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Characterization of an echinocandin B-producing strain blocked for sterigmatocystin biosynthesis reveals a translocation in the *stcW* gene of the aflatoxin biosynthetic pathway

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Echinocandin B (ECB), a lipopolypeptide used as a starting material for chemical manufacture of the anti-Candida agent LY303366, is produced by fermentation using a strain of *Aspergillus nidulans*. In addition to ECB, the wild-type strain also produces a significant level of sterigmatocystin (ST), a potent carcinogen structurally related to the aflatoxins. Characterization of a mutant designated A42355-OC-1 (OC-1), which is blocked in ST biosynthesis, was the result of a chromosomal translocation. The chromosomal regions containing the breakpoints of the translocation were isolated and DNA sequencing and PCR analysis of the chromosomal breakpoints demonstrated the translocation occurred within the *stcW* gene of the ST biosynthetic pathway, resulting in disruption of the open reading frame for this gene. Biochemical feeding studies indicate the involvement of this gene product in the conversion of averufin to 1-hydroxy versicolorone. This work demonstrates an effective synergy between classical strain improvement methods and molecular genetics. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 333–341.

Keywords: *Aspergillus nidulans*; aflatoxin; sterigmatocystin; mutagenesis; translocation; echinocandin B

Introduction

In *Aspergillus flavus* and *Aspergillus parasiticus*, ST has been identified as an intermediate in the biosynthetic pathway (Figure 1) for aflatoxin B_1. Both of these mycotoxins exhibit similar biological properties due to their bisfuranoid structure. Aflatoxin B_1 is recognized as an extremely toxic and carcinogenic compound [2,3,11,12,14]. In other species of *Aspergillus*, including certain strains of *A. nidulans*, ST accumulates and there is no evidence of further conversion to aflatoxin B1 [4,6]. The complete biosynthetic gene cluster has been cloned and sequenced and a number of gene functions determined by gene disruption methods ([1], Figure 2). *A. nidulans* A42355 produces echinocandin B (ECB), a lipopeptide, which may be chemically modified to produce derivatives that are potential therapeutic agents for treating fungal infections in man [7]. Production of ECB by *A. nidulans* A42355 has been improved considerably by selection of mutants and optimization of fermentation parameters. However, the improved productivity was accompanied by increased ST biosynthesis. For this reason, a program was initiated to isolate mutants incapable of ST production, while retaining high ECB productivity. Classical strain improvement methods were used to produce mutants blocked in the ST biosynthetic pathway. These mutants were isolated by first reducing the chromosomal copy number of the polyploid production strain C747.0 followed by random mutagenesis. One such mutant was given the designation OC-1 [5]. Although this mutant represents an improvement from the ST-producing strain, regulatory and manufacturing requirements for both the production of pharmaceuticals from this strain and disposal of waste streams from fermentations require greater understanding of the nature of the mutation. Characterization of this strain was critical to its utility in order to assess the potential for accumulation of other bis-furan-containing intermediates in the ST biosynthetic pathway and the potential for reversion of the mutation or low level expression of the pathway. Here we report the genetic characterization of the mutation in OC-1, which reveals a disruption of the *stcW* gene of the aflatoxin biosynthetic pathway.

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Materials and methods

Strains used

The A42355-OC-1 (OC-1) and A42355-GR14 (GR14) strains are mutants of the original soil isolate of *A. nidulans* (A42355, NRRL11440, ATCC 20600). Strain GR14 was mutagenized by UV light to produce the OC-1 strain. *E. coli* strain DB1317-2119 (I^-, recA983Colon;Tn9-200 (Cm^r), recD1014, hsdR zZjJ 202Colon;Tn10,Tc^r,$Cm25^r$) was used for construction of the OC-1 cosmid library. Strains DH5-alpha (Life Technologies, Gaithersburg, MD) or IVN-F′-alpha (Invitrogen, San Diego, CA) were used for routine cloning work. Strain TAHK79.4 is an *A. nidulans* PW1-derived strain with the *stcW* gene deleted (FGSC-A1050) [8].

Nucleic acid manipulations

Standard molecular techniques were used to manipulate DNA in vitro. [10]. Oligonucleotides were synthesized on an ABI 394 DNA synthesizer (Perkin-Elmer, Foster City, CA) and purified on PDP-10 columns before use. PCR was performed on a Perkin-Elmer 9600 thermal cycler and sequencing was carried out on an ABI 373 DNA sequencer as suggested by the manufacturer.

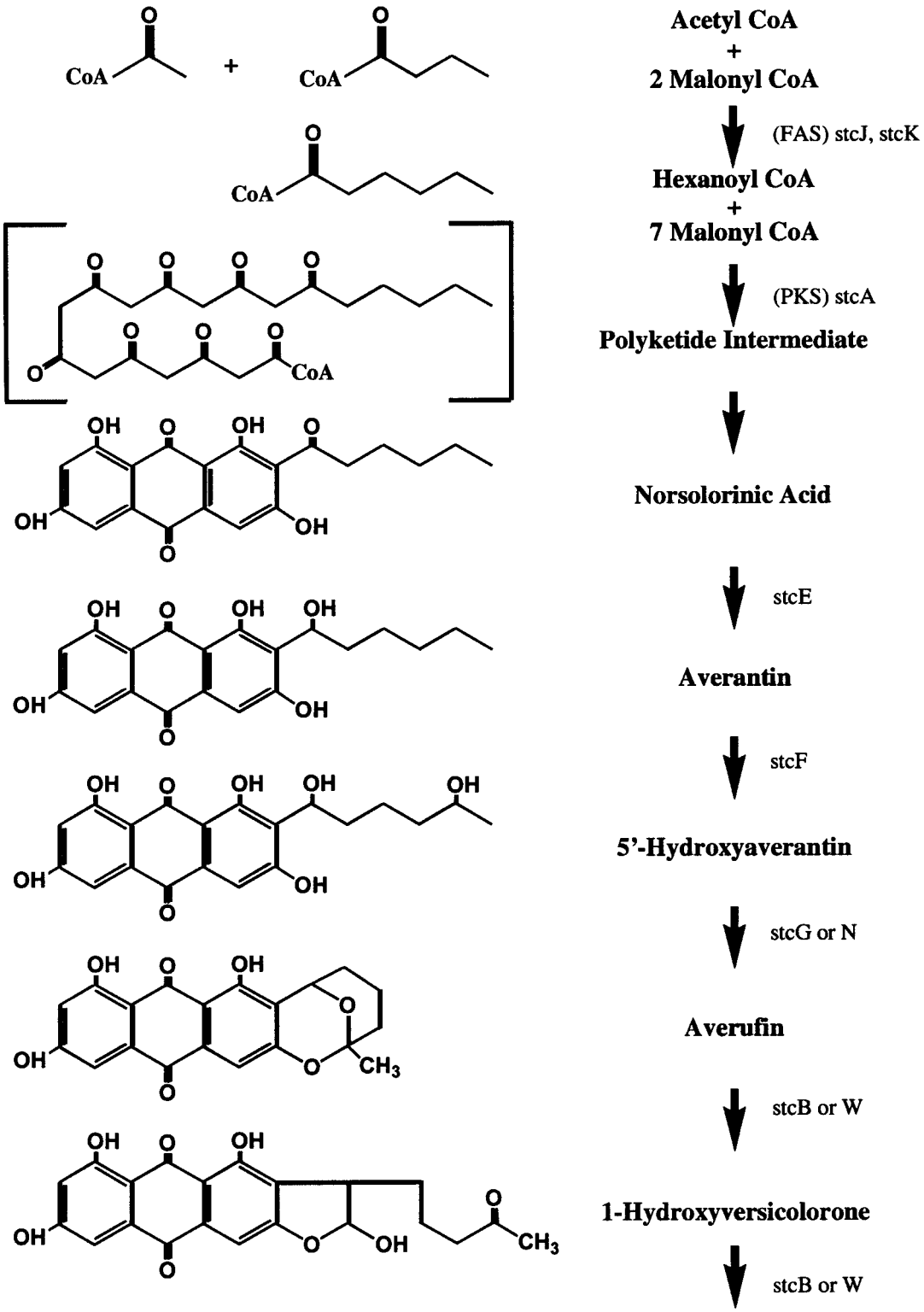

Figure 1a Biosynthetic pathway for sterigmatocystin.

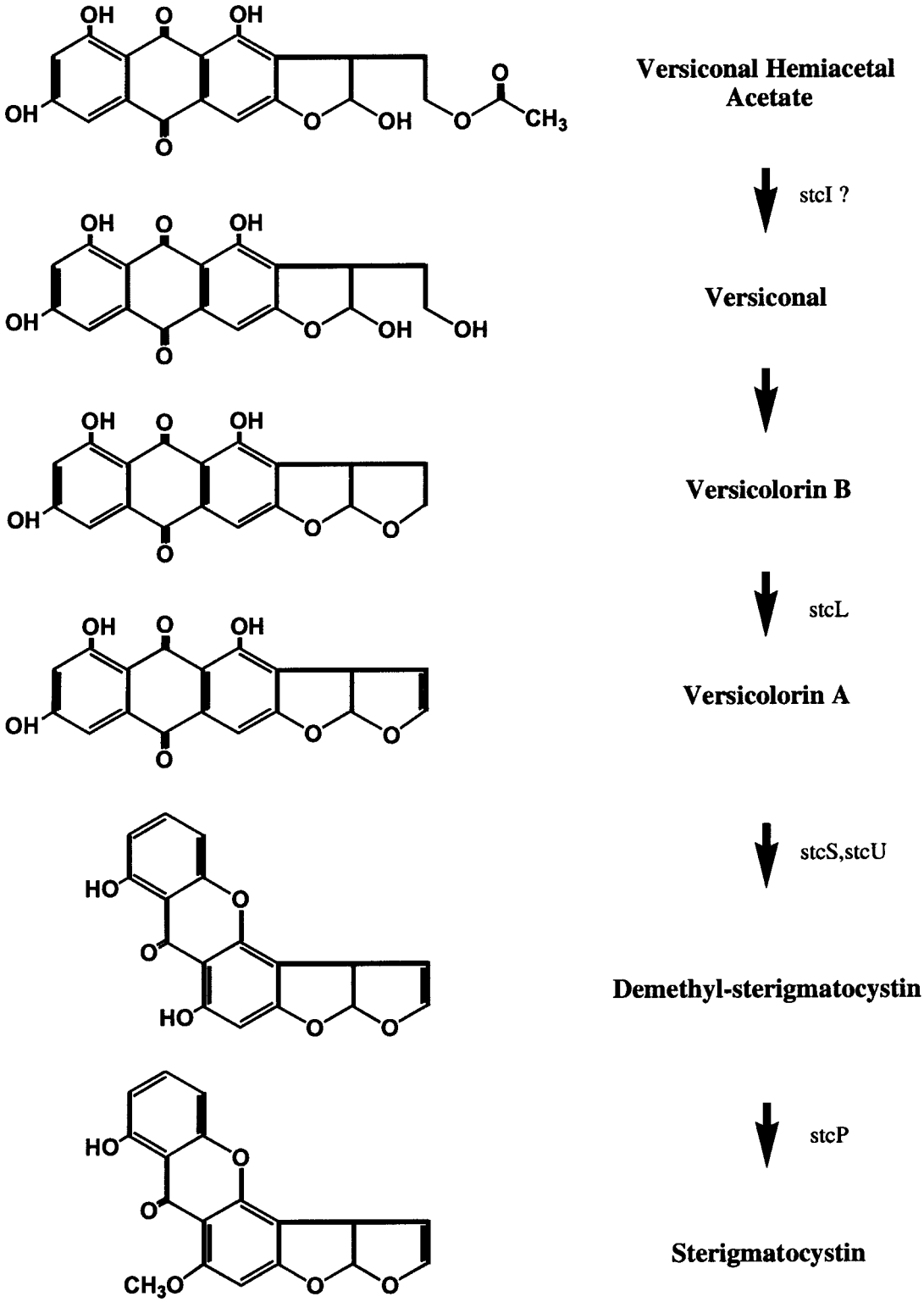

Figure 1b (continued).

Fungal manipulations

Fungal DNA isolation and transformations were performed essentially as described elsewhere [9,13]. Biochemical precursor feeding studies were performed by addition of 2 mg of each precursor to a 50-ml static culture of strain OC-1 or TAHK79.4 in a 250-ml flask. Additions were performed after 2 days growth at 37°C and cultures were allowed to grow an additional 8 days. The mycelial mat was removed and extracted with 25 ml methanol.

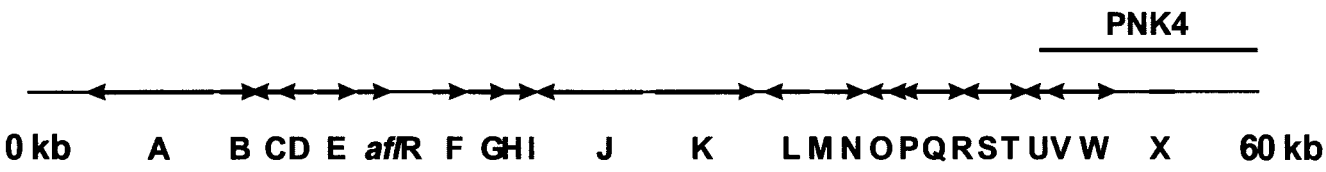

Figure 2 Sterigmatocystin biosynthetic cluster showing region contained in the plasmid PNK4 (reprinted with permission of PNAS, Brown *et al*, 1996 [1]).

Detection of ST

Detection of ST and its precursors and quantitation of ST was accomplished using high performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization (APCI) mass spectrometric detection. A Finnigan triple quadrupole (TSQ-700) mass spectrometer was adapted with a Finnigan APCI source. The APCI source temperature was maintained at 500°C and the capillary was set at 250°while the nitrogen sheath gas was set at approximately 60 psi. Corona discharge was turned on for all analyses. For quantitation of ST, single ion monitoring for the $(M+H)^+$ ion of ST in Q1 was performed. For other experiments, the mass spectrometer (Q1) was scanned from m/z 100–1000 in 2 s while in the centroid mode of operation. The mass spectrometer was calibrated with a solution of MRFA tetrapeptide and 6 pmol/μl apomyoglobin (Sigma) in 50%ACN/5%HOAc in H_2O. The resolution and calibration parameters were set with the instrumental "GUIDE" routine under an approximate resolution of 1000.

The HPLC used for this analysis was a Waters 600-MS gradient system. Two different HPLC assays were utilized: the first for quantitation of ST and the second for attempts to identify production of precursors to ST. The flow rate was 1.0 ml/min for both assays and the analytical column was a Vydac, C18 (0.46×15 cm×5 μm) column. Linear gradients were run between all time points. The effluent from the HPLC column flowed directly into the APCI source with no postcolumn splitting. The HPLC gradient used for detection of ST was made from 50:50, ACN:10 mM NH_4OAc (buffer A) and 50:50 ACN:20 mM NH_4OAc/H_2O (buffer B). The gradient started with 25%A:75%B and went to 100%B by 10 min followed by a transition back to starting conditions at 13 min and ending at 14 min. The HPLC gradients used for detection of ST precursors was made from 10 mM

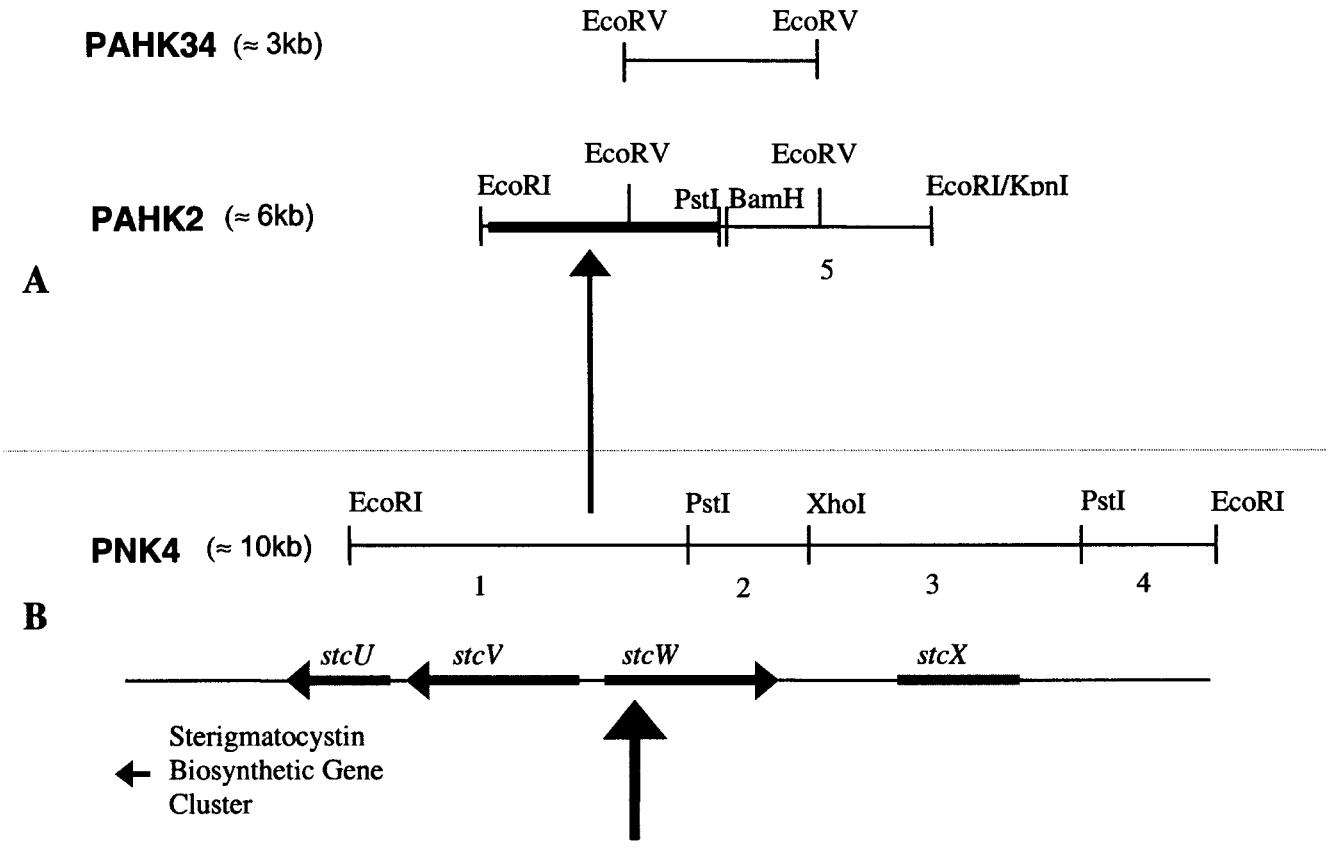

Figure 3 Physical map of hybridizing fragments. (A) DNA related to OC-1. (B) DNA related to FGSC-4. Bold lines indicate fragments that hybridize to PNK4 DNA fragments. Shaded arrows indicate location and direction of transcription of some of the biosynthetic genes. Heavy arrow indicates location of the chromosomal translocation within the *stcW* gene. Approximate insert sizes in parentheses.

```
            1                                                                 60
FGSC-4      GATATCTAAA GGCGATTCAT ATCCGTTTTA GACCATACAG CACACAATAC CCCCCGCA
GR14        .......... .......... .......... .......... .......... ...CCGCA
OC1         GATATCTAAA aGCGATgCAT ATCCGTTcTg GAaCATACAG CACACAAcA. CCCCCGCA

FGSC-4
GR-14

            61                                                                108
FGSC-4      ATG ACT GTG CAC TAT GTT CAC GAA GGC CCA GAG CCT CAA GAG TCA CGG
GR14        ATG ACT GTG CAC TAT GTT CAt GAA GGC gCA GAG CCT CAA ccG TCA CGG
OC1         ATG ACT GTG CAC TAT GTT CAt GAA GGC gCA GAG CCT CAA ccG TCA CGG

FGSC-4      Met Thr Val His Tyr Val His Glu Gly Pro Glu Pro Gln Glu Ser Arg
GR-14       Met Thr Val His Tyr Val His Glu Gly Ala Glu Pro Gln Pro Ser Arg

            109                                                               156
FGSC-4      TAT AGC ATC CCG CAA CAT ACA ACA TGG ATG GAC CCT AAC AAC CGT CGC
GR14        TAT AGC ATC CCG CAg CAT ACA ACA TGG ATG GAt CCT AAC AAC CGT tGt
OC1         TAT AGC ATC CCG CAg CAT ACA ACA TGG ATG GAt CCT AAC AAC CGT tGt

FGSC-4      Tyr Ser Ile Pro Gln His Thr Thr Trp Met Asp Pro Asn Asn Arg Arg
GR-14       Tyr Ser Ile Pro Gln His Thr Thr Trp Met Asp Pro Asn Asn Arg Cys

            157                                                               204
FGSC-4      CTG CGG GTG ATT ACC ATT GGA GCT GGC TTT TCT GGA ATT TTG ATG GCG
GR14        CTG CGG GTG gTT ACC ATc GGA GCT GGC TTT TCc GGA ATc cTG ATG GCa
OC1         CTG CGG GTG gTT ACC ATc GGA GCT GGC TTT TCc GGA ATc cTG ATG GCa

FGSC-4      Leu Arg Val Ile Thr Ile Gly Ala Gly Phe Ser Gly Ile Leu Met Ala
GR-14       Leu Arg Val Val Thr Ile Gly Ala Gly Phe Ser Gly Ile Leu Met Ala

            205                                                               252
FGSC-4      TAT CAA ATC CAG AAG CAA TGC GCA AAT ATC GAA CAT GTG GTG TAC GAG
GR14        TAT CAg ATC CAG AAG CAg TGC GCA AAT ATC GAA CAT GTG GTG TAC GAG
OC1         TAT CAg ATC CAG AAG CAg TGC GCA AAT ATC GAA CAT GTG GTG TAC GAG

FGSC-4      Tyr Gln Ile Gln Lys Gln Cys Ala Asn Ile Glu His Val Val Tyr Glu
GR-14       Tyr Gln Ile Gln Lys Gln Cys Ala Asn Ile Glu His Val Val Tyr Glu

            253                                                               304
FGSC-4      AAG AAT CAT GAT ATT GGA GGT ATGCCTCCT CCCACTCCCA TATGTGGATG ..
GR14        AAG AAT CAT GAT ATT GGA GGT ATGtCTCCT CCCACTCCCA TATGTaGATG gt
OC1         AAG AAT CAT GAT ATT GGA GGT ATGtCTCCT CCCACTCCCA TATGTgtccg at

FGSC-4      Lys Asn His Asp Ile Gly Gly                          ↓
GR-14       Lys Asn His Asp Ile Gly Gly

            305                                                               356
FGSC-4      AGTCTAATTA CGCGCAGGC ACT TGG CTC ACA AAT CGT TAC CCT AAT GCA GGC
GR14        AGTCTAATaA gGCGCAGGC ACT TGG CTC ACA AAT CGT TAC CCT AAT GCA GGC
OC1         tttagcttat ccatttaat ccc tca att ctg cgg ggt aat cct gca gat aga

FGSC-4                          Thr Trp Leu Thr Asn Arg Tyr Pro Asn Ala Gly
GR-14                           Thr Trp Leu Thr Asn Arg Tyr Pro Asn Ala Gly

            357                                                         398
FGSC-4      TGT GAT GTT CCC AGT CAT GCG TAT ACG TAT CGG TTT GCA CTT
GR14        TGT GAT GTT CCC AGT CAT GCA TAT ACG TAT CGG TTT GCA CTT
OC1         cta ttc ttt tct ctg ggc aaa caa gcc aag atc gac gcc atc

FGSC-4      Cys Asp Val Pro Ser His Ala Tyr Thr Tyr Arg Phe Ala Leu
GR-14       Cys Asp Val Pro Ser His Ala Tyr Thr Tyr Arg Phe Ala Leu
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Figure 4a Sequences of the FGSC-4, GR-14, and OC1 *stcW* gene. Bold underlines indicate the location of PCR primers #10 and #H. Double underlines denote intron sequences. Changes in DNA sequence are indicated by using lower case. Boxes indicate changes in amino acids. Heavy arrow indicates the location of the translocation.

NH_4OAc (buffer A) and 90:10 ACN:100 mM NH_4OAc/H_2O (buffer B). The gradient started with 50%A:50%B and went to 100% by 10 min followed by a transition back to starting conditions at 13 min and ending at 14 min.

For quantitation of ST in methanol extracts from the precursor feeding studies, a stock solution at a concentration of approximately 100 μg/ml ST prepared in methanol was used for the standard curve. The ST was obtained from Sigma Chemical and accurately

```
            399                                                        452
FGSC-4      GTTTGTATTC CTGTCTGTTG ATATACATAG AGTCCATCTG ACTTGGCTTG CAAG
GR14        GTTTGTATCC TTGTCTGTTG ATATGCATGG AGTCCATCTG ACTTGACTTG CAAG
OC1         cgccagaaaa tgataagggg agccaggatt atcaacgagt cggacgccaa tttg

FGSC-4
GR-14
```

```
            453                                                        500
FGSC-4      TAC CCC GAT TGG CCT CGT TAC TTC TCC TAC GCG TCG GAT ATC TGG GAG
GR14        TAC CCC GAT TGG CCT CGC TAC TTC TCC TAC GCG TCG GAT ATC TGG GAG
OC1         tag ctg gcc gcc ctc aga ata ggt cta gtc cat ttg tag aga ata tgt

FGSC-4      Tyr Pro Asp Tyr Pro Arg Tyr Phe Ser Tyr Ala Ser Asp Ile Trp Glu
GR-14       Tyr Pro Asp Tyr Pro Arg Tyr Phe Ser Tyr Ala Ser Asp Ile Trp Glu
```

```
            501 505
FGSC-4      TAT CT
GR14        TAT CT
OC1         ata at

FGSC-4      Tyr
GR-14       Tyr
```

Figure 4b (continued).

weighed for preparation of the solution. A 10-fold serial dilution (10 μg/ml) of the 100 μg/ml solution was prepared and used for all LC/MS analyses. Further serial dilutions were prepared in methanol of the 10 μg/ml solution for external calibration. The concentration of the stock solution was also verified spectrophotometrically using published extinction coefficients. In addition to quantitation of ST, full-scan LC/MS data for extracts of OC-1 and GR-14 were compared to determine whether A42355-OC-1 differs from A42355-GR14 in its profile, especially in terms of attempting to decide if a precursor to ST was accumulating in A42355-OC-1.

Results

Cloning of one of the chromosomal translocation breakpoints from OC-1

Previous hybridization experiments demonstrated that the chromosomal rearrangement was located near the *verA* (*stc*U) gene of the pathway because plasmid pNK4 hybridized to both chromosomes involved in the translocation event [5]. The chromosomal breakpoint that contains the 5′, or proximal, region of the *stcW* gene is referred to as the "left" breakpoint while the breakpoint containing the 3′, or distal, region is referred to as the "right" breakpoint. In order to isolate each of the breakpoints, a genomic cosmid library of OC-1 DNA was constructed from a *Sau*3A partial digest of OC-1 genomic DNA. Cosmids suspected of containing the translocation breakpoints were identified by colony hybridizations using an approximately 10-kb *Eco*RI fragment from the plasmid pNK4 as a probe. A cosmid, HK4, was isolated, which hybridized to pNK4. Subclones of HK4 were generated from an *Eco*RI digestion and the isolated plasmids hybridized to the *Eco*RI–*Pst*I fragment (fragment #1, Figure 3) from pNK4. A plasmid, pAHK2, was identified, which hybridized to regions of pNK4 homologous to the region which contained the left end of the breakpoint. Restriction mapping/hybridization studies (data not shown) showed fragment 1 hybridized to pAHK2 up to the *Bam*HI site. Based on this information, a subclone (pAHK34) was isolated from the internal *Eco*RV fragment in pAHK2 and sequenced. Sequence analysis of the two clones pAHK1 and pHK34 revealed that pHK34 did contain the left end of the break. Similar attempts were made to clone the right translocation breakpoint without success.

Characterization of the left end of the translocation

Comparison of the DNA sequence obtained from pAHK34 with the FGSC-4 wild-type *stcW* gene (Genbank Accession Number M29819) reveals a striking similarity of these two genes (Figure 4). A change in only four amino acids between the genes is evident within the first exon of the gene followed by a divergence in the sequences within the first intron. In order to conclusively prove and determine the exact location of the translocation, sequence information on the *stcW* gene in the ST-producing parent strain (GR-14) was necessary. To obtain this, oligonucleotide primers were synthesized based on the FGSC-4 and OC-1 gene sequences, which could be used to clone the region where the discontinuity occurs. PCR amplification of the GR14 strain with two of these primers resulted in amplification of a 449 bp fragment. This fragment was cloned into PCRII (Invitrogen, San Diego, CA) and subsequently sequenced.

The left end chromosomal breakpoint can readily be identified by the complete change in the DNA sequence occurring exactly 237 bp past the putative ATG start site for the gene. Thus, comparison of OC-1 and GR-14 sequences reveals that the breakpoint is located within the first intron for the *stcW* gene and results in disruption of the coding region.

Characterization of the right end of the translocation

Although attempts to clone the right end of the translocation proved unsuccessful, characterization of the right end of the translocation was accomplished using PCR. Primers were synthesized to amplify regions distal to the *stcW* gene. The primer sequences were based on the DNA sequence of the FGSC-4 strain because no sequence data were available for this

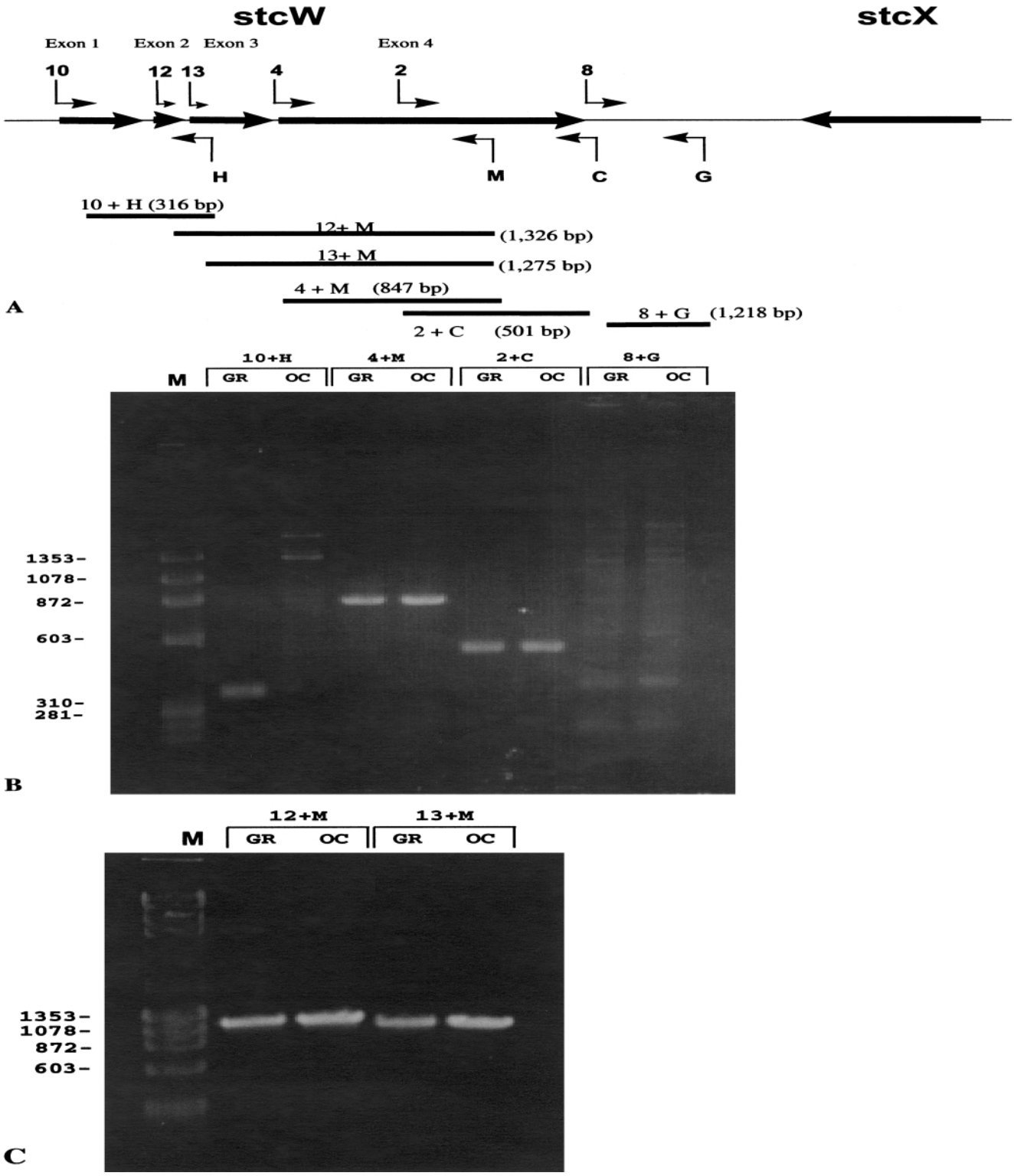

Figure 5 (A) PCR primer design and expected amplified fragments for characterization of the right end of the translocation. (B) Distal PCR analysis. (C) Proximal PCR analysis. M = marker lane, GR = amplification products using total genomic DNA from strain GR-14 as template for PCR, OC = amplification products using total genomic DNA from strain OC-1 as template for PCR.

region from the OC-1 strain. The regions amplified contain portions of the *stcW* gene, which are progressively closer to the region of the left end breakpoint (Figure 5A). Initial PCR amplification demonstrated the 3′ end of the *stcW* gene was present as evidenced by successful amplification of the correct size fragments with the primers 4+M and 2+C with both OC-1 and GR14 template DNA (Figure 5B). Also, failure of primers based on DNA sequence distal to the coding region of the *stcW*

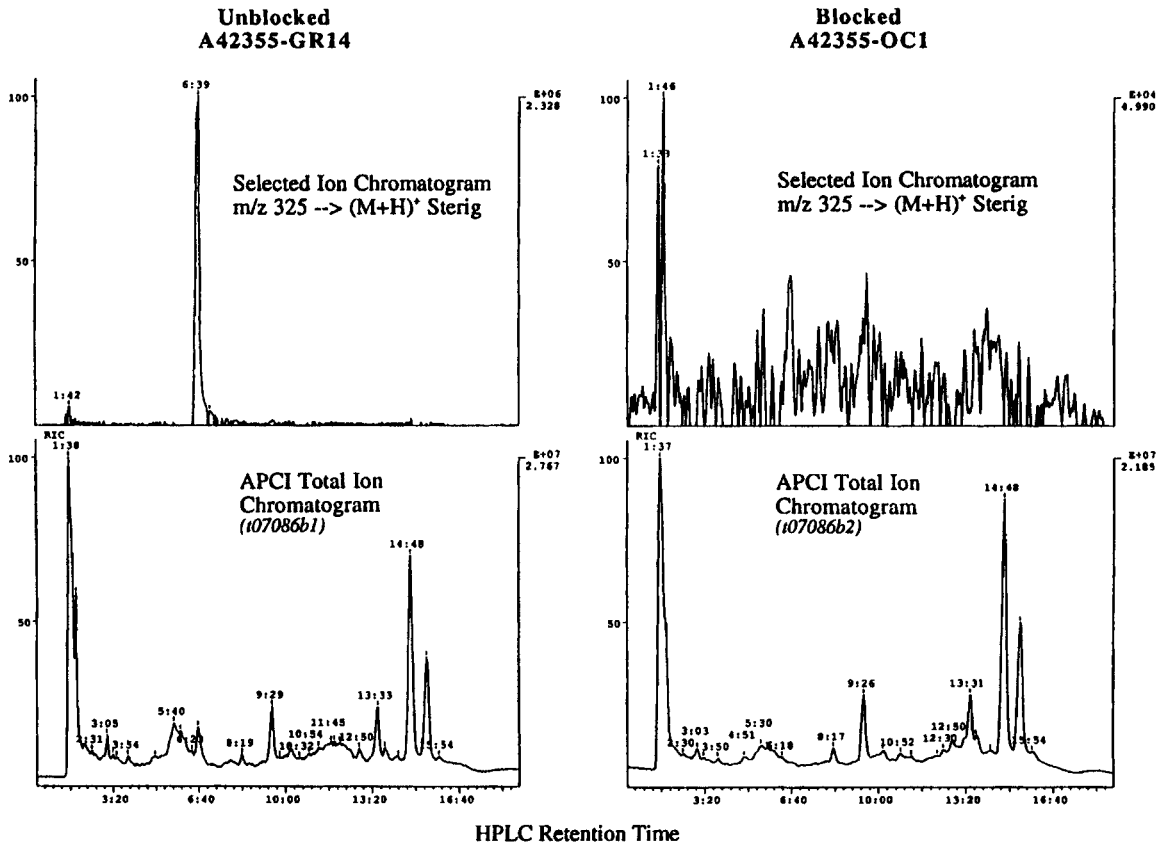

Figure 6 Mass spectral selected ion chromatograms of strains GR-14 (left) and OC-1 (right) with single-ion monitoring for ST (top) or total ion monitoring for precursors (bottom).

gene (8+G) suggests that this region is divergent from the FGSC-4 wild type because these primers produced the expected size fragments with wild-type DNA as the template. To determine the location of the right end breakpoint more specifically, PCR amplification was carried out using primers based on the starting sequences of exon 2 and exon 3. Amplification with these primers (12+M, 13+M) generated the expected size fragment with both of these primers (Figure 5C). The successful amplification using primers based on this DNA sequence indicates that the coding DNA sequence of the *stcW* gene is present in the OC-1 strain; however, it has been translocated to a new region.

However, a small loss of a few base pairs cannot be ruled out. The location of the first base of the amplifying primer (#12) is 16 bp from the known breakpoint of the left end of the gene.

Confirmation of disruption of sterigmatocystin pathway

To illustrate that the sterigmatocystin (ST) pathway had been disrupted, HPLC analysis of GR14 and OC-1 fermentations were studied using APCI/MS to determine the mass (and therefore the identity) of the fermentation products. In addition to qualitative identification of the fermentation products, ST was quantified by single-ion monitoring (SIM). Figure 6 shows the full-scan LC/APCI-MS for methanol extracts of GR14 and OC-1. The top chromatograms are selected ion chromatograms indicating mass spectral response for ST and the bottom chromatograms are the total ion responses.

It is clear in the selected ion chromatograms that ST is not present in A42355-OC-1 (right) but is clearly detected in A42355-GR14 (left). Quantitation of ST production in the two fermentations showed 5.7 mg/ml ST in GR-14 and nondetectable levels in OC-1. The detection limit for quantification of ST in this assay was approximately 1 ng/ml.

In order to determine whether an ST precursor product was present in A42355-OC-1, the expected mass for the protonated precursor molecule was searched in the LC/APCI-MS full-scan data using a wider gradient HPLC assay than used for the quantification work. Quality reference standards for the precursor molecules were not available. For the masses of the precursor molecules shown in Figure 1, there was no indication of higher levels of any specific precursor in A42355-OC-1 relative to A42355-GR14. Special attention was paid to determining if averufin was accumulating, though no indication of this ST precursor was observed.

Precursor feeding studies

To further evaluate the function of the *stcW* gene product, feeding studies were performed with two of the biochemical intermediates in the ST biosynthetic pathway. Versiconal hemiacetal acetate (VAOAc) and versicolorin A (VA) were fed to statically growing cultures of the OC-1 strain and the *stcW*-deleted wild-type strain TAHK79.4. The fermentation products from these feeding studies were again analyzed by LC/APCI-MS for levels of ST production. Both the VAOAc- and VA-fed fermentations showed production

Table 1 Precursor feeding study results

	OC-1		TAHK79.4	
	Trial 1	Trial 2 (ppb)	Trial 1 (ppb)	Trial 2 (ppb)
Control	nd	0	0	0
VAOAc	nd	31	126	32.9
VA	nd	51.3	39	18.9

VAOAc = versiconal hemiacetal acetate, VA = versicolorin A, nd = not done. Detection limits: Trial 1 = 0.6 ppb, Trial 2 = 5 ppb.

of ST relative to a non–precursor-fed OC-1 control fermentation (Table 1). The ability of the OC-1 strain to convert VAOAc and VA to ST proves that the action of the *stcW* gene product occurs before formation of either of these compounds.

Conclusions

The exact location of a chromosomal translocation in *A. nidulans* A42355-OC-1 has been determined. This translocation resulted in disruption of the *stcW* gene involved in ST biosynthesis. The *stcW* gene is thought to function at one of three possible steps in the pathway: the conversion of averantin to 5′-hydroxyaverantin, the conversion of averufin to 1-hydroxy versicolorone, or conversion of 1-hydroxyversicolorone to versiconal hemiacetal acetate. Disruption of any of these three genes should cause accumulation of an intermediate that does not contain a bis-furan ring structure and is therefore not carcinogenic. Additional feeding studies with strain TAHK79.4 have been conducted by other investigators using norsolorinic acid [8]. The resulting accumulation of averufin suggests that the most probable function for the *stcW* gene is the conversion of averufin to 1-hydroxy versicolorone. Final proof that the loss of ST production is due to translocation within the *stcW* gene would require restoration of function by complementation with an intact *stcW* gene. The data presented here, however, provide compelling evidence for an essential function of the *stcW* gene. Additionally, the use of molecular biology methods to characterize a strain derived from classical mutagenesis methods has proven to be a valuable approach for producing a nonrecombinant organism for production of a pharmaceutical with the level of understanding of the mutation necessary to satisfy stringent manufacturing and regulatory requirements. These efforts have resulted in a strain that is incapable of forming bis-furan–containing intermediates and unlikely to revert or produce low levels of ST. As a result it is significantly safer for the production of ECB analogues of pharmaceutical importance.

Acknowledgements

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