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Genes for the biosynthesis of spinosyns: applications for yield improvement in *Saccharopolyspora spinosa*

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Spinosyns A and D are the active ingredients in an insect control agent produced by fermentation of *Saccharopolyspora spinosa*. Spinosyns are macrolides with a 21-carbon, tetracyclic lactone backbone to which the deoxysugars forosamine and tri-*O*-methylrhamnose are attached. The spinosyn biosynthesis genes, except for the rhamnose genes, are located in a cluster that spans 74 kb of the *S. spinosa* genome. DNA sequence analysis, targeted gene disruptions and bioconversion studies identified five large genes encoding type I polyketide synthase subunits, and 14 genes involved in sugar biosynthesis, sugar attachment to the polyketide or cross-bridging of the polyketide. Four rhamnose biosynthetic genes, two of which are also necessary for forosamine biosynthesis, are located outside the spinosyn gene cluster. Duplication of the spinosyn genes linked to the polyketide synthase genes stimulated the final step in the biosynthesis — the conversion of the forosamine-less pseudoaglycones to endproducts. Duplication of genes involved in the early steps of deoxysugar biosynthesis increased spinosyn yield significantly. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 399–402.

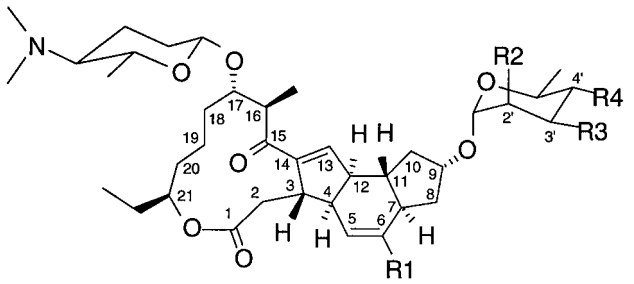
Keywords: forosamine; polyketide; *Saccharopolyspora spinosa*; spinosyn; tri-*O*-methyl rhamnose

Introduction

Spinosyns are the active ingredients in Naturalyte™ insect control products produced by the actinomycete, *Saccharopolyspora spinosa*. Spinosyns are macrolides (Figure 1) consisting of a 21-carbon, tetracyclic lactone, to which are attached two deoxysugars: tri-*O*-methylated rhamnose and forosamine [3]. The most active components of the spinosyn family of compounds are spinosyns A and D; other factors in this family have different levels of methylation and are significantly less active. Both the rhamnose and forosamine moieties are essential for the insecticidal activity of spinosyns [2,8].

Spinosyn A is derived from nine acetate and two propionate units to produce a non-glycosylated intermediate, the aglycone. The structure of the aglycone suggests that a linear polyketide molecule is made first, and then cyclized. Three carbon–carbon bonds are formed to obtain the tetracyclic aglycone. Attachment of the two deoxysugar residues, in the sequence tri-*O*-methyl rhamnose then forosamine, results in the final spinosyn molecule (Figure 2). The order of sugar additions and modifications was deduced from bioconversion studies of incomplete spinosyns fed to wild type and mutant strains blocked in spinosyn biosynthesis [1,3]. Rhamnose attachment must occur before forosamine addition. Methylation of the rhamnose hydroxyl groups can occur after the sugar has been attached to the polyketide, but it is only completed if it occurs in the sequence 2′, 3′ then 4′. The amino group of the forosamine must be methylated before its incorporation into the macrolide.

The sequence of steps involved in deoxysugar biosynthesis was predicted by the sequence similarity of spinosyn genes to genes whose functions have been determined in other pathways [9]. Both tri-*O*-methyl rhamnose and forosamine are believed to be synthesized from glucose-1-phosphate *via* the common intermediate NDP-4-keto-6-deoxy-D-glucose (Figure 2). The biosynthetic pathway for rhamnose has been elucidated in enteric bacteria, where the deoxysugar is an element of surface antigens [4]. The first step, activation of glucose by addition of a nucleosidyl diphosphate (NDP), is catalyzed by a NDP–glucose



Factor	R1	R2	R3	R4
A	H	OCH_3	OCH_3	OCH_3
D	CH_3	OCH_3	OCH_3	OCH_3
H	H	OH	OCH_3	OCH_3
J	H	OCH_3	OH	OCH_3
K	H	OCH_3	OCH_3	OH
P	H	OCH_3	OH	OH

Figure 1 The structure of the spinosyns. The dimethylamino sugar forosamine and tri-*O*-methyl rhamnose are shown on the left and right sides of the tetracyclic aglycone, respectively. Spinosyns A and D are the most active insecticidal molecules. *S. spinosa* also produces a number of other less active minor submethylated factors (H, J, K and P), as well as some others not shown here [2,3,9].

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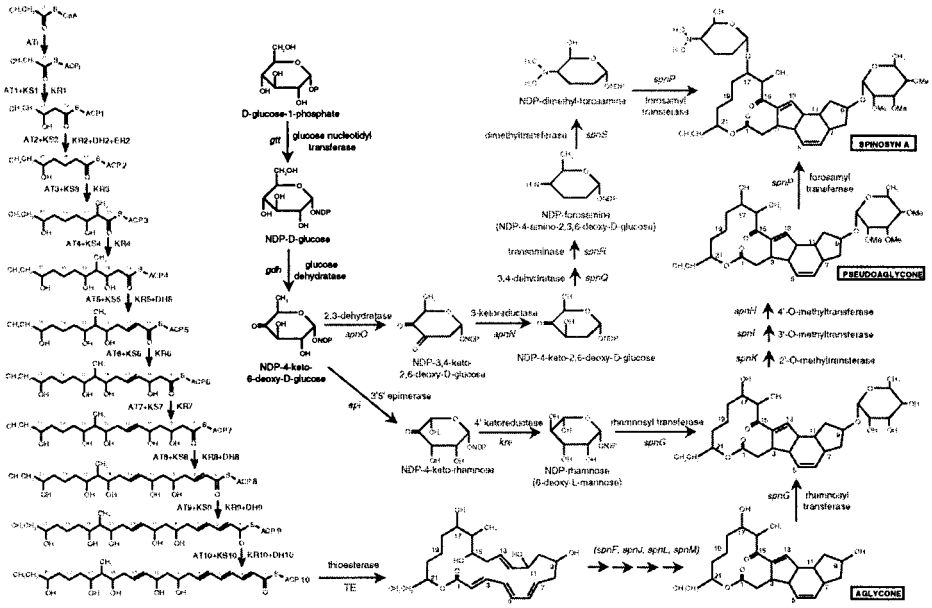

Figure 2 A proposed pathway for spinosyn biosynthesis. Key intermediates in the pathway are shown in bold inside boxes. The enzymes involved in the polyketide and deoxysugar biosynthesis and genes (in italics) that code for these enzymes are shown next to the arrows. NDP-4-keto-6-deoxy glucose serves as a common branch point intermediate for both rhamnose and forosamine biosynthesis.

synthase (designated *gtt*). The second step, dehydration to NDP-4-keto-6-deoxy-glucose, is catalyzed by glucose dehydratase (designated *gdh*). In *S. spinosa*, there is only a single copy of each gene, so these gene products must supply precursor for both deoxysugars [5]. Rhamnose synthesis requires two additional enzymes, a 3′,5′-epimerase (*epi*) and a 4′-ketoreductase (*kre*), that are unique to the pathway. Forosamine synthesis requires five additional genes that are specific for this sugar. In this report, we review the organization of the spinosyn gene cluster and describe the effects of duplicating certain spinosyn genes on spinosyn production.

Cloning and characterization of the spinosyn biosynthetic genes

The majority of the genes coding for spinosyn biosynthesis were isolated on three overlapping cosmids. Complete DNA sequence analysis of these cosmids indicated 23 open reading frames (ORFs) in a cluster spanning ~80 kb (Figure 3). Nineteen of these genes appear to be involved in spinosyn biosynthesis [9]. The *spn* genes were characterized initially by comparison to sequence databases at the National Center for Biotechnology Information. In most cases, they were further characterized by gene disruption and bioconversion of biosynthetic intermediates [9]. Five large genes encoding a type I polyketide synthase are located on the right side of the cluster. They span a 56-kb region, and include 50 enzymatic domains organized as a loading module and 10 extender modules. To the left of these genes is a cluster containing four genes apparently involved in carbon–carbon bond formation, four genes involved in rhamnose attachment and methylation and six genes involved in forosamine biosynthesis and attachment.

The four genes thought to play a role in the cross-bridging reactions to form the tetracyclic polyketide were identified by gene disruption and bioconversion analysis. Their individual functions were not easily identified by DNA homology searches [9]. Two of the genes are related to actinomycete methyltransferase genes and one to a family of *Candida albicans* lipase genes. All three of these genes lacked key amino acids conserved in the active sites for methlytransferase or lipase enzymes, suggesting that the *S. spinosa*

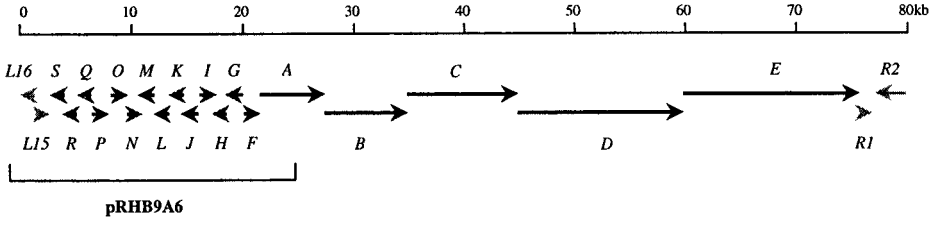

Figure 3 The organization of the spinosyn biosynthesis gene cluster. The five polyketide synthase genes (*spnA–E*) are located on the right side of the cluster and the genes coding for forosamine biosynthesis (*spnO*, *spnN*, *spnQ*, *spnR*, *spnS* and *spnP*), rhamnose methylation (*spnH*, *spnI* and *spnK*), and polyketide bridging (*spnF*, *spnJ*, *spnL* and *spnM*) are organized on the left side of the cluster. The genes *L15* and *R1* flank the spinosyn gene cluster and are not required for spinosyn production. The rhamnose biosynthesis genes (*gtt*, *gdh*, *kre* and *epi*) (not shown) are located in three different regions of *S. spinosa* genome and are not linked to the main cluster shown in this figure. The functions of individual genes are shown in Figure 2 and described in detail elsewhere [5,9].

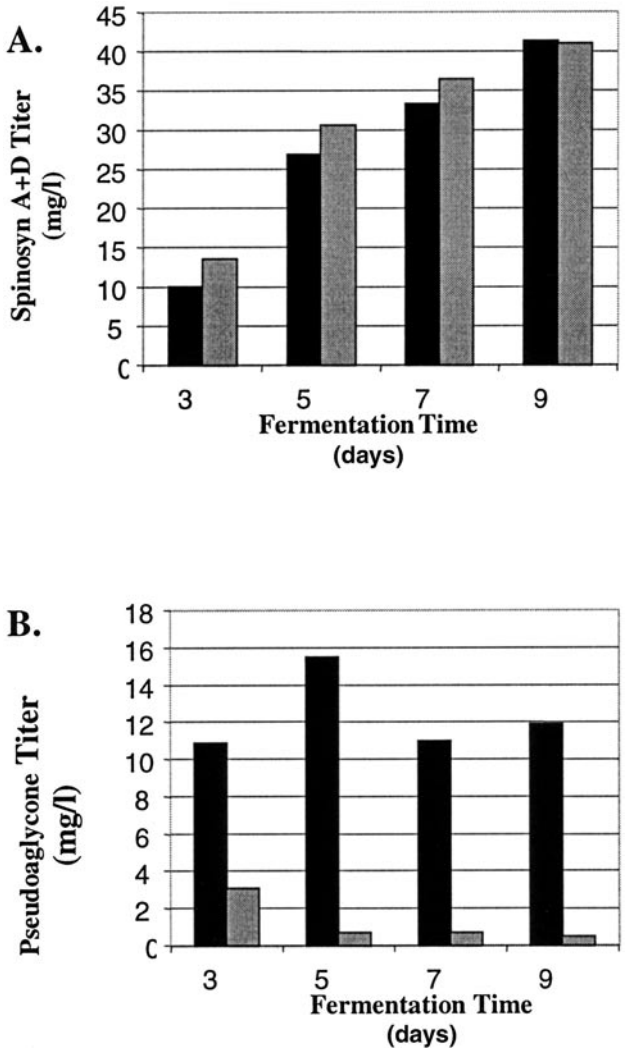

Figure 4 The effects of integrating cosmid pRHB9A6 on PSA conversion at different stages of fermentation. Cosmid pRHB9A6 was introduced into *S. spinosa* by conjugation from *E. coli* S17-1, using the method of Matsushima *et al* [6]. Black bars represent titers from the parent strain without pRHB9A6. Dark gray bars represent titers from the recombinant strain with pRHB9A6. The transconjugants and the parent strain were evaluated in 10 replicate fermentations and titers were averaged.

enzymes carry out new functions. The genes in the spinosyn cluster that are involved in deoxysugar biosynthesis, modification and attachment were much more similar to genes in other organisms. They encode the critical residues that are required for the enzymatic functions that have been experimentally demonstrated in other deoxysugar pathways [9].

The spinosyn gene cluster does not contain genes whose products would synthesize rhamnose. The first two steps in the biosynthesis of many deoxysugars, including rhamnose, are catalyzed by the products of *gtt* and *gdh* genes that are highly conserved in several bacteria [4]. Southern hybridization analysis indicated that *S. spinosa* contains homologues of the corresponding *S. erythraea* genes [5]. However, there is only one copy of each gene in the genome of *S. spinosa*, and they are not linked to the spinosyn cluster. A *kre* gene that codes for a ketoreductase involved in rhamnose synthesis is adjacent to the *gdh* gene. The *epi* gene, encoding the first unique enzyme of rhamnose synthesis, is not closely linked to either the spinosyn gene cluster or to the other rhamnose genes. Madduri *et al* [5] proposed that these genes are not clustered with the other spinosyn genes because they function in both primary and secondary metabolism. Rhamnose is essential for cell wall biosynthesis in *S. spinosa*, similar to that observed in mycobacteria [7], and is also needed for spinosyn production.

Manipulation of spinosyn production by gene duplications

The output from the spinosyn pathway could be increased by modifying regulatory genes that control the expression of the genes for the biosynthetic enzymes, or by duplicating those structural genes that encode rate-limiting enzymes. Unfortunately, there do not appear to be any regulatory genes within the spinosyn cluster that can be exploited. There is a homologue of the *lysR* transcriptional regulator just beyond the left end of the cluster, but its duplication or disruption had no effect on spinosyn production [9]. We therefore focused our efforts to enhance yield on duplicating the genes for critical biosynthetic enzymes.

During the early part of a spinosyn fermentation, *S. spinosa* accumulates pseudoaglycones (PSAs), the intermediates that lack forosamine. This indicates that the biosynthesis or attachment of forosamine can be limiting under certain conditions. We duplicated the genes involved in these processes by integrating a copy of the cosmid pRHB9A6 (Figure 3) into the chromosome by homologous recombination. The cosmid was introduced into *S. spinosa* by conjugation from *Escherichia coli* S17-1, using the method of Matsushima *et al* [6]. It is unable to replicate autonomously in *S. spinosa*, so integration must occur to generate stable, apramycin-resistant transconjugants. Such strains converted almost all of the PSAs they produced during the early stages of fermentation into fully glycosylated spinosyns A and D (Figure 4). The overall productivity was comparable to the parental strain by the end of the

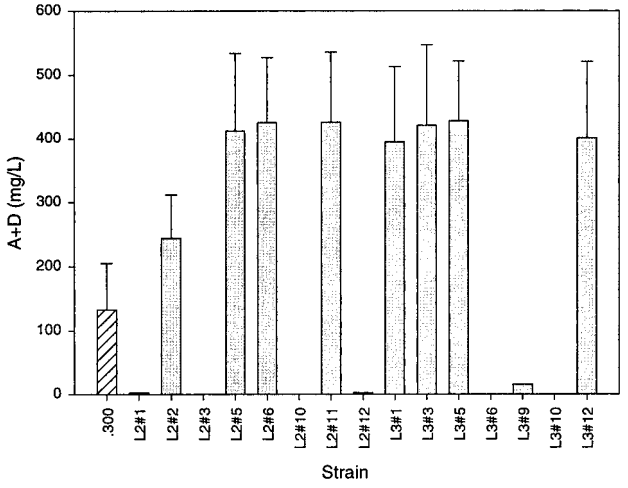

Figure 5 Spinosyn production in *S. spinosa* A83846.300 transconjugants. Production in the control parent strain A83846.300 (represented as 300) is shown by the hatched bar. The solid bars indicate production in transconjugants. Fifteen independent transconjugants, L2#1–12 and L3#1–12, and the parent strain were evaluated in 10 replicate fermentations and the titers were averaged. Error bars represent 1 SD above the mean.

fermentation. This approach could be particularly valuable if significant amounts of PSA remain at the end of the production phase in high-yielding production fermentations.

Several strains derived by nitrosoguanidine mutagenesis accumulate large amounts of PSA instead of spinosyns A and D. In fact, they accumulate twice as many molecules of polyketide as their parent. However, since each PSA molecule lacks the forosamine moiety, the total amount of deoxysugar incorporated into spinosyns did not change. This suggests that *S. spinosa* restricts its polyketide production to exploit fully the availability of the deoxysugars. To increase the supply of the common deoxysugar precursor 4-keto-6-deoxy-glucose, we combined the DNA fragments containing the *gtt* and *gdh* genes into a single plasmid, and integrated it into the *S. spinosa* chromosome [5]. This genetic modification created a dramatic increase in spinosyn yield (Figure 5). As higher-yielding strains are acquired, the problems with the deoxysugar supply may diminish, and other steps in the biosynthetic pathway could become limiting. Having the genes for these steps in hand should enable us to make further changes that will continue to improve the productivity of this important fermentation.

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