J Ind Microbiol Biotechnol (2006) 33: 94–104
DOI 10.1007/s10295-005-0016-9

ORIGINAL PAPER - JMBM

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Butenyl-spinosyns, a natural example of genetic engineering of antibiotic biosynthetic genes

Received: 13 April 2005 / Accepted: 5 July 2005 / Published online: 23 September 2005

Abstract Spinosyns, a novel class of insect active macrolides produced by *Saccharopolyspora spinosa*, are used for insect control in a number of commercial crops. Recently, a new class of spinosyns was discovered from *S. pogona* NRRL 30141. The butenyl-spinosyns, also called pogonins, are very similar to spinosyns, differing in the length of the side chain at C-21 and in the variety of novel minor factors. The butenyl-spinosyn biosynthetic genes (*bus*) were cloned on four cosmids covering a contiguous 110-kb region of the NRRL 30141 chromosome. Their function in butenyl-spinosyn biosynthesis was confirmed by a loss-of-function deletion, and subsequent complementation by cloned genes. The coding sequences of the butenyl-spinosyn biosynthetic genes and the spinosyn biosynthetic genes from *S. spinosa* were highly conserved. In particular, the PKS-coding genes from *S. spinosa* and *S. pogona* have 91–94% nucleic acid identity, with one notable exception. The butenyl-spinosyn gene sequence codes for one additional PKS module, which is responsible for the additional two carbons in the C-21 tail. The DNA sequence of spinosyn genes in this region suggested that the *S. spinosa spnA* gene could have been the result of an in-frame deletion of the *S. pogona busA* gene. Therefore, the butenyl-spinosyn genes represent the putative parental gene structure that was naturally engineered by deletion to create the spinosyn genes.

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Keywords Spinosyn · Macrolide · Polyketide · *Saccharopolyspora pogona*

Introduction

Recently, a new naturally occurring series of insect active compounds was discovered from a novel soil isolate, *Saccharopolyspora pogona* NRRL30141. The culture produced a unique family of over 30 new spinosyns [21]. These butenyl-spinosyns, also called pogonins, have a butenyl substitution at the 21 position on the spinosyn lactone (Fig. 1). The previously known spinosyns produced by *S. spinosa* [20] were substituted with ethyl or methyl at C-21 (Fig. 1).

The spinosyn molecules have a unique tetra-cyclic macrolide base with two reduced sugars, forosamine and tri-*O*-methylrhamnose, which are required for bioactivity [20]. Spinosyns are highly potent natural insect control agents which have been commercialized under the trademark Naturalyte®. Naturalyte® insect control has been used since 1997 for the control of chewing insects on a variety of crops [32]. Spinosyn formulations were recently approved for use on organic crops (Entrust®) and for animal health applications (Elector®).

The biosynthetic genes for spinosyn production include 19 genes encoded on 80 kb of *S. spinosa* genomic DNA [35]. The spinosyn gene cluster included five genes encoding a large PKS, four unique genes involved in cross-bridging of the polyketide lactone and ten genes involved in sugar biosynthesis. Because of the unique tetracyclic structure of spinosyns, the spinosyn genes have recently been the subject of a number of investigations into the mechanisms of polyketide biosynthesis [9, 15, 16, 22, 23, 29].

In addition to their butenyl tail at C-21, the butenyl-spinosyns have a number of distinct variations from the published spinosyn factors [21]. The unique spinosyns in the butenyl-spinosyn series include nonforosamine sugars at C-17, hydroxylation at C-8 and C-24, and a tridecenolactone spinosyn (14-membered lactone).

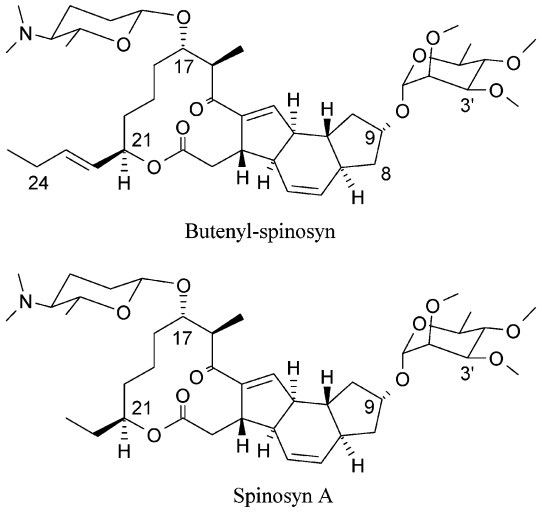

Fig. 1 Structures of spinosyn A and butenyl-spinosyn

Therefore, we expected that the biosynthetic genes could reveal some interesting variations from spinosyn biosynthesis. We report here the cloning and sequencing of the genes for biosynthesis of butenyl-spinosyns. The biosynthetic origin of the butenyl-spinosyn butenyl tail suggests an example of natural genetic engineering by homologous recombination.

Materials and methods

Microbial strains and growth conditions

Escherichia coli DH5α-MRC$^+$ (Gibco BRL, Gaithersburg, MD, USA) used for DNA cloning was grown on LB agar (BD, Franklin Lakes, NJ, USA) and Terrific Broth [2] + 0.4% v/v glycerol (TB). When used, apramycin (am, Sigma Chemical Co., St. Louis, MO, USA) was added to LB and TB at 100 mg/l. *E. coli* ATCC 47055 was obtained from ATCC (Manassas, VA, USA). *S. pogona* NRRL 30141 is a novel soil isolate [21], *S. pogona* NRRL 30421 was derived from *S. pogona* NRRL 30141 through mutagenesis [17]. For genomic DNA isolation, *S. pogona* NRRL 30141 was grown in INV-2 media (9.0 g/L dextrose, 30 g/L trypticase soy broth, 3.0 g/L yeast extract, 2.0 g/L magnesium sulfate. 7 H_2O), and for fermentation, *S. pogona* or derivative cultures were grown, extracted and analyzed by LC/MS according to Hahn et al. [17].

Molecular methods

Unless specifically listed, standard protocols for DNA manipulations were used [3]. Chromosomal DNA was isolated using a Genomic DNA purification kit (Qiagen Inc., Valencia, CA, USA) and cosmid DNA was isolated using the NucleoSpin Nucleic Acid Purification Kit (CLONTECH Laboratories, Inc., Palo Alto, CA, USA). *S. spinosa* or *S. pogona* DNA probes were PCR amplified using AmpliTaq DNA Polymerase Kit (Perkin Elmer/Roche, Branchburg, NJ, USA) in a 48-sample DNA Thermal Cycler (Perkin Elmer Cetus) under the following cycle conditions: (1) 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min; 25 cycles and (2) 72 °C, 10 min; 1 cycle. PCR products were gel-extracted utilizing Qiagen II Gel Extraction Kit (Qiagen Inc.). DNA probes were random-prime labeled with 50 μCi [α^{32}P]dCTP, 3,000 Ci/mMol using 4 µl High Prime reaction mixture (Boehringer Mannheim, Mannheim, GDR). Separation of unincorporated nucleotides from radiolabeled DNA probes was performed using NucTrap Push Columns (Stratagene, LaJolla, CA, USA). Approximately 2.0×10^7 cpm were added to membranes for all DNA hybridizations. Hybridization conditions for all probes were for 16 h in a 65 °C shaking water bath. Hybridization solutions containing radiolabeled probes *spnF*, *spnS*, and *spnE* (TE) were washed under medium stringency conditions: (1) Fifteen minutes room temperature in 300 ml 3× SSC/0.5% SDS, (2) 30 min, 65 °C shaking in 300 ml fresh 3× SSC/0.5% SDS, (3) 30 min, room temperature in 300 ml 1× SSC/0.5% SDS. Membranes screened with the radiolabeled probe derived from *S. pogona* cosmid 9D3 sequence were washed under stringent conditions: (1) 30 min, 65 °C shaking in 300 ml fresh 1× SSC/0.5% SDS, (2) 30 min, 65 °C shaking in 300 ml fresh 0.33× SSC/0.5% SDS, (3) 30 min, 65 °C shaking in 300 ml fresh 0.1× SSC/0.5% SDS.

Construction of *S. pogona* cosmid libraries

Total cellular DNA isolated from *S. pogona* was partially digested with *Sau*3AI and cloned into the *Bam*HI site of cosmid pOJ436 [5]. Insert size of the constructed cosmid clones ranged from 20 to 40 kb. Cosmid clones were packaged in vitro using Gigapack III Gold Packaging Extract (Stratagene) and *E. coli* transductants were spotted in duplicate onto Hybond N+ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) nucleic acid binding membranes. Membranes were supported on LB agar plates + am and incubated overnight at 37 °C. Membranes were processed according to the manufacturers' protocols and DNA was cross-linked to the membrane with 1,200 μJ using an UV Stratalinker 1800 (Stratagene).

Screening of *S. pogona* library and identification of cosmids containing butenyl-spinosyn biosynthetic genes

The 16S rRNA genes of *S. spinosa* and *S. pogona* have 98% DNA sequence identity (D.R. Hahn et al., manuscript in preparation). Because of this phylogenetic similarity between the producing strains and the high structural similarity between the two families of spinosyns, it was expected that the butenyl-spinosyn gene

cluster would be highly similar to the spinosyn gene cluster. Therefore, three DNA probes based upon unique spinosyn biosynthetic genes (*spn*) or domains from *S. spinosa* [35] were synthesized. The three probes were spaced to maximize the chance of cloning the entire butenyl-spinosyn biosynthetic gene cluster (Fig. 2): (1) the *spnS* gene probe (forward primer 5′-GTGCCGAATACGCGAAGGTC-3′; reverse primer 5′-TCCAGGAAGGTATTCCGCGC-3′) was at the left end of the spinosyn biosynthetic cluster, (2) the *spnE* thioesterase domain (TE) was at the right end (forward primer 5′-TCCCGATGCCTGGATTCATTG-3′; reverse primer 5′- CGTCCATCATCGAGAAGTGGTC-3′) and (3) the *spnF* gene was in the central portion of the *spn* genes (forward primer 5′-GCGACAACGCGATCCAGATC-3′; reverse primer 5′-CCATGTCGTGGGCATATTTCTC-3′). Eight cosmid clones were identified as positively hybridizing to *S. spinosa* probes *spnS*, *spnF* or *spnE* (TE) (Fig. 2).

The eight cosmid clones were further characterized by restriction digestion and the end of the insert of each cosmid was sequenced so that putative genes could be surmised from comparison to the *spn* genes. Cosmids, 8H3, 9D3, and 10C1 covered the maximal amount of the butenyl-spinosyn gene cluster (designated *bus* for butenyl-spinosyn) and the NRRL 30141 chromosome (Fig. 2). The insert in cosmid 8H3 was 40.3 kb and hybridization indicated homology to both the *spnS* and *spnF* genes. The right end sequence was in a ketosynthase (KS) domain which was similar to several *spn* KS domains. The left end sequence had no similarity to any known gene of *S. spinosa* indicating that this cosmid extended beyond the homology to the *spnS* gene. Cosmid 9D3 which hybridized to the *spnF* probe had a 31.7-kb insert. The left end sequence was homologous to the *spnG* gene and the right end was highly similar to the KS domain of module 5 in the *spnD* PKS gene. The insert in cosmid 10C1 was 40.6 kb and hybridized only to the *spnE* (TE) probe. The left end sequence of cosmid 10C1 was homologous to the KS6 domain of the *spnD* PKS gene and the right end had no *S. spinosa* homology. From this gene homology/domain order (*spnS, spnG, spnF*, KS?, KS5, KS6, TE) it appeared that the butenyl-spinosyn genes were collinear with the spinosyn genes (Fig. 2), although the 32-kb distance between the *spnG* gene and the KS5 domain (cosmid 9D3) was approximately 5 kb longer than in the *spn* PKS genes. It was also apparent that approximately 5 kb of the butenyl-spinosyn biosynthetic genes had not been cloned (between cosmids 9D3 and 10C1).

In order to identify a clone spanning the region between cosmids 9D3 and 10C1, one additional probe based on the end sequence of cosmid 9D3 was synthesized (forward primer 5′-CGTACGTGGCGATCAG-3′; reverse primer 5′-GTCCAAGTTTCGGTTGCGTTC-3′). Using high-stringency hybridization conditions, three additional cosmids were identified from the genomic library (Fig. 2). Cosmid 9F4 had a 36.9-kb insert and the right end sequence was homologous to the KS-AT domains of module 9 in the *spnE* gene. The left end had homology to the ER domain of module 2 of the *spnB* gene. The left end sequence also had some DNA bound by a *Sau*3AI site which was not similar to the *spn* genes. It is assumed that the 9F4 cosmid had a small second insert of noncontiguous *S. pogona* DNA.

DNA sequencing

Nucleotide sequence from the cosmid/vector junctions was obtained by fluorescent cycle sequencing according to the methods of Burgett and Rosteck [8] under thermal

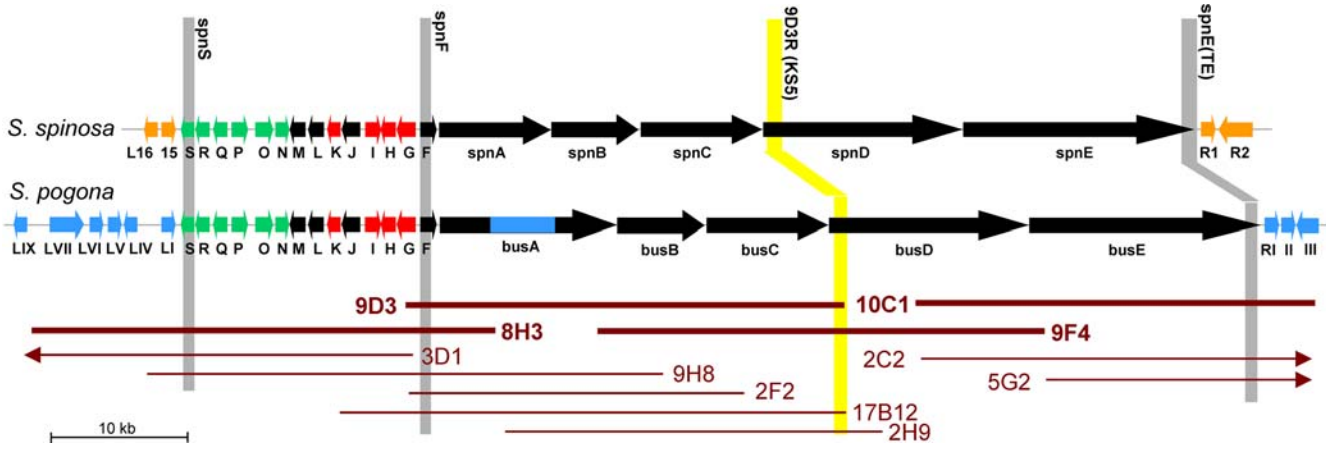

Fig. 2 A graphical comparison of the spinosyn biosynthetic genes from *S. spinosa* (*top*) and the butenyl-spinosyn biosynthetic genes from *S. pogona* (*bottom*). *Colors* of the genes involved in spinosyn or butenyl-spinosyn biosynthesis correspond to their function (polyketide synthase and aglycone bridging are shown in *black*; rhamnose biosynthesis are shown in *red*; forosamine biosynthesis are shown in *green*). Genes from *S. spinosa* which are not involved in spinosyn biosynthesis are shown in *orange*. Non-*bus* genes and sequences unique to *S. pogona* are shown in *blue*. Approximate extents of cosmid clones are indicated at the *bottom of the figure*. *Vertical bars* indicate the location of primers used for cloning: the three primers based on *spn* genes are designated in *gray*; the one primer based on *bus* genes is designated in *yellow*. Cosmids which were sequenced are shown in *bold* and cosmids which extended beyond the sequenced region are indicated as *arrows*

cycler conditions: 96 °C, 30 s; 50 °C, 15 s; 60 °C, 4 min; 25 cycles with a 377 ABI Prism Sequencer (Applied Biosystems, Inc., Foster City, CA, USA). The complete sequences of cosmids 8H3, 9D3, 9F4, and 10C1 were determined at SeqWright, Inc. (Houston, TX, USA). The cosmid clones represented over 110 kb of *S. pogona* genomic DNA. For ease of analysis, the sequence was divided into two segments [18]: Seq. ID no. 1 (GenBank accession number: AX600586) included the start codon of *busA* (+1 in Fig. 2) and all DNA to the 3′ of that, which included the five PKS genes. Seq. ID no. 2 (GenBank accession number: DQ087286) began at the base before the *busA* start codon and included all DNA to the 5′ side of that base.

Transformation of *S. pogona*

Cosmid 8H3 (Fig. 2) and plasmids derived from pOJ260 [5] were transferred from *E. coli* ATCC 47055 into *S. pogona* NRRL 30141 or NRRL 30421 by conjugal transfer [25].

Gene disruption of *busO* in *S. pogona*

A pair of oligonucleotides (*busO*a, 5′-TAGAAGGCCTGCAGGTCGAGAC-3′; and *busO*b 5′-TAGTTGGCCACACTGCACTGGACC-3′) were used to amplify a 912-bp region internal to the 1,457-bp *busO* gene using FailSafe PCR (Epicenter, Madison, WI, USA) and cloned into pCRII (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was digested with *Eco*RI and the *busO* fragment was cloned into the *Eco*RI site of pOJ260 [25]. The resultant plasmid was conjugated from *E. coli* ATCC 47055 into a derivative of *S. pogona* by conjugal transfer [25]. Six independent amR exconjugants were fermented and analyzed for production of butenyl-spinosyn and derivatives.

Table 1 Sequence of KS & KS^{Q} Domains in the *BusA* & *SpnA* polyketide synthases

BusA KS^{Q}	163*	GPSLTVDSG**Q**SSSLVG	178*
SpnA KS^{Q}	163*	GPSLTVDSG**Q**SSSLVG	178*
KS^{Q} Consensus†		GPS--VDSG**Q**SSSLVA	
BusA KS1b	1152*	GPAVTVDTA**C**SSSLVA	1168*
BusA KS1a	2899*	GPAVTVDTA**C**SSSLVA	2914*
SpnA KS1	1152*	GPAVTVDTA**C**SSSLVA	1168*
KS Consensus†		GPA-TVDTA**C**SSSLVA	

* amino acid in the translated sequence of the BusA or SpnA protein beginning at the N-terminous. KS active site cysteine (C) or glutamine (Q) are shown in bold. Amino acids conserved in all listed KS domains are highlighted in yellow, residues conserved in extender KS and KS^{Q} domains are highlighted in green and blue, respectively

† consensus sequences were derived from KS^{Q} & KS active sites listed by Brautaset, *et al.* [6].

Results and discussion

The DNA sequence of the butenyl-spinosyn biosynthetic genes

The polyketide synthase (PKS) genes

The *S. pogona* DNA sequence AX600586 included a region of about 60 kb with striking homology to the DNA encoding the polyketide synthases of known macrolide producers [11, 14, 26]. The butenyl-spinosyn PKS DNA region consisted of five large open reading frames (ORFs) with in-frame stop codons at the end of ACP domains, similar to the PKS ORFs in the other macrolide-producing bacteria. The five butenyl-spinosyn PKS genes were arranged head-to-tail (see Fig. 2), without any intervening non-PKS functions such as the insertion element found between the erythromycin PKS genes AI and AII [12]. The PKS genes are designated *busA*, *busB*, *busC*, *busD*, and *busE*.

The boundaries and functions of the 12 modules and 55 domains identified in the *bus* PKS genes were predicted based on similarities to the conserved amino acid sequences of the domains in other polyketide synthases, particularly the erythromycin polyketide synthase [13]. Each of the five *bus* PKS genes encodes one or more PKS modules (Fig. 3a). Each module has all the functional domains required for addition of a single two carbon ketide unit to the polyketide chain. It would require 12 ketide units to construct the putative butenyl-spinosyn polyketide precursor (Fig. 3b, c). The modules and domains of the *bus* PKS are encoded in the same order in which they would be used in the biosynthesis of the polyketide (Fig. 3).

Like the *spn* PKS, the *bus* PKS has a KSQ domain at the amino terminus of the loading module. It is expected that this KSQ domain cannot function as a *β*-ketosynthase because it contains a glutamine residue at amino acid 172 (Table 1), in place of the cysteine required for *β*-ketosynthase activity [31]. It has been reported that KSQ domains function to decarboxylate malonyl-ACP and are chain initiation factors [7]. None of the other butenyl-spinosyn PKS domains contains the sequence characteristics of the inactive domains found in the erythromycin and rapamycin PKS genes [1, 14].

Although *busB-E* are comparable in size and highly homologous to *spnB-E* (Table 2) , *busA* is significantly larger (by 5,244 bp) than *spnA*. The first 4,245 bp (module L) and the last 3,486 bp (module 1a) of *busA* have many similarities to *spnA*. These similarities are readily picked up in a BLAST search using the *busA* gene in which *spnA* is detected as the sequence in GenBank most similar to *busA*. However, bases 4,246–9,548 (module 1b) do not have direct counterparts in the *spnA* gene. The similarity between the module 1b domains and the *spn* genes is comparable to the similarity of module 1b to the domains of other PKS domains such as erythromycin. This 5-kb region codes for an additional

Table 2 Similarity of *bus* PKS and *spn* PKS genes

Butenyl-spinosyn gene	*bus* ORF length bp (aa)	Bus functional domain[a]	Best match in A83543 spinosyn PKS	*spn* ORF length bp (aa)	Spn functional domain[a]	ORF percentage of identity (DNA) (%)	ORF percentage of identity (aa) (%)
BusA	13,032 (4,344)		*spnA*	7,788 (2,595)			
1–4,245	4,245 (1,415)	KSQ-KS1b	21,111–25,214	4,245 (1,415)	KSQ-KS1	92	91.2
4,246–6,548	5,301 (1,767)	AT1b-KS1a	None[b]	NA			
9,549–13,032	3,486 (1,162)	AT1a-ACP1a	26,407–28,896	3,486 (1,162)	AT1-ACP1	91	87.6
BusB	6,450 (2,149)	KS2-ACP2	*spnB*	6,459 (2,152)	KS2-ACP2	93	93.1
BusC	9,546 (3,167)	KS3-ACP4	*spnC*	9,513 (3,170)	KS3-ACP4	94	93.5
BusD	14,805 (4,935)	KS5-ACP7	*spnD*	14,787 (4,928)	KS5-ACP7	94	93.6
BusE	16,692 (5,564)	KS8-ACP10	*spnE*	16,767 (5,588)	KS8-ACP10	94	90.6

bp base pairs, *aa* amino acids
[a]functional domain names correspond to Fig. 3
[b]Similarity to *S. spinosa* PKS genes was in the same range as similarity to other like domains of the *bus* & *spn* PKS genes

module with five functional domains: KS1b, AT1b, DH1b, KR1b, and ACP1b. These functions together with the preceding initiation domain appeared to be responsible for the biosynthesis of the butenyl side chain, characteristic of butenyl-spinosyns relative to spinosyns.

The PKS proteins, which perform similar reactions in the biosynthesis of spinosyns, share 87–93% amino acid identity and the genes range from 93–94% DNA sequence identity (Table 2). It should be noted that the *spn* PKS enzymes SpnB-E and the similar *bus* PKS enzymes BusB-E must maintain distinct substrate specificity because, although the reactions performed by the enzymes are identical, each of the substrate polyketides are slightly different. Formation of the lactone intermediate from the butenyl-spinosyn polyketide is catalyzed by the thioesterase in module 10 of BusE. This cyclization takes place between the hydroxyl at C-21 and the terminal hydroxyl attached to the ACP of module 10 (Fig. 4a). Action of the thioesterase (TE) on the projected butenyl-spinosyn polyketide would yield the tetracyclic butenyl-spinosyn aglycone which has a 12-membered lactone (Fig. 4a). One minor factor in butenyl-spinosyn fermentation the tridecenolactone spinosyn A (TDL) has the 5-6-5 ring system characteristic of the spinosyns, but a 14-membered lactone [21]. The butenyl-spinosyn polyketide required to form the lactone precursor to TDL spinosyn would require a hydroxyl at C-23. This polyketide precursor could be formed by an allelic rearrangement of the C-21 hydroxyl with the double bond at C-22/C-23; the hydroxyl would be displaced to C-23 and the double bond to C-21/C-22 (Fig. 4b). It is likely that this allelic rearrangement would be favored in vitro under acidic conditions (P. Graupner, personal communication); however, it is unclear how this is accomplished in vivo.

Genes adjacent to the PKS responsible for additional modifications

In the DNA upstream of the PKS genes (GenBank accession number DQ087286) there were 20 ORFs, with the features of genes: each consists of at least 100 codons, beginning with ATG or GTG and ending with TAA, TAG or TGA, and each has the codon bias expected of protein-coding regions in an organism whose DNA contains a high percentage of guanine and cytosine residues [4]. These 20 ORFs are represented graphically in Fig. 2. The ORFs were compared directly to the sequence of the spinosyn biosynthetic genes from *S. spinosa* (Genbank accession number AY007564). The high degree of similarity in both the DNA and protein sequence was a strong indication that the genes performed similar functions in biosynthesis of spinosyns. Therefore, 14 of the ORFs have been designated as butenyl-spinosyn biosynthetic genes, namely: *busF*, *busG*, *busH*, *busI*, *busJ*, *busK*, *busL*, *busM*, *busN*, *busO*, *busP*, *busQ*, *busR*, and *busS* (labeled F through S in Fig. 2). The letter designation of these *bus* genes was made to correspond to their *spn* gene counterparts (Table 3). Genes, *busG*, *busH*, and *busI* were highly similar to *spn* genes involved in tri-methyl rhamnose biosynthesis [16, 35]. Likewise genes *busN*, *busO*, *busP*, *busQ*, *busR*, and *busS* are putatively involved in forosamine biosynthesis like their *spn* gene counterparts [35, 37]. The remaining four genes, *busK*, *busF*, *busJ*, *busL*, *busM*, are projected as carbon-bridging genes [23, 29, 35]. The *spn* counterparts of these genes have been examined in depth elsewhere [16, 23, 29, 35, 37].

In addition, there were a number of ORFs found immediately downstream of *busS* (in cosmid 8H3) and 3 ORF's downstream of the PKS genes (in cosmid 2C10). To assign functions to the polypeptides identified, the amino acid sequences of the predicted polypeptides were compared to sequences deposited in the databases at the National Center for Biotechnology Information (NCBI, Washington, DC, USA), using the BLASTX algorithm to determine how well they are related to known proteins. After BLAST analysis the significant protein matches presented in Table 4 were selected as the sequence with the highest BLAST score for which there was direct experimental evidence supporting the stated function. In a few cases, no such confirmed sequences were available; those scores are presented in parenthesis (Table 4).

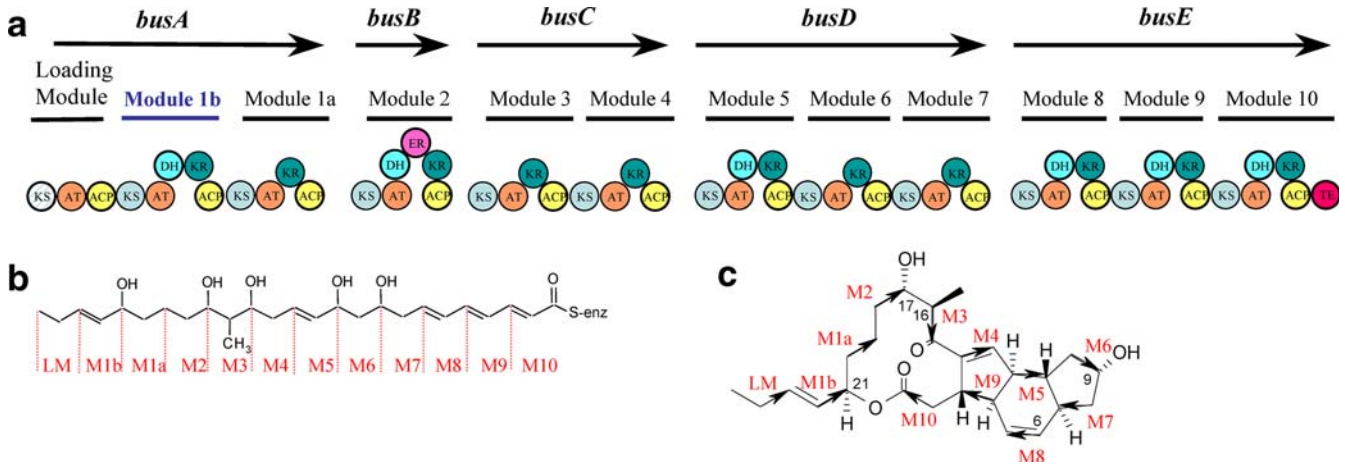

Fig. 3 Model of the butenyl spinosyn polyketide synthase and its polyketide product. *a* Bus polyketide synthase. The extent of the five PKS genes encoding the five proteins of the butenyl-spinosyn PKS is represented by arrows at the top and the extent of each of the 12 PKS modules is indicated by *bars* below. Functional domains are represented by *circles* which are color-coded and labeled by the functions. Abbreviations of domains: *KS* ketosynthase, *AT* acetyltransferase, *ACP* acyl carrier protein, *DH* dehydratase, *KR* ketoreductase, *ER* enoylreductase, *TE* thioesterase. The first KS is distinctive in that it is the KS_Q loading domain for the PKS. *b* The putative polyketide predicted from the *bus* PKS is shown. The carbons added and reduction due to each module is indicated (*M2* module 2, etc.). *c* The butenyl-spinosyn aglycone. Carbons resulting from each module are indicated with *arrows*. Important carbons where modification or variations occur are numbered

Complementation of a butenyl-spinosyn *O*-methylation mutation by Cosmid 8H3

In an experiment to test whether the genes cloned were indeed responsible for butenyl-spinosyn biosynthesis, cosmid 8H3 was transformed into a previously isolated butenyl-spinosyn biosynthetic mutant (NRRL 30421) with altered rhamnose methylation. If the cloned genes were able to complement the mutation, then the genes were highly likely to be involved in butenyl-spinosyn production. *S. pogona* strain NRRL 30421 is a mutant of *S. pogona* NRRL 30141 which was unable to fully methylate the rhamnose on butenyl-spinosyns [17]. Strain NRRL 30421 accumulated 3′-*O*-des-methylrhamnosyl butenyl-spinosyn (3′-ODM; Fig. 5) and several additional butenyl-spinosyn factors which lack *O*-methylation at the 3′ position of the rhamnose [17]. This methylation defect is presumed to be the result of a mutation in one of the *O*-methyltransferases encoded by the *busH*, *busI* or *busK* genes. All three of these genes were cloned on cosmid 8H3 (Fig. 2).

Cosmid 8H3 was transferred from *E. coli* ATCC 47055 into strain NRRL 30421 by conjugal transfer. Although this cosmid has a ϕC31 *att* site, cosmids transferred into *S. spinosa* by this method are preferentially integrated into the chromosome by homologous recombination [25]. Therefore, the *S. pogona* transformants are likely to have a duplication of the cloned

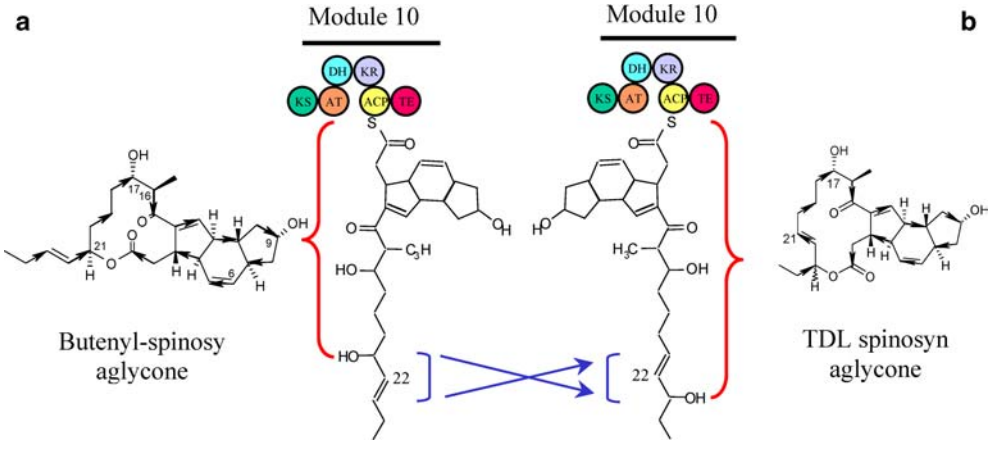

Fig. 4 Cyclization of the Butenyl-Spinosyn Polyketide. (**a**) The putative polyketide product of module 10 is shown covalently attached to the ACP10 cysteine. The residues involved in cyclization to form the 12-membered lactone are indicated by the *red bracket*. (**b**) The polyketide required for TDL spinosyn formation is shown at *right* with the residues required for 14-membered lactone formation indicated by the *red bracket*. The postulated allelic rearrangement around C-22 is indicated in *blue*

Table 3 DNA similarity of *bus* and *spn* biosynthetic genes

Pogonin gene	Bus ORF length bp (a.a.)	Spinosyn gene	Spn ORF length bp (a.a.)	BLAST score	ORF percentage of identity (DNA) (%)	ORF percentage of identity (aa) (%)	Function reported in GenBank
busF	828 (275)	*spnF*	828 (275)	1,247	94	91	C-methylation
busG	1,173 (390)	*spnG*	1,173 (390)	1,844	95	90	Rhamnose glycosyltransferase
busH	753 (250)	*spnH*	753 (250)	1,328	97	97	Rhamnose methylation
busI	1,188 (395)	*spnI*	1,188 (395)	1,966	96	92	Rhamnose methylation
busJ	1,620 (539)	*spnJ*	1,620 (539)	2,587	95	83	Oxido-reduction
busK	1,194 (397)	*spnK*	1,194 (397)	2,163	96	88	Rhamnose methylation
busL	852 (283)	*spnL*	852 (283)	2,274	94	94	C-methylation
busM	933 (310)	*spnM*	963 (320)	1,909	95	96	C-bridging
busN	999 (332)	*spnN*	999 (332)	1,772	96	91	Forosamine synthesis
busO	1,461 (486)	*spnO*	1,461 (486)	2,319	95	92	Forosamine synthesis
busP	1,314 (437)	*spnP*	1,368 (455)	2,004	94	89	Forosamine glycosyltransferase
busQ	1,344 (447)	*spnQ*	1,389 (462)	2,355	94	81	Forosamine synthesis
busR	1,137 (378)	*spnR*	1,158 (385)	1,852	95	89	Sugar transamination
busS	750 (249)	*spnS*	750 (249)	1,255	96	93	Aminosugar methylation

Table 4 Putative functions of open reading frames linked to the *bus* genes

Gene	Significant protein match	GenBank accession	BLAST Score[a]	Reported function
ORF LI	*ngt N*-glycosyltransferase (*Saccharothrix aerocologenies*)	AB023593	221	Glycosyltransfer
ORF LIV	*urdR* hexose-4-ketoreductase (*Streptomyces fradiae*)	AF080235	243	Hexose ketoreduction
ORF LVI	*fkbM*, FK506 O-methyltransferase	U65940	100	Methyltransfer
ORF LVII	*oleP*, P450 monooxygenase (*Streptomyces antibioticus*)	L37200	387	Monooxygenase
ORF LVIII	Transposase (*Mycobacterium avium*)	AF107207	(180)	Transposition
ORF LIX	*mmcR* (*Streptomyces lavendulae*)	AF127374	124	Methyl transfer
ORF RI	resolvase-like protein (*Acidithiobacillus ferrooxidans*)	U73041	(97)	Transposition
ORF RII	hypothetical protein *yvmC* (*Bacillus subtillus*)	AF017113	(120)	Unknown
ORF RIII	alcohol dehydrogenase [*Streptomyces coelicolor* A3(2)]	AL133236	(155)	Alcohol dehydrogenase

[a]Greater similarity is associated with higher BLAST scores (Altschul et al. 1990)

segment in cosmid 8H3, separated by the plasmid. Two independent isolates transformed with cosmid 8H3 were fermented and analyzed for production of butenyl-spinosyn and 3′-ODM.

While NRRL 30421 produced predominantly 3'-ODM, strains of NRRL 30421 containing cosmid 8H3 produced mostly butenyl-spinosyn (Table 5). The production of butenyl-spinosyn and 3′-ODM in NRRL 30421 containing cosmid 8H3 was similar to the production in nonmutant culture NRRL 30141 (Table 5). It has, therefore, been demonstrated that the genes present on cosmid 8H3 were able to complement the methylation defect in strain NRRL 30421 to restore production of fully methylated butenyl-spinosyn.

Accumulation of butenyl-spinosyn precursor and shunt product caused by disruption of *busO*

In a second experiment to test if the genes cloned were indeed responsible for butenyl-spinosyn biosynthesis, the cloned genes were used to construct a knock-out mutation in *S. pogona* NRRL 30141. As in *S. spinosa*, it

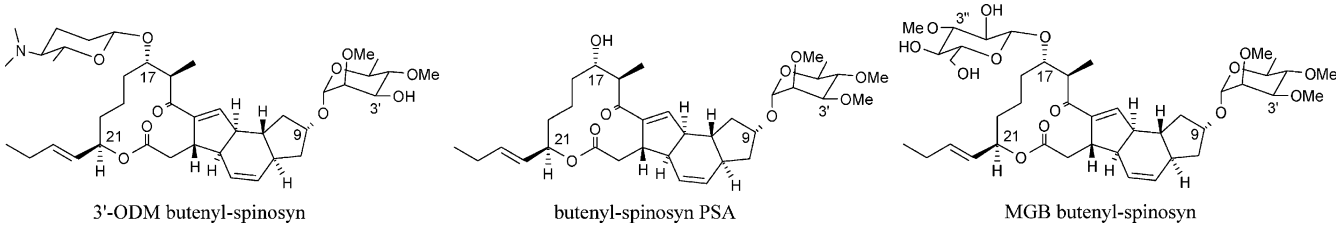

Fig. 5 Structure of butenyl-spinosyns produced by NRRL 30141 and NRRL 30421. 3′-*O*-desmethylrhamnosyl butenyl-spinosyn (3′-ODM) is the primary metabolite of NRRL 30421. The butenyl-spinosyn pseudoaglycone (PSA) and 17-(3″-*O*-methylglucosyl)-butenyl-spinosyn (MGB) are both minor metabolites of NRRL 30141

Table 5 Butenyl-spinosyns produced by *S. pogona* transformants

Strain (genotype)	Pogonin μg/ml	3′-ODM μg/ml	Ratio of compounds[b]
NRRL 30421 (3′-ODM[a])	0.7	1.0	0.7
NRRL 30421 (3′-ODM)/8H3-42	8.9	0.5	17.8
NRRL 30421 (3′-ODM)/8H3-45	3.0	0.1	30.0
NRRL 30141	9.7	0.4	24.3

[a] 3′-ODM = mutation preventing methylation of rhamnose at 3′ position. The numbers 42 and 45 represent different isolates transformed with cosmid 8H3

[b] The ratio of compounds was determined by dividing the concentration of butenyl-spinosy in each fermentation by the concentration of 3′-ODM

Table 6 Butenyl-spinosyns produced by *S. pogona* mutants

Strain (genotype)	Butenyl-spinosyn[a]	PSA[a]	MGB[a]
NRRL 30141	366.3	1.0	0.4
NRRL 30141 *busO65*	ND	13.8	1.7
NRRL 30141 *busO67*	ND	12.3	3.7
NRRL 30141 *busO68*	ND	6.7	3.8
NRRL 30141 *busO70*	ND	9.3	1.3
NRRL 30141 *busO71*	ND	12.3	2.4
NRRL 30141 *busO72*	ND	5.4	1.6

ND not detected

[a]Amounts reported are relative to the concentration of PSA in NRRL 30141

is projected that *S. pogona* requires six genes, *busN*, *busO*, *busP*, *busQ*, *busR*, and *busS* for biosynthesis of forosamine and its addition to the butenyl-spinosyn pseudoaglycone (PSA; Fig. 5). Inactivation of any of these genes would be expected to disrupt formation of forosamine and prevent production of butenyl-spinosyn. The *busO* gene was inactivated by integration of a cloned internal fragment of the *busO* gene which resulted in partial duplication of the *busO* gene, to yield two truncated copies of the gene flanking the plasmid and antibiotic resistant gene.

The parental strain, *S. pogona* NRRL 30141 produced high levels of butenyl-spinosyn and low levels of 17-hydroxy buthenyl-spinosyn (PSA; Table 6) and 17-(3″-*O*-methylglucosyl)-butenyl-spinosyn (MGB; Fig. 5). Although butenyl-spinosyn was produced at high levels in NRRL 30141, butenyl-spinosyn could not be detected in any of the six *busO* mutants by LC/MS. This demonstrated that *busO* was required for biosynthesis of butenyl-spinosyn. The isolation of PSA from all *busO* mutants indicated that all butenyl-spinosyn biosynthetic genes not required for forosamine biosynthesis were functional. Levels of PSA were increased in all six mutants (Table 6), as would be predicted from a deficiency in forosamine supply. The levels of MGB, which has a sugar (3″-*O*-methyl glucose) other than forosamine at C-17, also increased in the *busO* mutants. This suggested that the forosamyltransferase (BusP) encoded by the *busP* gene was functional in these *busO* mutants and could transfer other sugars to the butenyl-spinosyn PSA.

Genes responsible for minor butenyl-spinosyn metabolites

In spite of the high degree of DNA and amino acid similarity between some *bus* and *spn* genes, it should be noted that some of the *bus* gene products catalyze different reactions in the biosynthesis of butenyl-spinosyns relative to spinosyns. These differences are manifested in the distinct butenyl-spinosyn compounds that have been isolated from *S. pogona*.

All natural spinosyns are substituted at C-17 with forosamine or a specific forosamine isomer [20]. Butenyl-spinosyns, on the other hand, are substituted at C-17 with a wider range of forosamine isomers, as well as neutral sugars like amicetose, *O*-methyl-glucose, and *O*-methyloleandrose [21]. This C-17 glycosylation diversity relative to spinosyns requires biosynthetic enzymes to make the sugars and a glycosyltransferase capable of catalyzing these glycosylations. These sugars might be synthesized by specific synthase genes located near the *bus* genes or elsewhere in the chromosome, or they may be synthesized by alternate substrate specificity of the listed butenyl-spinosyn biosynthetic genes. Amicetose could be produced by genes outside of the *bus* gene cluster or it may be a shunt product of an intermediate in the biosynthesis of forosamine as outlined in Fig. 6. Methyl-oleandrose (mole) could be synthesized from NDP-4-keto-2,6-deoxy-D-glucose, an intermediate in the biosynthesis of forosamine (Fig. 6). Ketoreduction (putatively *ORF LIV*) and *O*-methylation of this precursor by the *bus* genes (*busH, busI* or *busK*) and/or other *S. pogona* genes (putatively *ORF LVI*) could lead to the biosynthesis of butenyl-spinosyn derivatives containing methyloleandrose (Fig. 6).

The spinosyn forosamyl transferase, *spnP*, cloned into *S. erythraea* SGT2 (*ery* PKS-deleted strain) was shown to add the alternate sugars mycarose and D-glucose to the C-17 position of spinosyn [15]. Other glycosyl transferases exogenous to *S. erythraea* were unable to glycosylate spinosyn, indicating that the inherent spinosyn specificity of SpnP was required [15]. Likewise, we expect that the forosamyl glycosyltransferase, BusP, was responsible for attaching multiple sugars to the butenyl-spinosyn pseudoaglycone. This was supported by enhanced addition of methyl-glucose to butenyl-spinosyn in six *busO* mutants of *S. pogona*, all of which have an unaltered *busP* gene. This natural ability of the BusP glycosyl transferase to transfer both amino and neutral sugars is unique in secondary metabolite biosynthesis [15]. However, this evidence does not firmly rule out the involvement of a glycosyl transferase other than BusP, such as ORF LI, in the attachment of alternate sugars at C-17.

Several butenyl-spinosyn analogs produced by *S. pogona* are hydroxylated at C-8 or C-24 (Fig. 1) [21]. Macrolides can be hydroxylated postsynthesis by P-450 monooxygenases as in hydroxylation at C-6 in erythro-

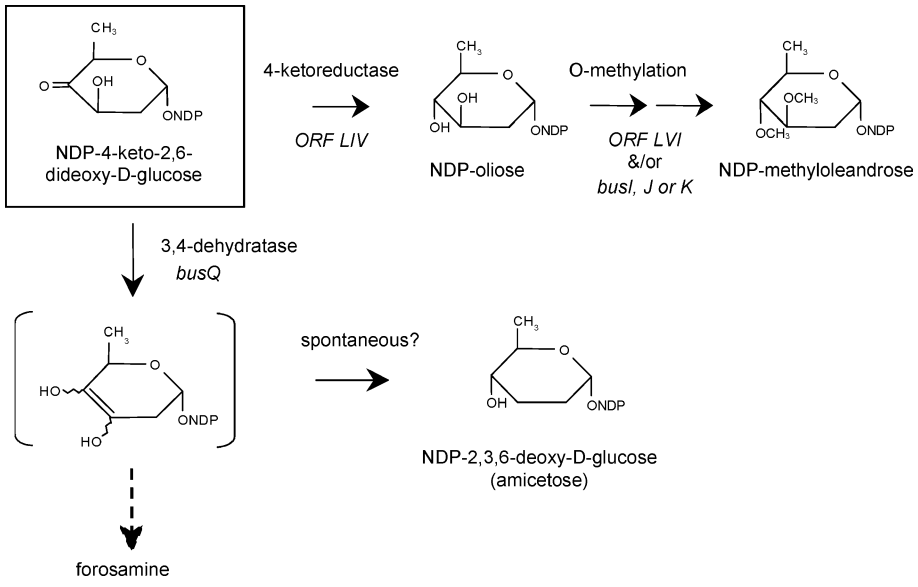

Fig. 6 Putative biosynthesis of alternate sugars using *bus* & linked genes. NDP-4-keto-2,6-dideoxy-D-glucose, shown in the box, is an intermediate in the biosynthesis of forosamine (the product of BusN or SpnN) [35]. The product of the BusQ or SpnQ proteins is a putative unstable intermediate (brackets) based on deoxyhexose biosynthesis [33, 35]

mycin biosynthesis [36]. ORF LVII was highly similar to *oleP* a P-450 monooxygenase involved in polyketide hydroxylation in oleandomycin production in *Streptomyces antibioticus* (Table 4). Therefore, it may be responsible for the hydroxylations at C-8 or C-24 of butenyl-spinosyns. Alternatively, hydroxylated precursors such as glycolate or glycerol can be incorporated during polyketide synthesis, as in leukomycin [30]. It has been reported that the AT domain specific for addition of glycolate in the niddamycin producer (*nid* AT6) is similar to methyl-malonyl-CoA specific AT domains of the erythromycin and rapamycin PKS genes [19]. PKS module 7 is responsible for the addition of carbons 8 and 9 of the butenyl-spinosyn polyketide; however, the sequence of the *busD* AT7 domain is not similar to the putative glycoate specific sequences of *nid* AT6. Although this seems to indicate that bus AT7 is not specific for glycolate, there are other unique sequences in *bus* AT7 relative to other AT domains and *nid* AT6 which could denote alternate specificity. It seems likely that a monooxygenase such as ORF LVII would be responsible for the C-8 or C-24 hydroxylations. No C-8 hydroxylated spinosyns are produced by *S. spinosa*, therefore, the butenyl-spinosyn biosynthetic genes responsible for these modifications are unique to *S. pogona*.

In addition, rhamnose methylation is altered in *S. pogona* relative to *S. spinosa*. Mutants of *S. spinosa* which exhibited altered methylation of the rhamnose on spinosyn [27, 28, 34], typically produced mono-desmethylated rhamnose derivatives of spinosyns. Di-desmethyl rhamnose derivatives of spinosyns were only detected in the presence of methyltransferase inhibitors like sinefungin. No tri-desmethyl rhamnose derivatives of spinosyns were ever isolated. Mutants of *S. pogona* with altered methylation of rhamnose [17], produced di- and tri-desmethyl rhamnose derivatives of butenyl-spinosyns in high amounts, in the absence of methyltransferase inhibitors.

Putative origins of the spinosyn and butenyl-spinosyn genes

Eleven of the twelve PKS modules of the spinosyn and butenyl-spinosyn biosynthetic genes were highly similar (Table 2). However, module 1b of the *busA* gene was markedly different from any *spn* PKS module. A comparison of the protein (or DNA) sequence of the *busA* and *spnA* genes showed strong similarity between the loading modules of both genes. This similarity was over 90% at both the DNA and amino acid sequence level and continued through the KS domains of *spnA* M1 and *busA* M1b (Fig. 7). However, the similarity between *busA* M1b and *spnA* M1 dropped significantly in the AT domains and low similarity continued through the end of *busA* M1b. The strong similarity between *busA* and *spnA* M1 resumed 150 amino acids into the

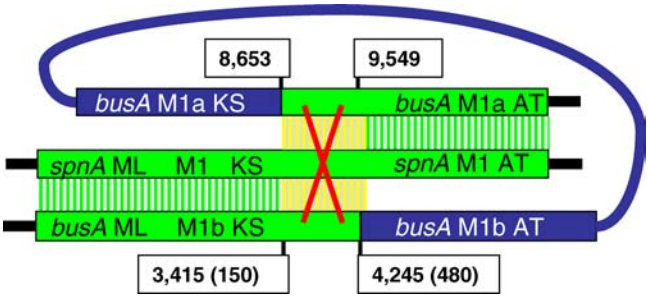

Fig. 7 Illustration of putative natural genetic engineering of the *spnA* gene from the *busA* gene. *Green boxes and lines* indicate regions of *busA* and *spnA* genes with >90% DNA identity, *blue boxes and lines* indicate unique regions of *busA* with <90% DNA identity to *spnA*. *Yellow* indicates the region of homology between all three domains where the postulated recombination crossover (represented by the *red* "*X*") would occur. *Numbers on the flags* correspond to the nucleotide in the *busA* gene (AX600586); *numbers in parenthesis* indicate the amino acid number in the KS domain of *busA* M1b

M1a KS domain and continued through the end of each gene (Fig. 7).

Therefore, all three modules (*busA* M1b and M1a and *spnA* M1) showed strong similarity over the last 350 amino acids of the KS domains. If the KS domain of *busA* M1b and the KS domain of *busA* M1a were lined up as shown in Fig. 7, there appears to be sufficient similarities to support homologous recombination. The product of such a recombination would result in cross-over in the KS domain and an in-frame deletion of one entire PKS domain. The resulting module would have the arrangement found in the *spnA* gene. It could, therefore, be postulated that the *spnA* gene was derived from the *busA* gene by homologous recombination across the highly similar KS domains of M1b and M1a.

Conclusions

Analysis of the *bus* gene cluster revealed a high degree of conservation with the *spn* cluster from *S. spinosa*. The gene order and gene orientation was totally conserved between *S. spinosa* and *S. pogona*. DNA flanking the *bus* gene cluster, on the other hand, was completely diverged from the *spn* cluster. As in *S. spinosa*, no regulatory genes nor genes for biosynthesis of rhamnose were directly linked to the *bus* biosynthetic cluster. Several of the unique genes flanking the *bus* cluster may be involved in formation of some of the unique butenyl-spinosyn factors, but these genes need further investigation.

In this analysis, we found that the origin of the butenyl tail in butenyl-spinosyns was due to an additional PKS module in the *bus* PKS relative to the *spn* PKS. The functional domains of module 1b in the *busA* gene have the functions necessary to synthesize this unique addition. We found a high degree of similarity between the KS domains of the *busA* gene which contains this additional module and the *spnA* gene. An in-frame deletion between the homologous module 1b KS and module 1a KS within the *busA* gene would result in a gene with very similar structure to the *spnA* gene. Thus the spinosyn biosynthetic cluster may have been derived as an in-frame deletion from an ancestral cluster which produced butenyl-spinosyns, analogous to the *bus* cluster.

The butenyl-spinosyn-producing strain *S. pogona* NRRL 30141 has a number of significant differences from *S. spinosa*: a hairy rather than spiny spore coat, bacteriophage sensitivity and different 16S rRNA secondary structure. However, *S. pogona* was very similar to *S. spinosa* in its growth characteristics and biochemical tests. The 16S rRNA sequence similarity between the two strains was 98% identity (D. Hahn, manuscript in preparation) and BLAST analysis indicated that the two 16S rRNA gene sequences were nearest neighbors within the *Saccharopolyspora*. Therefore the strains, although different, are so closely related that the proposed common origin of spinosyn genes is feasible.

Acknowledgements We would like to acknowledge the assistance of Dennis Duebelbeis and Paul Lewer who provided LC and LC/MS analysis of fermentations. We also acknowledge Dow AgroSciences Discovery management for enthusiastic support of this work.

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