Identification of Peptaibols from *Trichoderma virens* and Cloning of a Peptaibol Synthetase*

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The fungus *Trichoderma virens* is a ubiquitous soil saprophyte that has been applied as a biological control agent to protect plants from fungal pathogens. One mechanism of biocontrol is mycoparasitism, and *T. virens* produces antifungal compounds to assist in killing its fungal targets. Peptide synthetases produce a wide variety of peptide secondary metabolites in bacteria and fungi. Many of these are known to possess antibiotic activities. Peptaibols form a class of antibiotics known for their high ε-aminoisobutyric acid content and their synthesis as a mixture of isoforms ranging from 7 to 20 amino acids in length. Here we report preliminary characterization of a 62.8-kb continuous open reading frame encoding a peptaibol synthetase from *T. virens*. The predicted protein structure consists of 18 peptide synthetase modules with additional modifying domains at the N- and C-termini. *T. virens* was shown to produce a mixture of peptaibols, with the largest peptides being 18 residues. Mutation of the gene eliminated production of all peptaibol isoforms. Identification of the gene responsible for peptaibol production will facilitate studies of the structure and function of peptaibol antibiotics and their contribution to biocontrol activity.

Peptaibols, a class of linear peptides of fungal origin with 7–20 residues, have three structural characteristics, (i) a high proportion of ε,ε-dialkylated amino acids with an abundance of ε-aminoisobutyric acid (Aib), (ii) an N-terminal residue, usually acetyl, and (iii) a C-terminal amino acid, such as phenylalaninol or leucinol. Peptaibols naturally occur as mixtures of isoforms, and more than 250 sequences are now known (public-1.cryst.bbk.ac.uk/peptaibol/search.html). They are divided into three subclasses, which are (i) the long sequence peptaibols with 18–20 amino acid residues exemplified by alamethicins (1) or trichorizanins (2–4), (ii) the short-sequence peptaibols with 11–16 residues exemplified by harzianins (5–7) or zervamincins (8, 9), and (iii) the lipopeptoids (10) with 7 or 11 residues, the N terminus of which is acylated by a short fatty acid chain such as octanoic acid instead of acetic acid represented by trichoginAIV. Peptaibols generally exhibit antimicrobial activity against Gram-positive bacteria and fungi (11). Their biological activities are thought to arise from their membrane-modifying properties and their ability to form transmembrane voltage-dependent channels (12, 13). The producing fungi, mainly of the genus *Trichoderma* and related genera such as *Emericellopsis* and *Gliocladium*, have antagonistic activity against fungal phytopathogens, which has led to their use as biocontrol agents. Mycoparasitic strains of *Trichoderma* produce cell-wall-hydrolyzing enzymes in addition to antibiotics. Peptaibols are thought to act on the membrane of the target fungus to inhibit membrane-associated enzymes involved in cell wall synthesis. Indeed, peptaibols have been shown to act synergistically with cell wall-degrading enzymes to inhibit the growth of fungal pathogens (14, 15). Peptaibols may also elicit plant resistance to pathogens. The exogenous application of peptaibols has been shown to trigger a defense response in lima bean and to reduce the susceptibility of tobacco to tobacco mosaic virus (16, 17).

Because of the potential importance of peptaibols in the biological control of plant diseases, we sought to clone the gene(s) responsible for their synthesis. With their unusual amino acid content, we expected peptaibols to be the product of non-ribosomal peptide synthetases (NRPSs). These large multifunctional enzymes assemble components from a remarkable range of precursors (including nonproteinogenic amino acids and hydroxy or carboxylic acids), which can be N-methylated, acylated, reduced, or epimerized (18, 19). NRPSs have a modular structure in which each module is a semi-autonomous unit that recognizes, activates, and modifies a single residue of the final peptide (20). Each module can be further partitioned into distinct adenylation (A), thiolation (T), and condensation (C) domains, which together represent a minimal repeating unit of NRPSs (21). Analysis of the phenylalanine activation domain of gramicidin synthetase, GrsA, has been used to determine the key residues responsible for A-domain specificity in substrate recognition (22, 23). These have been referred to as signature sequences. It is hoped that a sufficiently large collection of verified signature sequence/amino acid substrate combinations will provide an NRPS “codon” table allowing prediction of amino acid substrates based on the signature sequences in uncharacterized NRPSs.

Using a PCR-based approach, we identified a peptide synthetase of *Trichoderma virens*. Here we report the cloning of a gene responsible for the production of peptaibols in *T. virens*. The predicted open reading frame represents the largest known NRPS and contains modules for the incorporation of 18 amino acids as well as the domain we predict is involved in acetylation of the N terminus and reduction of the C terminus of the peptaibol products.
Peptide Synthetase Producing Peptaibols

EXPERIMENTAL PROCEDURES

Fermentation—Conidial suspensions of T. virens strains TV29-8, TVP223, and TVP234 were inoculated into 120 Roux flasks, each containing 170 ml of the sterilized synthetic medium having the composition glucose (0.5%), KH₂PO₄ (0.08%), KNO₃ (0.07%), Ca(H₂PO₄)₂ (0.02%), MgSO₄·7H₂O (0.05%), MnSO₄·7H₂O (0.001%), CuSO₄·5H₂O (0.0008%), FeSO₄·7H₂O (0.0001%). Each flask was inoculated with a 2 ml of conidial suspension, and the stationary cultures were incubated at 27 °C for 18 days.

Isolation of Peptoids—The fermentation broth was filtered, and the filtrate was extracted 3 times with n-butyl alcohol, whereas the mycelium was extracted 3 times with methanol, yielding 0.9 and 1.1 g of crude fraction, respectively. These two fractions were subjected to gel filtration and anion exchange HPLC column eluted with MeOH to yield crude peptide mixtures in the head fractions. We recovered 512 mg of crude peptide mixtures from the culture filtrate fraction and 12 mg from the mycelial fraction of TV29-8. These peptide mixtures were each chromatographed over a silica gel column (Kieselgel 60 H Merck) by elution with a CH₃CN/MeOH gradient (from 9:1 to 1:1). Each fraction yielded two groups of peptides, TVA and TVB. The culture filtrate yielded 272 mg of TVA and 120 mg of TVB. Lower amounts of TVA (14 mg) and TVB (19 mg) were obtained from the mycelium. The peptides from the mycelium were not analyzed further.

Column chromatographies were monitored by TLC (SiO₂, Merck 60 F254; CH₃CN/MeOH, 7:3) and visualized by spraying with anisaldehyde reagent (p-anisaldehyde:sulfuric acid:acetic acid, 1:1:50) followed by heating performed with a Girdel 3000 chromatograph on a Chirasil-L-Val (Kromasil, 5 μm, 4.6 × 250 mm) with MeOH:H₂O:CH₃CN (86:14 as the system eluent (flow rate, 1 ml/min) to separate peptaibol subfractions.

Amino Acid Analysis—The peptides (1 mg) were hydrolyzed (HCl 6 n, 110 °C, N₂), and the amino acids and amino alcohols were derivatized to produce N-trifluoroacetylisopropyl ester derivatives as previously described (2, 3, 6). The gas-liquid chromatography analyses of the N-trifluoroacetylisopropyl esters were performed with a Girdel 3000 chromatograph on a Chirasil-L-Val (N-propionyl-L-valine tert-butyramidine polysiloxane) quartz capillary column (Chrompack, 25-m length, 0.2-mm internal diameter) with He (0.7 bar) as the carrier gas with the following temperature program: 50 to 130 °C, 3 °C min⁻¹ followed by 130 to 190 °C, 10 °C min⁻¹. The separation factors (α) were (α;β) for the L and D enantiomers: Aib, 10.4; t-Ala, 14.3 (1.16); t-Glu, 33.2 (1.05); Gly, 17.8; L-Leu, 24.2 (1.11); t-Ser, 23.0 (1.05); t-Val, 17.7 (1.08); t-Valol, 18 (0.98). A different temperature program was used for the separation of proline enantiomers: 50 to 110 °C, 3 °C min⁻¹, then a plateau at 110 °C (10 min), followed by 110 to 190 °C, 10 °C min⁻¹; Rt (α;β) in seconds: L-Pro, 25.1 (1.02).

LSI Mass Spectrometry—Positive LSIMS were recorded on a ZAB2-SQX-70 (Siemens, Manchester, UK) mass spectrometer equipped with a standard fast atom bombardment source and a cesium ion gun equipped with a standard fast atom bombardment source and a cesium ion gun equipped with a LSI mass spectrometer equipped with a cesium ion source. The data was acquired using a Mass Spectra Link (MSL) software package and analyzed by DISNMR software. The adduct ions [M+H]⁺ and [M+HNa]⁺ were observed, allowing the molecular mass to be determined. The NMR spectra of TVA I, II, and IV were characteristic of peptaibols, showing amide protons between 6.50 and 8.70 ppm, a high proportion of them being singlets, typically of α,α-dialkylated amino acids. Several methyl singlets of Aib between 1.30 and 1.60 ppm and a sharp singlet at 2.05 ppm were assigned to the N-terminal acetyl. The TVA fraction was much more complex, and no pure compound was isolated (Fig. 1B).

Sequences of Trichorzins TVB I, II, and IV—The amino acid composition of TVBI was Aib [7] t-Ala [2], Gly [1], t-Gln [2], L-Valol [1], and the amino alcohol was t-valol. Peptide TVBI had an additional t-leucine and no valine, and TVB IV differed from TVB II by the replacement of one Aib by a D-isovaline. When analyzed by (+) liquid secondary ion mass spectrometry, the adduct ions [M+H]⁺ and [M+HNa]⁺ were observed, allowing the molecular mass to be determined. As generally described for these peptides (7), fragmentation events leading to acylion ions were detected and, thus, allowed the sequences of TVB I, II, and IV to be determined (Fig. 1C).

The [M+Na]⁺ ion of TVB I was observed at m/z 1727 (Table I). A main fragmentation at the Aib-Pro amide bond led to the b₁₂ acylion, which generated a series of acylion fragments at m/z 1009 (b₃₋₄), 896 (b₆₋₇), 809 (b₉), 724 (b₁₂), 653 (b₁₅), 568 (b₁₈), 440 (b₂₁), 355 (b₂₄), 256 (b₂₇), 185 (b₂₉), 128 (b₃₀), leading to the sequence Ac-Aib-Gly-Ala-Vib-Aib-Gln-Ala-Aib-Ser-Leu-Aib. An ammonium ion was observed at m/z 612 (g₁₂), which underwent fragmentation, leading to a series of acylion ions at m/z 509, 381, 296, and 211 from which the C-terminal sequence Pro-Leu-Aib-Gln-Valol was deduced (Table I). From their sequence analogy with the 18-residue trichorizin peptaibols (28–30), the name trichorzins TVB is proposed for this group (Table II).

Similarly, the mass spectrum of TVB II and TVB IV depicted the sodium adduct ions at m/z 1741 and 1755, respectively. The g₂₀ ion was observed at m/z 612 for the two compounds, and it underwent the same fragmentation, suggesting the 13–18-residue C-terminal sequence to be the same as TVB I, Pro-Leu Aib-Aib-Gln-Valol. The b₁₂ acylion was at m/z 1108 for TVB II, and its further acylion fragmentation allowed the sequence Ac-Aib-Gly-Ala-Vib-Aib-Gln-Ala-Aib-Ser-Leu-Aib to be determined for the 1–12-residue N-terminal moiety. This differs from TVBI by the Val/Leu substitution at position 1. The b₁₂ acylion for TVB IV was observed at m/z 1122, yielding acylion fragments giving the sequence Ac-Aib-Gly-Ala-Vib-Gln-o-Iva-Aib-Ser-Leu-Aib (Fig. 1C).

Mass spectral analysis of TVB III and V showed they were micro-heterogeneous. The main component of TVB III had the same quasi-molecular ions and fragmentation as TVB II and, therefore, may be an isomer. The pattern for TVB V was most similar to that of TVB IV. Because no pure peptide was ob-

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2 C. M. Kenerley, unpublished information.
tained from the TVA group, the mixture was analyzed by (+) liquid secondary ion mass spectrometry. Two quasi-molecular [M+Na]+ ions were observed in the high mass region. The more abundant class contained ions at m/z 1183, 1197, 1211, and 1227, suggesting a mixture of 11-residue peptides similar to harzianins HB (5) (Table II). The less abundant class contained ions at m/z 1452, 1466, and 1480, suggesting a mixture of 14-residue peptides similar to harzianins HC (6) (Table II).

Cloning of a Peptide Synthetase from T. virens—Degenerate primers (“Experimental Procedures”) successfully amplified a single PCR product of 886 bp, and subsequent sequence analysis of this product revealed clear homology to known peptide synthetases. The PCR product was used as a probe to identify a cosmid clone, 40B7, of T. virens genomic DNA. Two adjacent BamHI fragments of 6.8 and 8.3 kb (Fig. 2B) were sequenced and revealed a continuous 15.1-kb open reading frame with homology to 4 complete and two partial peptide synthetase modules. Sequence analysis of an additional 3.9-kb region in the 5′ direction of the gene revealed that the single open reading frame continued to the end of the cosmid insert. A T. virens bacterial artificial chromosome library was constructed to facilitate cloning of the peptide synthetase (25). A bacterial arti-

![HPLC chromatogram of peptaibols from T. virens.](image)

**Fig. 1. HPLC chromatogram of peptaibols from T. virens.** A, TVB peptaibols (Kromasil C18, 5 µm, 1.6 × 250 mm; MeOH/H2O (86:14), flow rate 1 ml/min, absorption monitored at 220 nm). B, peptaibol mixture TVA (Kromasil C18, 5 µm, 1.6 × 250 mm; MeOH/H2O (86:14), flow rate 1 ml/min, absorption monitored at 220 nm). C, sequence of the 18 residue trichorzins TVB I, II, and IV. Variant residues are indicated in bold.

|                 | TVB I | TVB II | TVB IV |
|-----------------|-------|--------|--------|
| [M+Na]+         | 1727 (16) | 1741 (24) | 1755 (10) |
| [M+H]+         | 1705 (9)  | 1719 (6)  | 1733 (10)  |
| b₁₂            | 1094 (28) | 1108 (23) | 1122 (27)  |
| y₆             | 612 (37)  | 612 (39)  | 612 (37)  |
| b₁₁            | 1009 (1)  | 1023 (1)  | 1037 (1)  |
| b₁₀            | 896 (6)   | 910 (4)   | 924 (5)   |
| b₉             | 809 (14)  | 823 (11)  | 837 (11)  |
| b₈             | 724 (11)  | 738 (9)   | 752 (9)   |
| b₇             | 653 (23)  | 667 (23)  | 681 (24)  |
| b₆             | 568 (4)   | 582 (3)   | 582 (4)   |
| C              | 509 (6)   | 509 (5)   | 509 (5)   |
| b₅             | 440 (60)  | 454 (54)  | 454 (58)  |
| C              | 381 (18)  | 381 (14)  | 381 (14)  |
| b₄             | 355 (61)  | 369 (53)  | 369 (62)  |
| C              | 296 (31)  | 296 (24)  | 296 (62)  |
| b₃             | 256 (100) | 256 (100) | 256 (100) |
| C              | 211 (22)  | 211 (18)  | 211 (18)  |
| b₂             | 185 (65)  | 185 (68)  | 185 (65)  |
| b₁             | 128 (50)  | 128 (52)  | 128 (50)  |

*Percent of base peak.*
ficial chromosome clone, pDXG70, was identified through Southern hybridization that contains the entire coding sequence for the gene, called \textit{texas-1} (\textit{tex1}). From the sequences obtained from subclones of pDXG70, we deduce that the gene encodes a single open reading frame of 18 complete peptide synthetase modules and additional domain homologies at the N and C termini of the predicted protein (Fig. 2). An additional 4.5 kb of sequence downstream of \textit{tex1} was determined. Within this region, two sequence homologies were identified; one displayed strong homology to a retrograde regulation protein of \textit{Saccharomyces cerevisiae} (Rtg2p) and the other to a calcium/proton exchanger of \textit{Neurospora crassa}. The sequence displaying Rtg2p homology (35\% identity) is convergent to \textit{tex1} beginning 2.2 kb from the N terminus and ending 0.7 kb from the C terminus. The calcium/proton exchanger homolog (29\% identity) is in the same orientation as \textit{tex1}, beginning 3.25 kb from the end of \textit{tex1} and extending to the end of the cosmid insert.

Conserved sequences in peptide synthetase modules are involved in ATP binding and amino acid thioesterification and transfer (31). These conserved domains are present in each of the modules (Fig. 2). BLAST alignments comparing the complete modules of \textit{tex1} to each other showed 35–58\% identity over the entire length of the modules. A comparison of the signature sequence residues showed that all modules have the

### Table II

| Organism       | Peptaibol       | Sequence                               |
|----------------|-----------------|----------------------------------------|
| \textit{T. virens} | TVB I           | Ac-UGAVQUAUSLUPLUUQV-OH                 |
| \textit{T. virens} | TVB II          | Ac-UGALQUSAUSLUPLUUQV-OH               |
| \textit{T. virens} | TVB IV          | Ac-UAGUSQAUSLUPLUUQV-OH               |
| \textit{T. harzianum} | Trichorzin_HA_I | Ac-UAGAVQVUVGLPLUUQV-OH               |
| \textit{T. harzianum} | Trichorzin_MA_I | Ac-UAGAVQVUVGLPLUUQV-OH               |
| \textit{T. harzianum} | Harzianin_HB_I  | Ac-UAGAVQVUVGLPLUUQV-OH               |
| \textit{T. harzianum} | Harzianin_HC_I  | Ac-UAGAVQVUVGLPLUUQV-OH               |
| \textit{T. harzianum} | Hypomuricin_B   | Ac-UAGAVQVUVGLPLUUQV-OH               |
| \textit{T. koningii}  | Trichokonin_KA_V | Ac-UAGAVQVUVGLAPUUQVQ-H               |
| \textit{T. koningii}  | Trichokonin_La  | Ac-UAGAVQVUVGLAPUUQVQ-H               |

**FIG. 2.** Modular structure of \textit{tex1} and schematic of four modules. A, modular organization of \textit{tex1} as revealed by DART (domain architecture search). Modules of the \textit{tex1} peptide synthetase are indicated by numbers 1–18. The domains within the modules are indicated. A domain with homology to a portion of ketoacyl synthase domains (pfam00109) and acyltransferase domains (pfam00698) is found at the N terminus. At the C terminus is a motif with similarity to 3 β-hydroxysteroid dehydrogenase/isomerase family (pfam01073). Assignment of the amino acids to the module is indicated (code: Ac, acetyl group attached to the N-terminal amino acid of the peptide; U, Aib; J, isovaline; V-OH, valinol). Scale represents amino acid position in \textit{tex1}. B, the 6.8- and 8.3-kb \textit{Bam}HI fragments of the high quality sequence are shown. Restriction enzyme sites are indicated (\textit{B}, \textit{Bam}HI; \textit{E}, \textit{Eco}RI; \textit{H}, \textit{Hin}dIII). The location of the 886-bp PCR product is indicated by the solid bar above module 11. The 0.4-kb \textit{Eco}RI-\textit{Hind}III fragment (*) that was replaced by the hygromycin phosphotransferase gene to generate a gene disruptant mutant is shown above module 11.
expected aspartate residue at position 235. Aspartate residues are invariant at this position for modules incorporating amino acids, differing only in modules that incorporate carboxylic acids (32). Modules 6 and 17 have identical residues in the remaining 8 positions (Table III). Modules 1, 5, 9, 12, 15, and 16 share identical residues at positions 236, 239, 299, and 322. These modules also have aromatic residues at position 278. Modules 3 and 8 have identical residues at position 299, 301, and 330 and similar residues at positions 236, 278, and 331. The comparison of signature sequences of the tex1 modules with the sequences of trichorzins TVB I, II, and IV are consistent with the view that the signature sequences determine amino acid specificity.

**Disruption of tex1 Abolishes Peptaibol Production**—We generated mutant alleles of tex1 by replacing a 0.4-kb segment of tex1 with a selectable marker conferring resistance to the antibiotic hygromycin B (Fig. 2B). The replacement vector (pPSK2) contained 4.5- and 2.6-kb segments of the peptide synthetase gene flanking the resistance marker (Fig. 3A). The linearized plasmid was used to transform strain TV29-8 and 324 transformants were tested for stable integration of the resistance gene marker (33). These transformants were screened by Southern blot analysis to verify the disruption at the tex1 locus. Two strains, TVP223 and TVP234, were clearly disrupted for the gene contained at least one additional ectopic copy of pPSK2. As predicted, bands in the mutant strains all hybridized to a probe obtained from the hygromycin cassette (data not shown).

The two gene disruption transformants were tested in parallel with TV29-8 for peptaibol production. In contrast to the wild type, TV29-8, no peptaibols were found. Strain TV29-8 produces a second antibiotic, gliotoxin, that is also thought to contribute to mycoparasitic activity. Gliotoxin production was examined in all 324 transformed strains, and all produced wild type levels of gliotoxin. This indicates that disruption of tex1 specifically eliminates peptaibol synthesis and does not cause a general defect in secondary metabolite production.

**DISCUSSION**

Here we demonstrate that T. virens, widely recognized as a biocontrol agent, produces peptaibol antibiotics. Several classes of peptaibols are produced by T. virens strain TV29-8 including 18-, 14-, and 11-amino acid residue peptides. The sequences of 3 of the 18 amino acid residue peptaibols, trichorzins TVB I, II, and IV, were determined. We have shown here that a large peptide synthetase (2.3 MDa) is responsible for production of all classes of peptaibols in T. virens. The evidence for this is (i) disruption mutants of tex1 failed to make peptaibols, (ii) domain homologies at both the N and C termini are consistent with the predicted functions necessary for acylation and reduction, respectively, and (iii) the gene sequence indicates that tex1 contains modules for the incorporation of 18 amino acid residues.

The intron-less gene will encode a mature protein of 20,925 residues (~2.3 MDa) encoded by a ~63-kb mRNA. This gene would specify the largest mRNA and the largest continuous coding region known. Given these large sizes (a linear 63-kb mRNA would span 10 μm and exceed the diameter of the cell), it is of interest to understand how such large mRNAs and proteins are processed. For example, it is conceivable that translation of the peptide synthetase could initiate before completion of transcription of the gene.

Because genes for secondary metabolite production are often found to be clustered (34), additional genes required for synthesis or export of a mature peptaibol may be identified by further analysis of the flanking regions. Identification of homologs of a retrograde regulation protein (Rtg2p) and a calcium/proton exchanger downstream of tex1 suggests that a gene cluster for peptaibol production does not extend beyond the 3′ end of tex1. Preliminary sequence in a 2-kb region upstream of tex1 has not revealed homology to known proteins.

The A domain of peptide synthetases contains 10 conserved motifs (22) that are present in each of the modules of tex1. Likewise, the known conserved motifs in the T domains and C domains were also identified within each module. Amino acid residues 16–196 of the tex1 protein aligned over 80% of the length of ketoacyl synthase domains (pfam00109), typically found in polyketide synthases, with BLAST E value of 1e−11 (35). Residues 416–703 aligned over 91% of the length of acyltransferase domains (pfam00698) (E value = 2e−43). We propose that these domains are responsible for the acetylation of the peptaibol N terminus in accordance with the colinearity of the domains with that of their products (32). Amino acid residues 20552–20781 aligned over 51% of the length of the 3 β-hydroxysteroid dehydrogenase/isomerase family (pfam01073) (E value = 3e−6) and aligns over 88% of the length of alcohol dehydrogenase domains (pfam00106) (E value = 0.003). We propose that this domain plays a role in the reduc-

**TABLE III**

**Signature sequences of putative amino acid activating domains of tex1**

| Amino acid | Module | 235 | 236 | 239 | 278 | 299 | 301 | 322 | 330 | 331 |
|------------|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Q          | 6      | D   | G   | G   | M   | V   | G   | G   | N   | Y   |
| Q          | 17     | D   | G   | G   | M   | V   | G   | G   | N   | Y   |
| J/U        | 7      | D   | C   | G   | W   | V   | V   | G   | V   | V   |
| U          | 1      | D   | L   | G   | Y   | L   | A   | G   | V   | F   |
| U          | 9      | D   | L   | G   | Y   | L   | A   | G   | C   | F   |
| U          | 12     | D   | L   | G   | Y   | L   | A   | G   | V   | F   |
| U          | 15     | D   | L   | G   | F   | L   | A   | G   | V   | F   |
| U          | 16     | D   | L   | G   | F   | L   | A   | G   | L   | F   |
| U/A        | 5      | D   | L   | G   | W   | L   | C   | G   | V   | F   |
| A          | 3      | D   | V   | G   | F   | V   | A   | G   | V   | L   |
| A          | 8      | D   | I   | F   | V   | V   | A   | G   | V   | I   |
| L          | 11     | D   | F   | L   | Y   | F   | G   | G   | V   | V   |
| L/V        | 14     | D   | A   | A   | L   | I   | G   | A   | V   | F   |
| V          | 4      | D   | M   | G   | F   | L   | G   | G   | V   | C   |
| V-OH       | 18     | D   | A   | I   | I   | I   | V   | G   | V   | T   |
| G          | 2      | D   | I   | G   | M   | V   | G   | V   | T   | L   |
| S          | 10     | D   | V   | G   | Y   | L   | A   | A   | V   | Y   |
| P          | 13     | D   | V   | L   | F   | C   | G   | L   | I   | C   |

J, 2-aminoisobutyric acid; U, isovaline; V-OH, valinol.

Amino acid Module 235 236 239 278 299 301 322 330 331 331
tive cleavage of the final amino acid to generate the C-terminal alcohol. Therefore, the mature peptaibol synthetase may contain all of the enzymatic activities necessary to produce peptaibols.

Nine residues in the active site of peptide synthetases have been proposed to play a major role in defining substrate specificity for incorporation of amino acids based on structural data (22). These residues define the signature sequences specifying amino acid incorporation. The signature sequences from the modules of \textit{tex1} are unique and do not exactly match the signature sequences found in other characterized NRPSs. Therefore, we could not use the signature sequences to make amino acid substrate assignments for the \textit{tex1} modules. However, we noted a very strong pattern in which the signature sequences of the 1st, 9th, 12th, 15th, and 16th modules were very similar (Table III). These may correspond to Aib residues found at positions 1, 9, 12, 15, and 16 of the TVB peptaibols. Modules 6 and 17 have identical residues at all 9 positions, and it is likely that these two modules incorporate the glutamate residues found in positions 6 and 17 in the peptaibols.

Peptaibols are among the largest products known that are synthesized by NRPSs, and a tremendous variety of peptaibol sequences has been found. Indeed, \textit{T. virens} and other peptaibol-producing organisms take advantage of combinatorial chemistry by producing a complex mixture of peptaibol compounds in culture filtrates. This is likely to be a result of the potential of the module to bind multiple substrates. In certain cases, the complexity can be manipulated by supplementing cultures with a specific amino acid (6). This suggests that the incorporation of amino acids into peptaibols reflects the availability of the cognate substrates rather than the existence of multiple NRPSs, each responsible for production of one member of the mixture. Our finding that a single mutation can eliminate all forms of peptaibols confirms this view.

A recent publication reported the partial cloning and characterization of a peptide synthetase from \textit{T. virens} with a sequence identical to the 3' terminal 5 kb of \textit{tex1} (36). Disruption of this gene (\textit{psy1}) was reported to cause partial or complete loss of hydroxymate siderophore production. The authors concluded that \textit{psy1} encodes an NRPS responsible for hydroxymate siderophore production. We believe this to be in error for the following reasons. First, the full sequence of the \textit{tex1} (\textit{psy1}) gene indicates that it synthesizes an 18-amino acid peptide. This is far larger than expected for an enzyme producing hydroxymate-containing siderophores. Second, \textit{Neurospora} and \textit{Aspergillus} species are also known producers of hydroxymate siderophores. The genome sequences of \textit{N. crassa} and \textit{Aspergillus fumigatus} are now available, and based on BLAST searches, neither genome contains a NRPS with 18 modules or strong sequence similarity to \textit{tex1} (\textit{psy1}). In addition, preliminary growth experiments with our \textit{tex1} gene disruption strains did not reveal significant growth defects or deficiency in siderophore production on iron-deficient medium relative to the growth on iron-sufficient medium (37). Although unlikely, it is possible that certain alleles of \textit{tex1} affect siderophore production directly or indirectly.

Here we report that peptaibols are produced by a peptide synthetase and the first characterization of a peptaibol synthetase gene. Cloning of additional peptaibol synthetases will facilitate generation of novel peptaibols by precisely replacing...
modules or making specific changes in the signature sequences. Manipulation of the abundance or sequence of peptaibols and heterologous expression of the *T. virens* peptaibol synthetase in other biocontrol fungi may prove to be useful tools in enhancing biocontrol activity.

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