu_4$, and $hyAsn_{10}$. Generally, hydroxylations are mediated by three enzyme classes: FAD-dependent monooxygenases, nonheme Fe(II)-oxygenases, and heme Fe(II)-oxygenases. Especially nonheme Fe(II)/α-KG-dependent monooxygenases have been shown to be involved in the biosynthesis of nonribosomal peptides. It was demonstrated that either free amino acids or PCP-tethered substrates were subjected to hydroxylation (Strieker et al., 2007, 2009). In the case of the *lyb* gene cluster, no putative hydroxylases could be identified in the genetic region surrounding both synthetases. As the genes required for the biosynthesis of a natural product are usually clustered, it can only be speculated that monooxygenases encoded in the *Lysobacter* genome are responsible for the generation of $hyPhe_3$, $hyLeu_4$, and $hyAsn_{10}$, but gene cluster cross-talk has been described for natural product biosynthesis (Lazos et al., 2010).

Adenylation Domain Specificities Confirm LybA and LybB as Lysobactin Synthetases

To verify that LybA and LybB are involved in the biosynthesis of lysobactin, the A-domains from module 1 of LybA (A_1) and modules 6 and 9 of LybB (A_6 and A_9) were cloned into the expression vector pET28a. Heterologous production of the proteins in *Escherichia coli* BL21 and Rosetta(DE3) cells, followed by purification using Ni-NTA agarose affinity chromatography (Figure 4A) afforded 1.44 (A_1), 0.85 (A_6), and 2.09 (A_9) mg/L media of

recombinant protein. A-domain specificities were studied via ATP/PPi-exchange assays. Analysis of the assays employing LybA A_1, which is predicted to activate L-Leu in vivo, showed L-leucine and D-leucine to represent the preferred substrates. Structurally related amino acids, such as isoleucine, valine, and alanine, showed reduced activities relative to D-Leu (ranging from 0.76 to 0.25; Figure 4C). Interestingly, various other amino acids (serine, phenylalanine, and lysine) also showed reduced activities ranging from 0.56 to 0.24 relative to D-Leu. A-domain selectivity is considered to be the gate-keeper ensuring the correct incorporation of building blocks into the final product. Nevertheless, in vitro analysis of TycA A_1, involved in the biosynthesis of tyrocidine, showed the recombinant protein to display an unexpected flexibility in substrate tolerance (Schaffer and Otten, 2009). In contrast, LybB A_6 exhibited a high degree of specificity toward L-arginine and D-arginine (Figure 4C), which is consistent with the prediction of Arg to be the cognate substrate for LybB A_6. Activities of all other tested substrates did not exceed 0.006, relative to L-Arg. The results obtained for LybB A_9 clearly show that solely Gly is recognized by the A-domain in vitro, which is also in full agreement with the biosynthetic model. Analysis of adenylation domain activities corroborates the predicted specificities and confirms the synthetases LybA and LybB to mediate lysobactin assembly following a linear logic. Interestingly, both LybA A_1 and LybB A_6 display an almost equal affinity for the L- and the D-configured substrate in vitro. The correct incorporation of the L-configured moiety is suggested to be the result of the low cytoplasmic abundance of the corresponding D-isomer or to be restricted by the specificity of downstream C-domains at their respective donor sites (Linne and Marahiel, 2000).

Characterization of LybB Thioesterase Activities

Product release in NRPS- or PKS-systems is generally mediated by C-terminally located thioesterase domains, responsible for cyclization of the intermediate or hydrolytic release of the linear carboxylate. These internal TE domains contain a characteristic GXSXG-motif, constituting a catalytic triad with highly conserved Asp and His residues. The formation of this catalytic triad consisting of Ser-His-Asp leads to the deprotonation of the active-site serine and generates a highly reactive oxyanion species. TE-mediated cyclization is initiated by the nucleophilic attack of the serine oxyanion onto the adjacent PCP-bound peptidyl thioester leading to the conversion of the peptidyl thioester to an acyl-O-TE oxoester intermediate (Sieber and Marahiel, 2005). The attack of either an internal nucleophile or of an external water molecule onto the oxoester results in the formation of a macrocyclic species or gives rise to the linear carboxylate. In addition to internal thioesterases, which are incorporated in the assembly line, external stand-alone thioesterases, also referred to as TEIIs, are associated with the biosynthesis of polyketides or nonribosomal peptides. Type II thioesterases are responsible for the regeneration of misprimed PCP-domains via hydrolytic cleavage of noncognate intermediates from the 4′-ppant cofactor (Linne et al., 2004; Schwarzer et al., 2002; Yeh et al., 2004). contrast to the well studied single TE domain modular systems, LybB ends with an unusual tandem thioesterase architecture, which has only been reported for few NRPS systems, namely arthrofactin, massetolide and syringopeptin (de Bruijn et al., 2008; Roongsawang et al., 2003; Scholz-Schroeder et al., 2003). In vivo characterization of arthrofactin tandem TEs via site-directed mutagenesis and the generation of deletion mutants gave first insights into the functionality of both domains (Roongsawang et al., 2007). It was shown that the Ser/Ala mutant of ArfC TE1 gave rise to arthrofactin nonproducing mutants. Deletion of ArfC TE2 or inactivation of the domain led to a drastically decreased arthrofactin production (5%), confirming ArfC TE2 to be directly involved in the efficient production of the natural product.

Phylogenetic analysis of LybB TE1 and LybB TE2, compared to thioesterases from NRPS- or PKS-systems, revealed the TEs to cluster with several other tandem TEs (Figure S2). LybB TE1 clusters with the TE1 domains found in the arthrofactin (ArfC TE1), massetolide (MassC TE1), and syringopeptin (SypC TE1) biosynthetic systems, which is also in full agreement with the phylogenetic analyses conducted earlier (Roongsawang et al., 2007). It was speculated that this subclass of TEs represents a novel cyclase family as all products of this group contain a macrolactone moiety. LybB TE2, MassC TE2, ArfC TE2, and SypC TE2 also constitute a discrete subclass of thioesterases. In earlier studies, it was suggested that these TE2-domains do not resemble variants of external TEIIs as they form a separate branch within the phylogenetic tree.

Taken together, the results derived from sequential analysis suggest both TEs to exhibit either cyclase or hydrolase activity. To investigate the role of both thioesterase domains in the process of lysobactin cyclization and product release, LybB PCP-TE1-TE2, LybB PCP-TE1, LybB TE1, and LybB TE2 were subcloned into the expression vector pET28a and heterologously produced in *E. coli* BL21 (DE3). Purification via Ni-NTA agarose affinity chromatography (Figure S3) afforded 7.4 (PCP-TE1-TE2/PCP-TE1), 3.0 (PCP-TE1), 1.6 (TE1), and 6.4 (TE2) mg/L media recombinant protein. Interestingly, heterologous production of LybB PCP-TE1-TE2 gave rise to proteolytic degradation of the full length peptide, resulting in the simultaneous purification of LybB PCP-TE1-TE2 and LybB PCP-TE1 (Figure S3D). The identity of the individual proteins was confirmed via peptide mass fingerprinting. Peptide fragments obtained from tryptic digestion were identified via HRMS-analysis and are shown in Figure S3D and demonstrate the release of the entire TE2 domain. To investigate if the cleavage of the full length peptide is an autocatalytic event, the mixture of PCP-TE1-TE2 and PCP-TE1 was incubated for 2 days at various pH-values (see Supplemental Experimental Procedures). SDS-PAGE analysis of the reactions did not reveal an increased formation of PCP-TE1. LybB PCP-TE1-TE2 was also purified in the presence of protease inhibitors to investigate the influence of protease inhibition on protein cleavage. Addition of inhibitors (EDTA, AEBSF, leupeptin, bestatin, aprotinin, and E-64 for the inhibition of serine proteases, cysteine proteases, and metalloproteases) did not reduce the cleavage of LybB PCP-TE1-TE2 (Figure S3E and Supplemental Experimental Procedures). These results suggest an intracellular *E. coli* protease to be involved in the posttranslational cleavage of the enzyme during the heterologous production of the protein in vivo. It can be suggested that LybB PCP-TE1-TE2 is also cleaved in the native strain *Lysobacter sp*. ATCC 53042 before lysobactin synthesis although it can only be speculated which specific protease is

involved in the cleavage process. The resulting lone-standing TE domain could then function as an external type II TE to regenerate misprimed PCP-domains via hydrolytic cleavage of the PCP-bound noncognate substrates. Sequential analysis of LybB TE1 and TE2 based on TE-core motifs, secondary structure elements and multiple sequence alignments with tandem TE-domains revealed a region between α6 (TE1) and β1 (TE2) lacking secondary structure motifs (Supplemental Experimental Procedures). The 13 residues located in this proline-rich region are considered to constitute the linker between LybB TE1 and TE2 and could contain a putative protease cleavage site, although it can only be speculated if a specific protease is involved in the cleavage process (Figure S4). The conserved proline residue that was considered to be the N terminus of ArfC TE2 is also located within this region (Roongsawang et al., 2007). To investigate the function of the individual LybB thioesterases, a biochemical characterization was carried out in vitro. This biochemical characterization of the thioesterases relied on the potential of dissected TE domains to mediate either hydrolysis or macrocyclization of artificial peptidyl-substrates (Sieber et al., 2004). TE-domains remain catalytically active units when separated from the enzymatic template and can be incubated with synthetic substrates for the evaluation of their inherent biocombinatorial potential (Kopp and Marahiel, 2007b). These substrates are synthesized via solid-phase peptide synthesis (SPPS), substituting the entire enzymatic machinery (Kopp and Marahiel, 2007b; Trauger et al., 2000). Recognition and cyclization of the synthetic substrate requires the *C*-terminal activation of the oligopeptide as a thioester, imitating the physiological situation of the naturally occurring PCP-ppant-bound substrate (Kohli et al., 2002). Investigation of the individual LybB TE domains required the synthesis of a cognate linear lysobactin undecapaptide with minor modifications of the peptidic backbone. The synthetically demanding amino acids $hyLeu_4$ and $hyAsn_{10}$ were substituted with nonhydroxylated L-Leu and L-Asn, but stereochemistry was conserved throughout the oligopeptide. C-terminal activation of the peptide was accomplished via thioesterification with thiophenol (Figure S5).

In vitro analysis of the TE domain activities was carried out by incubating the recombinant enzyme with the artificial substrate. All reactions were monitored via liquid chromatography-mass spectrometry (LC-MS). In the control reaction, lacking the enzymes, slight hydrolysis (Figure 5, Hy, t_R = 4.2 min) and nonenzymatic cyclization (Cy, Cy′, and Cy″, t_R = 7.5, 9.4, and 9.9 min) were observed (Figure S6). These macrocyclic products are suggested be derived from the intramolecular attack of three different nucleophiles (the primary amino group of $D\text{-}Leu_1$ and the secondary hydroxyl groups of $hyPhe_3$ and $aThr_8$) onto the activated C terminus of the peptide.

Incubation of the substrate (Su, t_R = 10.2 min) with recombinant LybB PCP-TE1-TE2/PCP-TE1 resulted in the complete conversion of the undecapeptidyl-thioester to the macrocyclic product (Cy, t_R = 8.28 min) and the hydrolyzed linear peptide (Hy, t_R = 5.27 min). Cy′ and Cy″, as observed in the control reaction were not detected (Figure 5). This suggests that Cy is the expected enzymatically generated lysobactin derivative resulting from ester bond formation between $hyPhe_3$ and Ser_{11}. In addition, MS^2-analysis of the macrocyclic product Cy revealed the formation of b-series fragment ions carrying a dehydro-

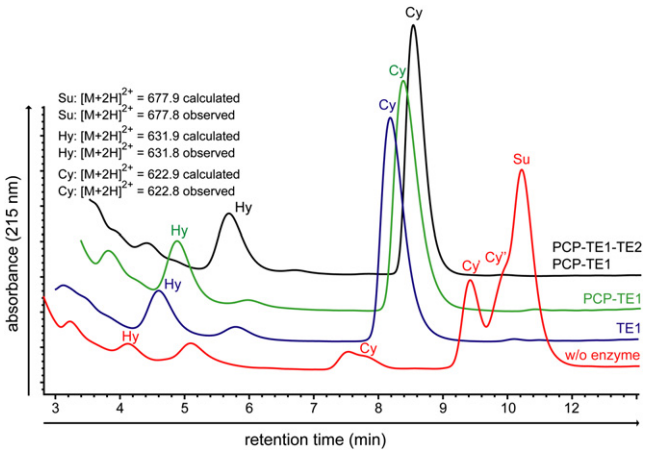


Figure 5. Cyclization of the Thiophenol-Activated Substrate Mediated by LybB PCP-TE1-TE2, LybB PCP-TE1, and LybB TE1

The HPLC traces correspond to the incubation of the substrate (Su, 100 μM) with the recombinant enzymes for 2 hr at 25°C. The red HPLC trace corresponds to the control lacking the enzyme. The formation of nonenzymatically cyclized peptides Cy′ and Cy″ is observed in the absence of the thioesterase. Enzymatic conversion of the substrate (Su, t_R = 10.2 min) leads to the hydrolytically cleaved peptide Hy at t_R = 5.2 min and the macrolactone Cy at t_R = 7.5 min. See also Figures S3–S7.

phenylalanine species. This formation of dehydro-phenylalanine species resulting from a loss of one molecule of water is characteristic for the fragmentation of lactones(Crotti et al., 2004). Additional fragment ions of the y- and the b-series strongly support the macrocycle to be the expected lysobactin derivative (Figure S7). The determination of kinetic parameters for LybB PCP-TE1-TE2-mediated macrocyclication revealed the enzyme to follow Michaelis-Menten-kinetics with K_M = 1.03 mM and k_{cat} = 11.1 s^{-1}, leading to a catalytic efficiency of k_{cat}/K_M = 10.8 $s^{-1} \times mM^{-1}$.

In addition, LybB PCP-TE1 was employed to investigate the potential influence of the C-terminal TE2 domain on the macrocyclization reaction. Analysis of the assay revealed the additional domain to have no influence on product formation or hydrolysis (Figure 5). The kinetic parameters were also determined for this reaction and are the following: K_M = 0.86 mM and k_{cat} = 15.8 s^{-1}, leading to a catalytic efficiency of k_{cat}/K_M = 18.4 $s^{-1} \times mM^{-1}$. Comparison of the kinetic profiles of LybB PCP-TE1-TE2 and LybB PCP-TE1 reveals only minimal differences in the affinity for the substrate or substrate turnover. In comparison with recombinant thioesterases derived from NRPS pathways, LybB PCP-TE1-TE2 and LybB PCP-TE1 display lower affinities toward the substrate and exhibit higher turnover rates. DptD PCP-TE, involved in daptomycin biosynthesis, displays a K_M = 50.1 μM and a k_{cat} = 0.003 s^{-1}, whereas A54145-PCP-TE, mediating macrolactonization of A54145-type acidic lipopeptides, shows a K_M = 80.2 μM and a k_{cat} = 0.0036 s^{-1} (Grunewald et al., 2004; Kopp et al., 2006). LybB TE1 was investigated for its macrocyclization potential and it was shown that the enzyme also catalyzes the predominant formation of the macrolactone (Figure 5). The reactions catalyzed by LybB PCP-TE1-TE2, LybB PCP-TE1, and LybB TE1 exhibited comparable cyclization/hydrolysis ratios of 5.7, 4.6,

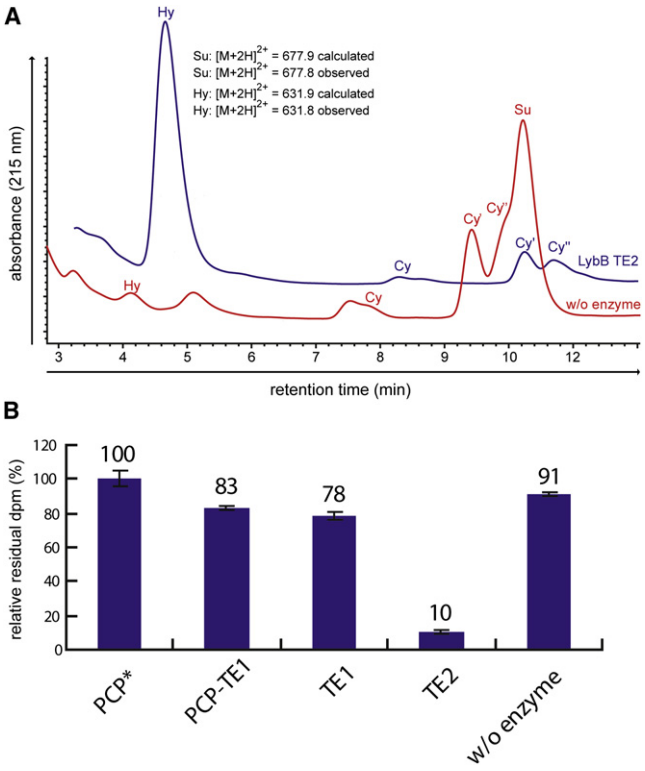


Figure 6. Hydrolysis of the Thiophenol-Activated Substrate Mediated by LybB TE2

(A) The HPLC traces correspond to the incubation of the substrate (100 μM) with the recombinant enzymes for 2 hr at 25°C. The red HPLC trace corresponds to the control lacking the enzyme. The formation of nonenzymatically cyclized peptides Cy′ and Cy″ is observed in the absence and presence of the thioesterase. Enzymatic conversion of the substrate (Su) leads to the predominant formation of the hydrolytically cleaved peptide Hy at t_R = 4.2 min. (B) Results of the deacylation studies for LybB TEs and the stand-alone *holo*-PCP SrfA-A PCP_1. Analysis of residual radioactivity confirms LybB TE2 to efficiently deacylate ^{14}C-acetyl labeled PCP with 10% relative radioactivity remaining. In contrast, LybB PCP-TE1 and LybB TE1 gave rise to 83% and 78% remaining radiolabel. Nonenzymatic hydrolysis afforded 91% residual radioactivity. The quantification of radioactivity was based on the immediate measurement of ^{14}C-acetyl *holo*-PCP after loading in vitro (PCP*). One-hundred percent (100%) represent 100,000 dpm.

See also Figures S3–S7.

and 4.9 after 2 hr at 25°C. Therefore the presence of TE2 in LybB PCP-TE1-TE2 does not lead to an increase in hydrolysis or influence the yields of the macrocyclization reaction. In all cases, the formation of the nonenzymatically cyclized peptides Cy′ and Cy″ was completely suppressed. Based on these results, it is evident that the first thioesterase of LybB is mediating the stereospecific macrolactonization of the linear lysobactin precursor.

As the in vivo characterization of tandem TEs in the arthrofactin synthetase ArfC confirmed both TE domains to be functional and involved in arthrofactin biosynthesis (Roongsawang et al., 2007), the role of the second thioesterase LybB TE2 was also investigated in vitro. Incubation of the enzyme with the substrate gave rise to substantial conversion of the thioester to the corresponding hydrolysis product (Hy, t_R = 4.2 min) (Figure 6). In addition, the three cyclization products (Cy, Cy′ and Cy″) were detected with comparable ratios. LybB TE2 displayed a cyclization/hydrolysis ratio of 0.04 and is therefore considered to solely mediate hydrolytic cleavage of thioester-activated peptidyl-substrates similar to external type II thioesterases. To further corroborate the function of LybB TE2 as a TEII-like protein, deacylation studies, employing LybB TEs and the stand-alone SrfA-A *holo*-PCP_1, were carried out (Kraas et al., 2010). SrfA-A PCP_1 was artificially misprimed in vitro utilizing Sfp and ^{14}C-acetyl-CoA. Hydrolytic cleavage of the acetyl-group was achieved by incubation of *holo*-PCP_1 with recombinant LybB TEs and residual radioactivity was quantified after precipitation of the enzymes. Analysis of the deacylation reactions demonstrated LybB TE2 to efficiently hydrolyze the PCP-bound acetyl group, thereby confirming the assigned catalytic function of LybB TE2 as a TEII-like enzyme (Figure 6B). In contrast, incubation of ^{14}C-acetyl labeled PCP with LybB PCP-TE1 or LybB TE1 did not result in substantial formation of deacylated PCP, excluding TE1 to be involved in the regeneration of misprimed PCPs.

The results of this study contradict the mechanism that was postulated based on the results of the in vivo characterization of ArfC TEs. Roongsawang et al. proposed the second thioesterase domain of ArfC to mediate the macrocyclization of the linear peptide, as the deletion and inactivation of this domain drastically reduced arthrofactin production by 95% (Roongsawang et al., 2007). It was postulated that, in a first step, the peptidyl-substrate is transferred from the adjacent PCP-domain onto the catalytically active serine residue of ArfC TE1. Subsequently, the lipoundecapeptidyl chain is transferred onto the active-site serine of ArfC TE2. Ultimately, the intramolecular attack of side-chain hydroxyl group of Thr leads to the formation and release of the macrolactone.

The results obtained in this study assign different functions to the individual TE domains. The in vitro characterization of TE1 clearly confirms this domain to be solely responsible for the formation of the macrolactone and the subsequent release of the product. Macrocyclization yields were not influenced by the presence of the additional *C*-terminal TE2 domain, confirming the formation of the macrolactone to be the preferred reaction. In contrast, LybB TE2 exclusively mediated the hydrolysis of the thioester-activated substrate and the misprimed PCP. It is suggested that LybB TE2 acts as a TEII-like thioesterase that could deacylate misprimed PCPs. This efficient deacylation of misprimed PCPs and ACPs by TEIIs has been reported for the bacitracin TEII, the surfactin TEII or the ScoT TEII in vitro (Kotowska et al., 2009; Schwarzer et al., 2002; Yeh et al., 2004). In addition, the proteolytic cleavage of the C-terminal TE2 domain has been observed and it cannot be ruled out that this posttranslational cleavage generates a lone-standing TEII-like protein in vivo. The reduced production of arthrofactin, observed during the in vivo characterization of ArfC TEs, could result from a decreased assembly line functionality due to misacylated PCPs, which cannot be regenerated by the TEII-like ArfC TE2 (Roongsawang et al., 2007). The disruption of TEII-encoding genes in NRPS- or PKS-gene clusters is commonly leading to a drastically decreased formation of the corresponding natural product, which can be complemented by a heterologous TEII (Kotowska et al., 2002; Schneider and Marahiel, 1998).

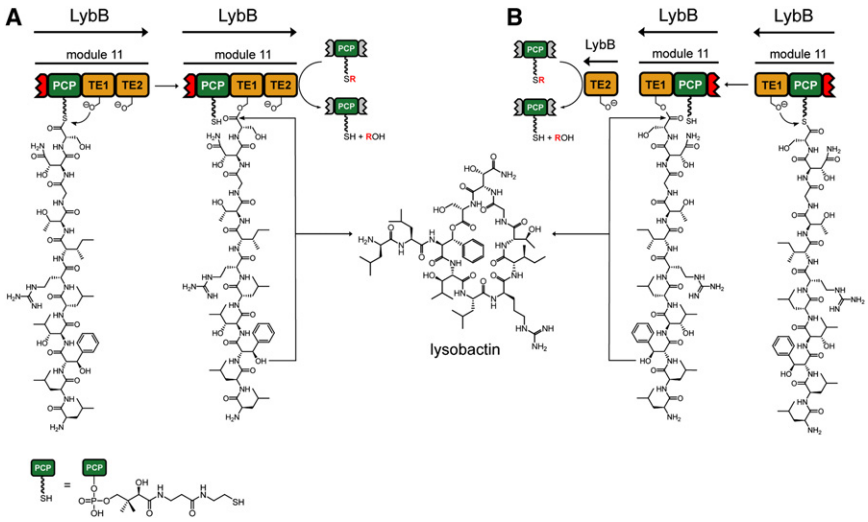


Figure 7. Proposed Biosynthetic Model for the Macrocyclization Reaction during Lysobactin Assembly

Before LybB TE1 catalyzed formation of the macrolactone, the undecapeptidyl-substrate is transferred from the adjacent PCP-domain onto the catalytically active serine residue. A subsequent intramolecular nucleophilic attack of the $hyPhe_3$ β-hydroxyl group onto the acyl-O-TE1 oxoester intermediate affords lysobactin. According to this model LybB TE2 acts as a TEII-like protein in *cis* (A) or as a lone-standing thioesterase in *trans* (B) to deacylate misprimed PCPs.

In summary it is concluded that LybB TE1 is catalyzing the macrocyclization reaction, whereas LybB TE2 deacylates misprimed PCPs and ensures a continuous production of lysobactin in *cis* (Figure 7A) or as a lone-standing TEII in *trans* (Figure 7B).

SIGNIFICANCE

Lysobactin represents a promising new agent for the treatment of infections caused by Gram-positive pathogens including MRSA and VRE, as its MOA differs from that of the last-line antibiotic vancomycin. In the study presented herein the gene cluster responsible for lysobactin assembly was identified via sequencing and annotation of the *Lysobacter sp.* ATCC 53042 genome. The *lyb* gene cluster encodes two multimodular NRPS and several proteins putatively involved in resistance and secretion of the natural product. The enzymatic assembly line consists of the two synthetases LybA and LybB, containing four and seven modules, respectively. Sequential analysis of the multimodular organization and the predicted adenylation-domain specificities suggested lysobactin assembly to follow a conventional linear logic. Biochemical analysis of A-domain specificities in vitro confirmed the predicted substrate preferences and proved the correlation between the primary sequence of lysobactin and the linear organization of the synthetases LybA and LybB. The termination module of LybB featured an unusual tandem TE architecture, observed in only few NRPS biosynthetic pathways. To determine the role of the individual thioesterase domains, both were characterized in vitro using a thiophenol-activated linear lysobactin analog. It was shown that LybB TE1 exclusively catalyzed the formation of a macrocyclic structure, whereas LybB TE2 solely mediated hydrolytic cleavage of the synthetic substrate. This work represents the first example of the biochemical characterization of a tandem TE termination module and enables the assignment of discrete catalytic activities for the individual TE-domains. Based on these results a chemoenzymatic approach utilizing LybB TE1 will enable a rapid access to lysobactin derivatives. In addition, the identification of the lysobactin assembly line offers the opportunity for the combinatorial biosynthesis of lysobactin analogs with improved pharmacological properties.

EXPERIMENTAL PROCEDURES

Cultivation of *Lysobacter sp.*, Fermentation, and Isolation of Lysobactin

Lysobacter sp. ATCC 53042 was purchased from LGC Standards GmbH and initially plated on soy agar slants (BBL Trypticase soy broth BD211768 15 g, Agar Roth 2266.2 7.5 g in 500 ml water). Fermentations were carried out as described earlier (Bonner et al., 1988; O'Sullivan et al., 1988). After centrifugation of the culture at 7000 rpm for 30 min, the supernatant was separated (pH-value was adjusted to 7.0) and extracted with butanol. The solvent was removed after combination of the extracts using rotary evaporator to yield a yellow solid. The residue was dissolved in methanol and the soluble portion was analyzed via LC-MS (Agilent/HP 1100 series, column: Macherey-Nagel cc 125/2 Nucleodur 100-3 c18 ec) with the following gradient: solvent A: water (0.1% trifluoroacetic acid), solvent B: acetonitrile (0.1% trifluoroacetic acid), flow rate: 0.3 ml/min, gradient: 0 min, 10% B, 30 min, 60% B, 33 min, 95% B, 35 min 95% B, 38 min, 10% B, 45 min, 10% B). Lysobactin showed a retention time t_R of 19.5 min under the conditions applied (m/z = 1276.8 $[M + H]^+$ observed, m/z = 1276.7 $[M + H]^+$ calculated; Figure S1). HRMS-analysis of lysobactin was performed via high resolution mass spectrometry on an LTQ-FT instrument (Thermo Fisher Scientific) connected to a microbore Agilent 1100 HPLC system.

Synthesis of the Thioester-Activated Peptidyl Substrate

L-threo-3-phenylserine hydrate (HyPhe) was purchased from Bachem. The primary amino group was protected with 9-fluorenylmethyloxycarbonyl (Fmoc) following a standard protocol (Watts et al., 2004). The undecapeptide $D\text{-}Leu_1\text{-}Leu_2\text{-}hyPhe_3\text{-}Leu_4\text{-}Leu_5\text{-}D\text{-}Arg_6\text{-}Ile_7\text{-}aThr_8\text{-}Gly_9\text{-}Asn_{10}\text{-}Ser_{11}$ was synthesized (Aapptec, Apex 396 peptide synthesizer) following the standard Fmoc solid phase peptide synthesis protocol. Thioesterification with thiophenol and the subsequent cleavage of side chain protecting groups were carried out following a protocol described earlier (Sieber et al., 2004). Purification of the thiophenol-activated peptide was carried out via preparative HPLC (Agilent/HP 1100 series, column: Macherey-Nagel VP 250/21 Nucleodur 100-5 c18 ec) with the following gradient: solvent A: water (0.1% trifluoroacetic acid), solvent B: acetonitrile (0.1% trifluoroacetic acid), flow rate: 10 ml/min, gradient: 0 min, 30% B, 30 min, 70% B, 32 min, 95% B, 35 min 95% B, 37 min, 30% B, 45 min, 30% B. After removal of the solvent via lyophilization, the peptidyl-SPh substrate was dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 100 mM.

In Vitro Characterization of Recombinant Thioesterases

The reaction mixture (final volume 50 μl) containing 25 mM HEPES, 50 mM NaCl, 10-400 μM peptidyl-SPh, 2 μl DMSO, and 1 μM enzyme was incubated at 25°C for a certain period of time (2 hr for activity analysis, 30 s for determination of kinetic parameters). The reaction was quenched by adding 50 μl MeOH to precipitate the enzyme. After centrifugation at 13,000 rpm for 10 min, the resulting supernatant was analyzed via LC-MS (Agilent/HP 1100 series, column: Macherey-Nagel cc125/2 Nucleodur 100-3 c18 ec, column temperature: 45°C) with the following gradient: solvent A: water (0.1% trifluoroacetic acid), solvent B: acetonitrile (0.1% trifluoroacetic acid), flow rate: 0.3 ml/min, gradient: 0 min, 30% B, 20 min, 50% B, 23 min, 95% B, 30 min 95% B, 33 min, 30% B, 40 min, 30% B. Experiments were performed in triplicate for the determination of kinetic parameters.

ACCESSION NUMBERS

The sequence of the lysobactin contig has been deposited in GenBank under accession number JF412274.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.chembiol.2011.02.012.

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