Identification of the nonribosomal peptide synthetase gene responsible for bassianolide synthesis in wood-decaying fungus *Xylaria* sp. BCC1067

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Intensive study of gene diversity of bioactive compounds in a wood-rot fungus, *Xylaria* sp. BCC1067, has made it possible to identify polyketides and nonribosomal peptides (NRPs) unaccounted for by conventional chemical screening methods. Here we report the complete nonribosomal peptide synthetase (NRPS) gene responsible for the biosynthesis of an NRP, bassianolide, using a genetic approach. Isolation of the bassianolide biosynthetic gene, *nrpsxy*, was achieved using degenerate primers specific to the adenylation domain of NRPS. The complete ORF of *nrpsxy* is 10.6 kb in length. Based on comparisons with other known NRPSs, the domain arrangement of NRPSXY is most likely to be C-A-T-C-A-M-T-T-C-R. The other ORF found upstream of *nrpsxy*, designated *efxy*, is 1.8 kb in length and shows high similarity to members of the major facilitator superfamily of transporters. Functional analysis of the *nrpsxy* gene was conducted by gene disruption, and the missing metabolite in the mutant was identified. Chemical analysis revealed the structure of the metabolite to be a cyclooctadepsipeptide, bassianolide, which has been found in other fungi. A bioassay of bassianolide revealed a wide range of biological activities other than insecticidal uses, which have been previously reported, thus making bassianolide an interesting candidate for future structural modification. This study is the first evidence for a gene involved in the biosynthesis of bassianolide.

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Abbreviations: ESITOF, electrospray ionization–time of flight; ESYN, enniatin synthetase; HTS, HC-toxin synthetase; KPR, ketopantoate reductase; MFS, major facilitator superfamily; NRP, nonribosomal peptide; NRPS, nonribosomal peptide synthetase.

The GenBank/EMBL/DDBJ accession numbers for the *nrpsxy* and *efxy* sequences of *Xylaria* sp. BCC1067 are EF456733 and EF456734, respectively.

A supplementary table showing ¹³C NMR data for substance A in CDCl₃, and three supplementary figures showing ¹H NMR and ¹³C NMR spectra of the substance A molecule purified from *Xylaria* sp. BCC1067 in CDCl₃ solution, ESITOF MS data for substance A, and an HPLC chromatogram of the acid hydrolysate of substance A, are available with the online version of this paper.

INTRODUCTION

Bassianolide is an octacyclodepsipeptide consisting of four molecules each of D-hydroxyisovaleric acid and L-N-methylleucine, and has been reported to be an insecticide obtained from *Beauveria bassiana* and *Verticillium lecanii* (Suzuki *et al.*, 1977). The structure of bassianolide is shown in Fig. 1. Bassianolide belongs to a family of structurally related cyclodepsipeptide compounds, which have emerged as a broad family of compounds characterized by at least one ester linkage. Great interest in this class of natural products has stemmed from their diverse range of biological activities, as has been shown for cryptophycins, didemnins, dolastatins, PF1022, enniatin, destruxin,

Fig. 1. Structure of bassianolide. The structure was obtained from PubChem Compound (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=pccompound>).

beauvericin and valinomycin (Scherkenbeck *et al.*, 2002; Sarabia *et al.*, 2004).

Cyclodepsipeptides are not synthesized by ribosomes in the same way as general peptides, but are instead produced by a nonribosomal peptide synthetase (NRPS). NRPSs are multimodular enzymes in which each module is responsible for the addition of a single amino acid. These enzymes produce nonribosomal peptides (NRPs) by a thiotemplate mechanism. NRPSs can incorporate proteinogenic and non-protein amino acids, as well as carboxy and hydroxy acids. Further modifications, such as *N*-methylation, epimerization, cyclization and heterocyclic ring formation, can also be carried out by these enzymes (von Döhren *et al.*, 1997; Marahiel *et al.*, 1997; Mootz *et al.*, 2002).

NRPs occur in a wide range of organisms, including bacteria, fungi, plants and marine organisms (Keller & Schauwecker, 2003). These compounds often possess desirable pharmaceutical characteristics, and have thus been used for many applications, including use as siderophores, antibiotics, immunosuppressants and anti-cancer drugs (Konz & Marahiel, 1999; von Döhren & Grafe, 1997).

So far, the majority of NRPSs and their products have been characterized in bacteria, especially *Bacillus* and *Streptomyces* species. Fewer fungal genes encoding NRPS have been fully sequenced and characterized experimentally. These genes include *acvA* from *Aspergillus nidulans* and *Penicillium chrysogenum*, which controls the production of the precursor of β -lactam antibiotics such as penicillin (Brakhage, 1997); *simA* from *Tolypocladium inflatum*, which controls the production of cyclosporin A and is used as an immunosuppressive drug in organ transplant surgery (Weber *et al.*, 1994); and *sidA*, which controls production of the siderophores *N'*,*N'*,*N'''*-triacetylfusarinine C (TAF) and ferricrocin, which are

virulence factors in *Aspergillus fumigatus* (Hissen *et al.*, 2005). To date, the genomic sequences of fungi appear to have the potential for faster discovery of novel NRPS genes. For example, 12 NRPS genes have been found in the *Cochliobolus heterostrophus* genome (Lee *et al.*, 2005); 15 putative NRPS genes have been found in *Fusarium graminearum* (Tobiasen *et al.*, 2007); and 14, 22, 14, 20 and 18 NRPS genes have been found in the genome sequences of *A. fumigatus*, *Aspergillus terreus*, *A. nidulans*, *Aspergillus flavus* and *Aspergillus oryzae*, respectively (Cramer *et al.*, 2006a). The functions of some genes have been determined by gene disruption; for instance, *NPS6* from *C. heterostrophus* is involved in virulence and tolerance to oxidative stress (Lee *et al.*, 2005; Oide *et al.*, 2006), and *gliP* from *A. fumigatus* is involved in gliotoxin production (Cramer *et al.*, 2006b). However, the functions of most NRPS genes in fungi remain unknown, requiring further investigation for greater understanding of NRPSs in these organisms (Stack *et al.*, 2007).

Xylaria sp. BCC1067 has been reported to be a rich source of bioactive secondary metabolites (Isaka *et al.*, 2000). One of the major compounds, 19,20-epoxycytochalasin Q, has revealed an interesting characteristic, as it contains polyketide and an amino acid in its core structure (hybrid polyketide–NRP). From our search for additional polyketide synthases (PKSs) and NRPSs in this fungus using a genetic approach via PCR, at least 10 PKS, one hybrid PKS–NRPS (Amnuaykanjanasin *et al.*, 2005) and seven NRPS (Paungmoung *et al.*, 2007) genes were found. These results implied that *Xylaria* sp. BCC1067 should have the genetic capacity to produce a number of natural products and that the total amount of these compounds is likely to be much greater than that which has already been reported for this fungus.

In this study, we identified and analysed the complete sequence of the bassianolide synthetase gene, *nrpsxy*, and confirmed its role in bassianolide biosynthesis by insertional mutagenesis, as shown by the absence of bassianolide production following disruption of the *nrpsxy* locus in *Xylaria* sp. BCC1067. Moreover, an ORF upstream of *nrpsxy* was also identified, revealing high similarity to members of the major facilitator superfamily (MFS) of transporters.

METHODS

Media and strains. *Xylaria* sp. BCC1067 (wild-type) and transformants of this strain were kept on malt extract agar (MEA) at 25 °C. Seven-day-old culture agar discs (3 mm diameter) of either the wild-type or the mutant strain were inoculated into 50 ml malt extract broth (Oxoid) for 14 days, before harvesting for DNA isolation. *Escherichia coli* LE392 was used as the host for lambda Fix II genomic library construction. Subclones were propagated in *E. coli* strain DH5 α (Woodcock *et al.*, 1989).

Construction of a lambda genomic DNA library. A genomic DNA from *Xylaria* sp. BCC1067 was prepared according to the method of Raeder & Broda (1985). The genomic library of *Xylaria* sp. BCC1067

was constructed following the protocol for Gigapack III XL Packaging Extract provided by the manufacturer (Stratagene).

Identification, cloning and sequencing of an NRPS gene.

Degenerate oligonucleotide primers targeting highly conserved motifs of known NRPSs (Turgay & Marahiel, 1994) were used for amplification and identification by PCR of putative NRPS gene fragments in *Xylaria* sp. BCC1067. The sequences of the oligonucleotides used were as follows: forward primer A3, 5'-TA(C/T) AC(T/C/G) TC(A/T/C) GGI (A/T)CI AA(G/A) GC-3'; and reverse primer A5, 5'-(C/T)TC (T/C/G)GT IGG (T/C/G)CC (A/G)TA (T/G)GC-3'. The NRPS PCR product (EN₁₁) labelled with [³²P]dATP was used as a probe for screening the genomic library of *Xylaria* sp. BCC1067 by plaque hybridization.

Construction of a bassianolide-deficient *Xylaria* sp. BCC1067 mutant.

Disruption of the *nrpsxy* gene was achieved by inserting a 2.05 kb phleomycin-resistance cassette (*ble*), which was derived from the vector pOBT (Cheevadhanarak *et al.*, 1991), into the first adenylation domain-coding region. The disruption construct was created by cloning a *HincII* fragment of the *nrpsxy* gene from the DNA insert of lambda λ XyENRPS I into vector pUC18. The resulting plasmid pEN3.8 was inserted by *ble* cassette into an *SmaI* site corresponding to the adenylation domain of the cloned fragment; as a result, a new plasmid, pDEN3.8, was obtained. The disruption plasmid pDEN3.8 was then cut with *EcoRI* to obtain a linear 4.554 kb DNA fragment. This fragment contained 1.048 and 1.451 kb segments of the *nrpsxy* gene flanking the *ble* cassette (Fig. 3a) and was used to transform protoplasts of *Xylaria* sp. BCC1067 according to the method of Tilburn *et al.* (1983).

Substrate specificity of the adenylation domain prediction. The substrate specificity-conferring amino acid in the adenylation domains was determined according to the method of Stachelhaus *et al.* (1999)

HPLC analysis of metabolite profile. HPLC was conducted using a reverse-phase column (Inertsil ODS-3, GL Sciences) and diode array detector (996 Photodiode Array Detector, Waters). An isolated culture of either the *Xylaria* sp. BCC1067 wild-type or the mutant NTT4 was grown in 250 ml malt extract broth. The cultures were incubated at 25 °C for 14 days. Subsequently, the harvested mycelia were extracted with 50 ml methanol for 2 days. After discarding the mycelium, the lipid portion of the extract was removed by extraction with 30 ml hexane, and the methanol layer was concentrated under reduced pressure to obtain a brown semisolid. This mycelial extract was dissolved in methanol to a final concentration of 100 mg ml⁻¹. Separation of metabolites from 20 μ l of extract was performed on an Inertsil ODS-3 reverse-phase column. Analysis was done at a flow rate of 1 ml min⁻¹ at 210 nm with a water–acetonitrile step gradient as follows: 0 min/5 % acetonitrile, 10 min/5 % acetonitrile, 15 min/50 % acetonitrile, 25 min/50 % acetonitrile, 30 min/80 % acetonitrile, 40 min/80 % acetonitrile, 45 min/100 % acetonitrile, and 60 min/100 % acetonitrile, following the method of Weckwerth *et al.* (2000). The water used in the analysis contained 0.05 % (v/v) trifluoroacetic acid (TFA).

Isolation and characterization of pure substance A. The mycelial extract was fractionated by Sephadex LH20 column chromatography (using methanol as an eluent) in order to obtain the fraction containing substance A. Subsequently, substance A was purified by step-gradient HPLC, as described above. Structural documentation was performed by NMR and MS analysis. Proton NMR (¹H NMR) and carbon-13 NMR (¹³C NMR) spectra were measured in CDCl₃ on a Bruker DRX400 spectrometer, and electrospray ionization–time of flight (ESITOF) mass spectra were obtained on a Micromass LCT mass spectrometer (Isaka *et al.*, 2005).

Determination of D and L configuration of amino acids in substance A by acid hydrolysis.

Substance A (0.5 mg) was hydrolysed with 6 M HCL (0.4 ml) at 110–120 °C for 15 h. After concentration to dryness, the residue was dissolved in 100 μ l methanol. Twenty microlitres of hydrolysed substance A was subjected to HPLC using a ligand-exchange-type chiral column (SUMICHIRAL OA-5000, 5 μ m bead size, 4.6 $\times$ 150 mm internal diameter $\times$ length; Sumika Chemical Analysis Service) with 5 % CH₃OH in 2 mM CuSO₄ as the system eluent (flow rate 1 ml min⁻¹, UV wavelength 235 nm). D and L configurations of α -hydroxyisovaleric acid and N-methylleucine were used as reference standards.

Biological assay. Biological activities of substance A against human epidermoid carcinoma (KB cells), human breast cancer (BC-1 cells), human small cell lung cancer (NCI-H187 cells), *Mycobacterium tuberculosis* H37Ra, *Plasmodium falciparum* K1, and African Green Monkey kidney fibroblasts (Vero cells) were performed by the Bioassay Laboratory at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Antibacterial activity was assessed using the disc diffusion assay (McGaw *et al.*, 2000). The bacterial strains tested included *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

RESULTS

Identification of the NRPS gene

To identify the NRPS gene(s) in *Xylaria* sp. BCC1067, degenerate PCR primers specific to conserved sequence motifs of NRPS genes were used in the amplification of genomic DNA from *Xylaria* sp. BCC1067. One product (~400 bp), EN₁₁, showed the highly conserved core motifs A3–A5 of typical NRPS adenylation domains. Probing the genomic library of *Xylaria* sp. BCC1067 with EN₁₁ allowed us to identify two overlapping phages, λ XyENRPS I and λ XyEN9.1, which covered 7.24 kb corresponding to a partial NRPS gene lacking its 3'-terminal region. To identify the remaining part of this gene, an additional step of chromosomal walking was performed. A *Sall* fragment of the 3'-terminal end of λ XyEN9.1, namely EN₆₄₃, was used as a probe to screen the genomic library (Fig. 2a). One positive phage, λ EN643T113, was isolated, and its DNA insert was subjected to sequencing. The results of sequence analysis revealed a complete ORF (10 641 bp), designated *nrpsxy*, which was interposed by a 61 bp intron located near the 3' end. In addition, we also found a smaller complete ORF (1806 bp), designated *efxy*, 5.7 kb upstream of *nrpsxy*, which was transcribed in the opposite direction.

By sequence analysis, we deduced that the *nrpsxy* gene encoded a single ORF of two complete peptide synthetase modules and an additional domain homologous to ketopantoate reductase (KPR) at the C terminus of the predicted protein (Fig. 2b). The predicted protein encoded by the *nrpsxy* gene contained 3546 amino acids with a calculated molecular mass of about 390 kDa. Almost the entire length of the amino acid sequence of NRPSXY (amino acid position 1–3135) displayed strong homology

Fig. 2. Schematic map of the genomic region containing the *nrpsxy* gene and modular structure of NRPSXY. (a) Restriction map and gene arrangement of *efxy* (GenBank accession no. EF456734) and *nrpsxy* (GenBank accession no. EF456733) in *Xylaria* sp. BCC1067. Restriction site abbreviations: D, *DraI*; E, *EcoRI*; H, *HincII*; S, *SmaI*; Sa, *SalI*; X, *XbaI*. The orientations of the genes are indicated by arrowheads, and the positions of introns are indicated by white bars. The *nrpsxy* gene is interrupted by a putative intron of 61 bp. The lambda clone arrangements isolated in this work and the probes used for chromosomal walking are marked (EN₁₁, EN₆₄₃). (b) Modular organization of NRPSXY as revealed by cDART (conserved Domain Architecture Retrieval Tool; <http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps>) from NCBI. The domains within the modules are indicated: A, adenylation domain; C, condensation domain; T, thiolation domain; M, *N*-methylation domain; R, KPR domain; A_{D-Hiv}, 2-D-hydroxyisovaleric acid-activating domain; A_{L-Leu}, *N*-methylleucine-activating domain. Both A_{D-Hiv} and A_{L-Leu} are annotated from the structure identification of substance A encoded by the *nrpsxy* gene.

to the enniatin synthetase (ESYN) encoded by the *esyn* gene of *Fusarium equiseti* (Q00869; 58 % identity and 74 % similarity) and to the cyclosporin synthetase encoded by the *simA* gene of *T. inflatum* (CAA82227; 48 % identity and 64 % similarity). The C-terminal domain of about 412 amino acids (position 3136–3546) did not show any homology to known NRPSs in the databases, but displayed significant similarity to several uncharacterized proteins from various microbes, including KPRs such as AbpA_C of *Magnaporthe grisea* 70-15 (XP_363700; 45 % identity and 66 % similarity), AbpA of *Pichia guilliermondii* ATCC 6260 (XP_001483630; 29 % identity and 50 % similarity), AbpA of *Ps. aeruginosa* C3719 (ZP_00965349; 30 % identity and 46 % similarity) and AbpA of *E. coli* F11 (ZP_00722740; 25 % identity and 42 % similarity). Detailed analysis of NRPSXY showed that it had a domain architecture (C-A-T-C-A-M-T-T-C-R) with corresponding regions of condensation (C) in amino acid residues 48–432, 1104–1442 and 2719–3082; adenylation (A) in amino acid residues 505–982 and 1582–2482; *N*-methylation (M) in amino acid residues 2083–2399, where this domain was inserted into the adenylation domain within the second module (between motifs A8 and A9); and thiolation (T) in amino acid residues 1017–1082, 2510–2571 and 2608–2670, as well as a KPR (R) domain in amino acid residues 3174–3507, which contained a putative NAD/FAD binding motif

(SRIHILGVGNLKGKVFV) (Fig. 2b). These conserved residues are indicated in bold type in Table 1.

To predict the substrate specificity-conferring amino acid in the NRPSXY adenylation domains, two alternative methods were used: (1) BLAST analysis of the amino acid sequence of each A domain against protein databases; and (2) signature sequence analysis according to the method of Stachelhaus *et al.* (1999). When using BLAST analysis via the NCBI website (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>), the first A domain of NRPSXY showed homology to the D-2-hydroxyisovaleric acid-activating domain from ESYN of *F. equiseti* (68 % identity and 81 % similarity), and the L-alanine-activating domain from HC-toxin synthetase (HTS) of *Cochliobolus carbonum* (38 % identity and 58 % similarity). The second A domain showed homology to the leucine-activating domain (63 % identity and 77 % similarity) and to the valine-activating domain (59 % identity and 77 % similarity) from cyclosporin synthetase of *T. inflatum*. It also showed homology to the valine-activating domain from ESYN of *F. equiseti* (54 % identity and 71 % similarity) and from cereulide synthetase B (CesB) of *Bacillus cereus* (38 % identity and 56 % similarity). However, the results from the second method revealed that the signature sequence of the first A domain showed significant matches to the signature

Fig. 3. Disruption of the gene encoding NRPSXY in *Xylaria* sp. BCC1067. (a) Schematic diagram of the insertion mutation of the *nrpsxy* gene. The disruption fragment, the genotype of the wild-type and the genomic rearrangement after a successful gene insertion by double-crossover integration in the mutant NTT4 are shown. The arrows marked with T6, T7, T10, T3, OliC and BleF represent the primers used for verifying the presence of the insertion in the mutant via PCR amplification. (b) PCR analysis of the mutant NTT4. Amplification of genomic DNA from the mutant NTT4 with the T6+T7 primer pair generated a PCR product of ~2.7 kb (lane 2) compared to the undisrupted size from wild-type genomic DNA (lane 1). OliC+T3 and T10+BleF primer pairs showed PCR products of the predicted sizes ~1.9 (lane 3) and ~3.5 kb (lane 4), which were absent when amplified from wild-type genomic DNA. The DNA ladder marker (lane M) shows the size in kilobases. (c) Southern blot analysis of the mutant NTT4. *DraI*-digested genomic DNA of the wild-type (lane 1) and the mutant NTT4 (lane 2) was hybridized to a 2.5 kb probe located at the 5' end of the gene. A homologous crossover into the *DraI* fragment yielded a band of ~5.2 kb and absence of the 3.1 kb wild-type band, as seen in mutant NTT4, indicating *ble* cassette insertion into the *nrpsxy* gene. Lane M, DNA ladder marker.

sequence for D-2-hydroxyisovaleric acid of ESYN (90 %) and to L-alanine of HTS (20 %). The signature sequence for the second domain of NRPSXY revealed the highest

similarity to the N-methylleucine-activating domain found in the second module of cyclosporin synthetase (CsaA) (90 %), the N-methylvaline-activating domain found in the

Table 1. Comparison of the conserved motifs of the catalytic NRPSXY domains with conserved core motifs previously defined by Konz & Marahiel (1999)

Conserved residues are indicated in bold type. ND, Not detected.

Domain	Consensus sequence (Konz & Marahiel, 1999)	NRPSXY module 1 (this work)	NRPSXY module 2 (this work)	
Adenylation				
A1	L(TS)YxEL	WTYNEL	LTYAEL	
A2 (core 1)	LKAGxAYL(VL)P(LI)D	LKTGRAFTLIDP	LKASLAYLPFDV	
A3 (core 2)	LAYxxYTSG(ST)TGxPKG	LAYVLFTSGSTGEPKG	LAYVIFTSGSTGRPKG	
A4	FDxS	FDAC	FDAA	
A5	NxYGPTE	NGYGQSE	NAYGPTE	
A6 (core 3)	GELxLxGxG(VL)ARGYL	GELVIESPGIARGYI	GELVVTGDGLARGYT	
A7 (core 4)	Y(RK)TGDL	YRTGDL	YRTGDR	
A8 (core 5)	GRxDxQVKIRGxRIELGEIE	GRRDSQVKIRGQRTSEVE	GRMDQQVKIRGHRIELAEVE	
A9	LPxYM(IV)P	LPQHSIP	LPSYMIP	
A10	NGK(VL)DR	TGKTDR	SGKVDR	
Thiolation				
T (core 6)	DxFFxLGG(HD)S(LI)	ASFFELGGDSI	[T2]: DNFFKLGGHSL	
Condensation				
C1	SxAQxR(LM)(WY)xL	TPFQSDVMDC	[C2]: SYAQGRIWFL	[C3]: YPATQMQRLE
C2	RHExLRTxF	RQTPALRTC	RHETLRTTF	QHFDMFRTV
C3	MHHxISDG(WY)xL	SHALVDNVLQE	MHHIISDGWSI	MSHALYDGIEL
C4	YxD(FY)AVW	NVQYANG	YRDFAVW	PPKFARY
C5	(IV)GxFVNT(QL)(~)xR	ND	IGFFVNTQCMR	VGPCTNTIPVR
C6	(HN)QD(YV)PFE	HDDAMHE	HQDVPFE	MQDQYLD
C7	RDxSRNPL	RDVARFLR	RDTSRNPL	REDQLANE
N-Methylation				
M1 (SAM)	VL(DE)GxGxG		VLEIGTGSG	
M2	NELSxYRYxAV		NELSAYRYAAV	
M3	VEsxARQxGxLD		VEVSCARQWSQSG	
Reductase				
R1 [NAD(P)H binding site]	V(LF)(LV)TG(AV)(TN)G(YF)LG		ILGVGNLGKFVA	
R2	VxxxVRA		WDAAVKA	
R3	GDL		VDL	
R4	VYPYxxLRx(PL)NVxxT		VYPTSPFSIVHAGRG	
R5	GYxxSKWxx		GYLALQGKRL	
R6	RPG		ND	
R7	LExx(VI)GFLxxP		IETLVNMVKQG	

fourth module of CsaA of *T. inflatum* (50 %), and the leucine-activating domain of bacitracin synthetase (BacA) (60 %) (Table 2). However, chemical analysis of the *nrpsxy* gene product has now confirmed that the first A domain of NRPSXY is responsible for the activation of D-2-hydroxy-isovaleric acid, while the second A domain activates *N*-methylleucine.

Regarding the *efxy* gene, its deduced amino acid sequence was composed of 602 amino acids, and was interrupted by four introns of 63, 62, 58 and 64 bp in length. This protein sequence showed similarity to many membrane transport proteins of the MFS, including those from Bcmfs1 of *Botrytis cinerea* (76 % similarity) (Hayashi *et al.*, 2002), AflT of *Aspergillus clavatus* (68 % similarity), TOXA of *Cochliobolus carbonum* (56 % similarity) (Pitkin *et al.*, 1996) and the MFS multidrug transporter of *A. fumigatus*

AF293 (66 % similarity). Secondary structure analysis by TMHMM (<http://www.cbs.dtu.dk/services/TMHMM>) revealed that EFXV contained 14 membrane-spanning domains.

Inactivation of the *nrpsxy* gene

To determine the function of the gene, insertional mutagenesis of the *nrpsxy* gene was performed. To interrupt the *nrpsxy* gene in the chromosomal locus of *Xylaria* sp. BCC1067, we constructed a linear DNA disruption fragment in which a phleomycin-resistance cassette, *ble*, was inserted by blunt-end ligation in the *Sma*I site of *nrpsxy* flanked by 1.048 kb *Eco*RI/*Sma*I and 1.451 kb *Sma*I/*Eco*RI fragments of the *nrpsxy* gene (Fig. 3a). Transformation of *Xylaria* sp. BCC1067 with the linear DNA fragment and selection on phleomycin led to the

Table 2. Signature sequences of amino acids in the putative activating domains of NRPSXY

Abbreviations: ESYN, enniatin synthetase; CsaA, cyclosporin synthetase; BacA, bacitracin synthetase; SrfA, surfactin synthetase; PhsC, phosphinotricin synthetase; Ent, enterobactin synthetase; LicD, lichenycin synthetase; CDA, calcium-dependent antibiotic synthetase; Com, complestatin synthetase; HTS, HC-toxin synthetase. Bold or italic type indicates the closest resemblance of NRPSXY modules to others present in the databases.

NRPS module	Activated substrate (source)	Amino acid residue at signature sequence position*									
		235	236	239	278	299	301	322	330	331	517
NRPSXY module 1	D-2-Hydroxyisovaleric acid (this work)	G	A	L	L	V	V	G	I	C	K
<i>NRPSXY module 2</i>	<i>N-Methylleucine (this work)</i>	<i>D</i>	<i>A</i>	<i>W</i>	<i>L</i>	<i>V</i>	<i>G</i>	<i>A</i>	<i>V</i>	<i>M</i>	<i>K</i> †
ESYN module 1	D-2-Hydroxyisovaleric acid	G	A	L	H	V	V	G	I	C	K
ESYN module 2	N-Methylvaline	D	G	W	F	I	G	I	I	I	K†
CsaA module 2	N-Methylleucine	D	A	W	L	Y	G	A	V	M	K†
CsaA module 4	N-Methylvaline	D	A	W	M	F	A	A	I	L	K†
CsaA module 9	Valine	D	A	W	M	F	A	A	V	M	K
BacA module 3	Leucine	D	A	W	F	L	G	N	V	V	K
SrfA module 2	Leucine	D	A	F	M	M	G	M	V	F	K
PhsC module 1	Proline	D	V	L	L	V	A	G	V	L	K
EntE module 1	2,3-Dihydroxybenzoate	N	Y	S	A	Q	G	Q	G	L	K
Com3 module 1	4-Hydroxyphenylglycine	D	I	F	H	L	G	V	E	V	K
HTS module 2	Alanine	D	A	G	G	C	A	M	V	A	K

*The residues correspond to the gramicidin S synthetase PheA numbering (Stachelhaus *et al.*, 1999).

†The *N*-methylation domain is integrated in the adenylation domain between the motifs A8 and A9.

isolation of 14 transformants. All transformants were screened by PCR and Southern blot analysis to verify disruption of the *nrpsxy* locus (data not shown). Only one positive transformant (mutant NTT4) revealed disruption of the *nrpsxy* gene. Mutant NTT4 was identified on the basis of a band shift in the mutant and the absence of similar products in the wild-type (Fig. 3a). Insertion of the *ble* cassette into the *nrpsxy* gene was verified using primer pairs specific to the *ble* cassette (BleF and OliC) and the region of the *nrpsxy* gene flanking the insertion site (OliC+T3 and T10+BleF; T10 and T3 are specific primers for the 5'- and 3'-flanking regions of the integration site, respectively). These primer pairs produced specific products, suggesting that the *nrpsxy* gene was disrupted by *ble* cassette insertion. Amplification of genomic DNA from mutant NTT4 with a T6+T7 primer pair generated a PCR product of ~2.7 kb, which may be compared to the nondisrupted size of genomic DNA from a wild-type strain, which generated a PCR product of ~720 bp. OliC+T3 and T10+BleF primer pairs showed PCR products of the predicted sizes of ~1.9 and ~3.5 kb, respectively (Fig. 3b), which were absent when amplified from genomic DNA from a wild-type strain. This result was confirmed by Southern blot analysis. *Dra*I-digested genomic DNA from a wild-type strain and mutant NTT4 was hybridized to a 2.5 kb probe located at the 5' end of the gene. A homologous crossover into the *Dra*I fragment would yield bands of ~4.5 and ~5.2 kb and the absence of the 3.1 kb wild-type band, as seen with mutant NTT4, indicating *ble* cassette insertion into the *nrpsxy* gene, as shown in Fig. 3(c).

Identification of a substance encoded by the *nrpsxy* gene

HPLC analysis of mycelium extracts from the wild-type was used as a reference in comparison to the mutant NTT4. The wild-type metabolic profile showed a visible peak related to substance A at a retention time of 49.3 min (Fig. 4a), whereas this peak disappeared in the profile of mutant NTT4 under the same conditions (Fig. 4b). Substance A was detected in the wild-type by methanol extraction of mycelia of *Xylaria* sp. BCC1067 separated by Sephadex LH20 column chromatography and purified by HPLC. The structure of substance A was elucidated using spectroscopic methods, especially ¹³C NMR, ¹H NMR and MS analysis. The ¹H NMR spectrum showed five *N*-methyl signals at 2.87, 2.90, 3.02, 3.06 and 3.27 p.p.m.; and the ¹³C NMR spectrum in the same solvent revealed a total of 60 signals. The NMR spectra are shown in Supplementary Fig. S1 and the ¹³C NMR spectra of substance A are summarized in Supplementary Table S1. The ESITOF mass spectra of substance A of *m/z* 931.5991 corresponded to [M+Na]⁺ (Supplementary Fig. S2), giving a molecular mass of 908.5991. HPLC analysis of its acid hydrolysate was performed using a chiral column. These analyses confirmed that substance A was a cyclodepsipeptide consisting of four residues of 2-D-hydroxyisovaleric acid (D-Hiv) and four residues of L-methylleucine (*N*-Me-L-Leu). HPLC analysis of the acid hydrolysate of substance A is shown in Supplementary Fig. S3. NMR spectra and mass analysis together with the amino acid composition are shown in Table 3.

Fig. 4. HPLC chromatogram of methanol extracts from mycelia of *Xylaria* sp. BCC1067 wild-type and mutant NTT4 strains. (a) HPLC results from the wild-type extract are presented as a peak with retention time 49.3 min, designated the substance A peak. (b) The substance A peak is missing in extracts from mutant NTT4. (c) The extracts from mutant NTT4 were spiked with extracts from the wild-type.

Biological activities of substance A

To evaluate the biological activity of substance A, pure substance A was subjected to several biological assays. The compound exhibited activity against *M. tuberculosis* H37Ra (MIC $6.25 \mu\text{g ml}^{-1}$), strongly inhibited proliferation of the human malaria parasite *Pl. falciparum* K1 (IC_{50} $1.65 \mu\text{g ml}^{-1}$), and also showed cytotoxic activity against Vero cells and three other cancer cell lines, KB, BC and NCI-H187 (Table 4). However, substance A exhibited no observable toxicity against the other tested bacteria.

DISCUSSION

Xylaria sp. BCC1067 has been shown to be a potential resource of bioactive secondary metabolites (Amnuaykanjanasin *et al.*, 2005; Isaka *et al.*, 2000). We have attempted to clone and analyse NRPS genes, which are expected to be involved in bioactive compound

formation in *Xylaria* sp. BCC1067. In this study, we identified an NRPS gene, *nrpsxy*, and showed that disruption of this gene resulted in the elimination of substance A production. Structural elucidation of substance A was carried out by NMR and MS. Finally, confirmation by a literature data comparison indicated that the structure of substance A was a cyclodepsipeptide consisting of four D- α -hydroxyisovaleryl-L-N-methyleucyl units, which was identical to bassianolide, a cyclooctadepsipeptide with a molecular formula of $\text{C}_{48}\text{H}_{84}\text{N}_4\text{O}_{12}$, previously isolated from *B. bassiana* and *V. lecanii* (Suzuki *et al.*, 1977). The results of gene disruption and metabolite structure elucidation confirmed the role of *nrpsxy* in the biosynthesis of an N-methyl-cyclooctadepsipeptide, bassianolide.

The NRPS encoded by the *nrpsxy* gene is an enzyme consisting of a single polypeptide chain. Its domain architecture was found to be highly similar to that of N-methyl-cyclohexadepsipeptide enniatin synthetase (ESYN)

Table 3. Comparison of chemical characteristics of substance A and bassianolide from Suzuki *et al.* (1977)

Characteristic or analytical result	Substance A (this work)	Cyclodepsipeptide bassianolide (Suzuki <i>et al.</i> , 1977)
Molecular mass (<i>m/z</i>)	908.5991	908
Total signal in ^{13}C NMR spectrum	60	60
N-Methyl signal in ^1H NMR spectrum (chemical shift in p.p.m.)	Five N-methyl signals (2.87, 2.90, 3.02, 3.06 and 3.27)	Five N-methyl signals (2.86, 2.89, 3.01, 3.05 and 3.25)
Acid hydrolysis analysis	N-Methyleucine and D-hydroxyisovaleric acid	N-Methyleucine and D-hydroxyisovaleric acid

Table 4. Antimycobacterial, antiplasmodial and cytotoxic activities of substance A

Target cells	Antimycobacterial, antiplasmodial or cytotoxic activity ($\mu\text{g ml}^{-1}$)
<i>M. tuberculosis</i> * (MIC)	6.25
<i>Pl. falciparum</i> † (IC ₅₀)	1.65
KB cells‡ (IC ₅₀)	3.64
BC cells‡ (IC ₅₀)	2.49
NCI-H187 cells‡ (IC ₅₀)	1.10
Vero cells‡ (IC ₅₀)	4.8

*MIC values of the standard drugs rifampicin, kanamycin and isoniazide were 0.0047, 2.5 and 0.05 $\mu\text{g ml}^{-1}$, respectively.

†The IC₅₀ value of the standard antimalarial compound dihydroartemisinin was 3.9 nM.

‡IC₅₀ values of the standard compound ellipticine were 0.670 $\mu\text{g ml}^{-1}$ for KB cells, 0.129 $\mu\text{g ml}^{-1}$ for BC cells, 0.273 $\mu\text{g ml}^{-1}$ for NCI-H187 cells and 0.5 $\mu\text{g ml}^{-1}$ for Vero cells.

from *Fusarium* species (Haese *et al.*, 1993, 1994). However, ESYN consists of two activation modules, which contain the catalytic binding sites for the substrates D-2-hydroxyisovaleric acid and the branched-chain L-amino acid, causing the substrates to assemble into three units of dipeptidol building blocks (von Döhren *et al.*, 1997). It has been reported that the N-methylated cyclodepsipeptides beauvericin and PF1022A are synthesized by NRPSs that are probably of similar domain architecture to ESYN (von Döhren *et al.*, 1997; Weckwerth *et al.*, 2000).

The domain arrangement of NRPSXY revealed a distinct characteristic, which was composed of two tandem T domains at the C-terminal module. The presence of the second T domain possibly represents the waiting position for repeating the enzymic reaction. Based on the domain/module organization of NRPSXY and the structure of substance A, the hypothetical mechanism of bassianolide biosynthesis by *Xylaria* sp. BCC1067 can be postulated to resemble the biosynthesis mechanism of enniatin, beauvericin and PF1022A. It appears likely that NRPSXY use their modules or domains more than once in the assembly of a single product and could be categorized as an iterative NRPS, as is the case for enterobactin synthetase and gramicidin S synthetase (von Döhren *et al.*, 1997; Mootz *et al.*, 2002).

The main difference between ESYN and NRPSXY is an additional putative KPR (EC 1.1.1.169) located at the C-terminal end of NRPSXY. Interestingly, in enniatin biosynthesis of *Fusarium sambucinum*, its 2-D-hydroxyisovaleric acid precursor is synthesized from 2-ketoisovalerate (2-KIV) catalysed by the D-hydroxyisovalerate dehydrogenase enzyme (Lee *et al.*, 1992), and it has been reported that the KPR enzyme from *Pseudomonas maltophilia* 845 can catalyse the biosynthesis of 2-D-hydroxyisovaleric acid from 2-KIV as an alternative substrate (Shimizu *et al.*, 1988). Furthermore, in the study of genes involved in AF-toxin biosynthesis in the plant-pathogenic fungus

Alternaria alternate, a gene designated AFTS1, which encodes a protein with similarity to enzymes of the aldoketoreductase superfamily, has been proposed to be the gene encoding an enzyme that catalyses the conversion of 2-KIV to 2-D-hydroxyisovaleric acid (Ito *et al.*, 2004). For cereulide biosynthesis in *B. cereus*, it has been shown that the A domain of cereulide synthetase B peptide specifically activates the α -keto acid (in this case 2-KIV), and is subsequently reduced to 2-D-hydroxyisovaleric acid by the keto-reductase domain embedded in its A domain before condensing to an adjacent amino acid precursor by the condensation domain (Magarvey *et al.*, 2006). However, the function of the R domain at the C-terminal of the NRPSXY peptide still warrants further analysis.

In conclusion, NRPSXY most resembles ESYN with respect to its domain organization. However, the end product of NRPSXY is a cyclooctadepsipeptide, a compound consisting of eight residues instead of the six residues synthesized by ESYN. In our case, comparative study of the amino acid specificity of the modules of NRPSXY, by predicting the signature sequence of the substrate-binding pocket together with chemical structure analysis of the NRPSXY product, revealed the specificity of 2-D-hydroxyisovaleric acid for the first A domain and of N-methylleucine for the second A domain. These results confirmed the hypothesis proposed by Stachelhaus *et al.* (1999). This finding may be useful for future prediction of amino acid-activating domains and could help as a guide to the characterization of newly discovered NRPSs.

A second ORF, the *efxy* gene, was located 5.7 kb upstream of the *nrpsxy* gene. This gene putatively encodes a transporter protein of the MFS. These proteins are usually of a membrane-bound type that may help prevent accumulation of toxic compounds in cells (Hayashi *et al.*, 2002). In filamentous fungi, a number of MFS transporters are known to mediate the secretion of endogenously produced toxins (Pitkin *et al.*, 1996), including cercosporin, HC-toxin and trichothecenes. These MFS genes are located in gene clusters together with genes that encode the enzymes involved in the biosynthesis of these toxins (Hayashi *et al.*, 2002; Pitkin *et al.*, 1996). Therefore, the protein encoded by *efxy* may serve as an efflux pump that transports bassianolide out of the cells of *Xylaria* sp. BCC1067.

We report here what is believed to be the first evidence of a biosynthetic gene for the NRP bassianolide. In this study, we have shown an *in vitro* bioassay of substance A, which exhibited antiplasmodial, antimycobacterial and antitumor activities beyond the previously reported insecticidal activity of bassianolide (Suzuki *et al.*, 1977; Champlin & Grula, 1979). These activities may be related to its cytotoxic actions; however, substance A showed no observable toxicity against bacteria such as *Staph. aureus* ATCC 29213, *B. subtilis* ATCC 6633, *E. coli* ACTT 25922 and *Ps. aeruginosa* ATCC 27853.

The potential of *Xylaria* sp. BCC1067 as a prolific resource for bioactive compounds has been demonstrated by

chemical and genetic approaches, and future identification and characterization of new natural products are in the pipeline. We expect that using genetic knowledge as a basic tool for further modification of biosynthetic genes in this micro-organism will result in novel compounds with biotechnological value for medical, agricultural and industrial exploitation in the near future.

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