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review

Novel Hybrid Polyketide Compounds Produced by Genetic Engineering of the Oxytetracycline Biosynthetic Pathway

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Summary

The whole oxytetracycline (OTC) gene cluster was cloned from the strain Streptomyces rimosus R6. Its restriction map is indistinguishable from that of the strain S. rimosus M15883, which means that results from the two strains can probably be combined. Constructions to induce gene disruptions of otcD1 and otcC were undertaken within the chromosome of S. rimosus R6. OtcD1 is thought to be involved in the folding, cyclization and aromatization of the hypothetical nonaketide intermediate in OTC biosynthesis (pre-polyketide), whereas OtcC hydroxylates the completed tetracyclic nucleus at C-6 (post-polyketide). The disrupted strains no longer produced OTC. OtcC::gmr produced three novel compounds, while otcD1::ermE produced a number of compounds, not detectable in the wild-type strain. These novel compounds contained 9, 15 and 17 C-atoms in their backbones instead of 19, as with OTC. The disrupted strains did not have the expected DNA structures, but carried DNA amplifications and deletions. This is probably due to the fact that the otc cluster is located within an amplifiable unit of DNA (AUD) which undergoes frequent spontaneous DNA amplifications and deletions. Spontaneous mutants (Class II) that have deleted the otc cluster are potential hosts for expressing new hybrid polyketide clusters. However, it was possible to isolate derivatives (Class IIR) that produce an antibiotic activity. It was shown that this compound was oxytetracycline and that the Class II mutants themselves also produce very small amounts of oxytetracycline.

Key words: Streptomyces rimosus, oxytetracycline, polyketides, biosynthetic pathway engineering

Introduction

Polyketides are a large, and structurally diverse, family of natural products with a broad range of biological activities and pharmacological properties. Some notable examples include antibiotics like oxytetracycline and erythromycin, anticancer drugs like tetracenomycin and doxorubicin, the antiparasitic avermectin, the immunosuppressant rapamycin, the growth promotant monensin, the cholesterol-lowering drug mevinolin and many others. The majority of polyketides are synthesized by *Streptomyces* and related filamentous bacteria,

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with fungi and plants making up the balance (*1*). Recently, our understanding of how streptomycetes synthesize these biologically active metabolites has advanced substantially, to the point where generation of novel therapeutics by this route is a viable proposition.

Polyketides are produced by polyketide synthase complexes (PKSs) through a specialized variation of fatty acid biosynthesis, which is mediated by fatty acid synthase complexes (FASs). Both FASs and PKSs catalyze the condensation of simple carboxylic acids, activated by coenzyme A, *via* a Claisen ester condensation reaction. Following each condensation, FAS's typically catalyze a complete reductive cycle of the β-oxoacyl-thioester including a ketoreduction, dehydration and enoylreduction. By contrast, PKSs either leave the reactive β-oxo groups substantially intact, or catalyze subsequent reduction to a variable extent. After the carbon chain has grown to a length characteristic of each specific product it is released from the synthase by thiolysis or acyltransfer. The choice of starter and extender units, the controlled variation in the chain length and differences in the reductive cycles lead to a huge variation among naturally occurring polyketides. The synthetic pathway leading to the polyketide backbone is called 'pre-polyketide' biosynthesis. Additional variability is achieved by a wide range of 'post-polyketide' modifications of the polyketide backbone (*2*).

Sequence and mechanistic similarities among many *Streptomyces* enzymes led to their classification into Type I and Type II PKS systems, following the classification system for FAS's. Type I PKS's, such as those responsible for the biosynthesis of complex polyketides like erythromycin, avermectin and rapamycin, are multifunctional enzymes with a modular structure. Each module is responsible for a single cycle of the synthesis reaction and contains domains for the necessary extension and reduction activities. By contrast, the Type II PKS's, which undertake the biosynthesis of actinorhodin, granaticin, frenolicin, griseusin, oxytetracycline and many others, are composed of three to six separate mono- or bifunctional proteins, the active sites of which are used iteratively for the assembly of the polyketide chain during pre-polyketide biosynthesis (*3*).

In complex polyketides, the one-to-one correspondence between the structure of the product and the active domains in the modular PKS has stimulated the rational design of novel complex polyketides by altering these genes by genetic manipulation. Thus in *Saccharopolyspora erythrea*, the producer of erythromycin, precise deletion of the ketoreductase domain of module 5 resulted in formation of the predicted 5-oxo-macrolactone, and deletion of the enoylreductase domain of module 4 resulted in the expected C-6, C-7 anhydromacrolactone. The size and shape of the polyketide product can be varied by designing mutants with the appropriate number and/or fusions of modules. For example, triketide lactone was produced by modules 1 and 2 alone, as well as by the fusion protein consisting of modules 1 and 2 with the thioesterase domain from module 6. Similarly, a new 12-membered macrolactone was produced by fusing the ACP-thioesterase di-domain of module 6 downstream of the ketoreductase domain of module 5 (*4*).

An approach to the rational design of novel simple aromatic polyketides cannot, however, be so straightforward. This is due to fundamental differences in the strategies by which complex (Type I) and simple (Type II) PKS's control product structure. In contrast to Type I PKSs, the Type II PKSs are structurally all very similar so it is not possible to predict polyketide structure from genetic architecture. For that reason, a specially designed host-vector system for the expression of engineered aromatic PKS clusters was constructed. It consists of *S. coelicolor* strain CH999, from which the entire actinorhodin gene-cluster has been deleted, together with the expression plasmid pRM5. By 'mixing and matching' of PKS genes from different polyketide producers, this system has been used to generate many novel products from aromatic PKSs and to develop rules and strategies for the rational design of novel aromatic polyketides (*5*).

This review describes the use of pathway engineering for the construction of genetically-modified strains of *S. rimosus* R6 (*6*). This strain has been already used in Croatia for a long time as a 'work-horse' for the commercial production of oxytetracycline (OTC). By engineering this strain, novel chemical entities of potential therapeutic value have been generated, based on the genetic potential of the *otc* pathway genes.

Results and Discussion

Cloning and restriction mapping of DNA from the S. rimosus R6 otc cluster

S. rimosus is genetically one of the best characterized species among industrially important streptomycetes. Two strains, M4018 studied in the United Kingdom and R6 studied in Croatia, have been used extensively. The gene cluster for OTC biosynthesis has been cloned from M15883, a derivative of strain M4018 and a restriction map derived. DNA sequencing of the cluster has identified the functions of many of the genes, although some have yet to have a function defined (*7*).

In order to clone the whole *otc* gene cluster from *S. rimosus* R6, cosmid gene banks were constructed in *Escherichia coli* using the cosmid vector sCos-1 (*8*) and total genomic DNA from strain R6-501 [a derivative of R6 (*9*)] and from MV17 (a mutant that overproduced OTC). Strain MV17 carries, along with the chromosomal copy, a plasmid-borne *otc* cluster. It resides on pPZG103, a 1 Mb linear plasmid prime derivative of linear plasmid pPZG101 (*10,11*). The copy number of pPZG103 was greater than that of the chromosome so it was expected that the cosmid gene bank of MV17 would be enriched for DNA fragments carrying *otc* genes. The *otr*A and *otr*B genes, that flank the chromosomally-located *otc* cluster in M15883 (*12,13*), were used as hybridization probes to isolate cosmid clones carrying DNA sequences from the *otc* cluster of the *S. rimosus* R6 lineage. In total, twelve hybridizing colonies were isolated. Eight hybridized to *otr*B, of which pPZG11 was taken as a representative (Fig. 1). Three colonies hybridized to *otr*A, and pPZG13 was taken as a representative. One colony, pPZG25, hybridized to both probes (Fig. 1). It was anticipated that pPZG11, pPZG13 and pPZG25 would con-

tain overlapping segments of DNA encompassing all of the *otc* biosynthetic genes of *S. rimosus* R6 and both resistance genes (*otr*A and *otr*B).

Restriction maps of pPZG11, pPZG13 and pPZG25 were constructed to compare the architecture of the *otc* cluster of *S. rimosus* R6 with the entire OTC biosynthetic pathway cloned from the *S. rimosus* M4018 lineage. Enzymes whose restriction sites flanked (*Eco*RI, *Eco*RV and *Hin*dIII), or were within the *otc* cluster of M15883 (*Bam*HI, *Bgl*II, *Pst*I and *Sac*I) (*14*) were used, singly or in pairs. Southern hybridization with *otc* resistance (*otr*A, *otr*B) and *otc* biosynthetic genes (*otc*C, *otc*D, *otc*Y2, *otc*Y1) (data not shown) facilitated the estimation of fragment sizes as well as the alignment of fragments into restriction maps (Fig. 1). The restriction maps of pPZG11, pPZG13 and pPZG25 and their cross-hybridization patterns, showed that pPZG11 and pPZG13 did not contain overlapping DNA, but that both hybridized with pPZG25 (data not shown). This was confirmed by hybridization of cosmid DNA's to *otc* resistance and *otc* biosynthetic genes. The insert of pPZG11 (17.1 kb) hybridized with the *otc*Y2, *otc*Y1 and *otr*B probes. The insert of pPZG13 (16.4 kb) hybridized with the *otr*A, *otc*C, and *otc*D probes, whereas that of pPZG25 (29.4 kb) hybridized with all of them (data not shown). From these data, the positions of the *Bam*HI, *Bgl*II, *Pst*I and *Sac*I restriction sites could be drawn (Figs. 1A and B).

The restriction map of pPZG25 (Fig. 1) suggested that not all of the *otr*A gene was present on this insert, as the *Sac*I site which lies adjacent to the 3' end of *otr*A was absent. To confirm this, Southern blots of R6 total chromosomal DNA and DNA of pPZG25 digested with appropriate enzyme combinations were hybridized with an *otr*A probe. This showed (Fig. 2) that a 5.2 kb fragment of *Eco*RI-*Bam*HI digested chromosomal DNA hybridized to the *otr*A probe (lane 2), whereas there is only a 1.9 kb fragment in pPZG25 (lane 3). The *Eco*RI restriction site in pPZG25 originated from the sCos-1 vector and is adjacent to the cloning site used to construct the *S. rimosus* gene banks. The *otrA* probe hybridises to a 2.7 kb *Sac*I-*Bam*HI fragment in chromosomal DNA (lane 4). Thus, around 800 bp of the chromosomal *Sac*I-*Bam*HI fragment including the 3' end of *otr*A was not present in pPZG25.

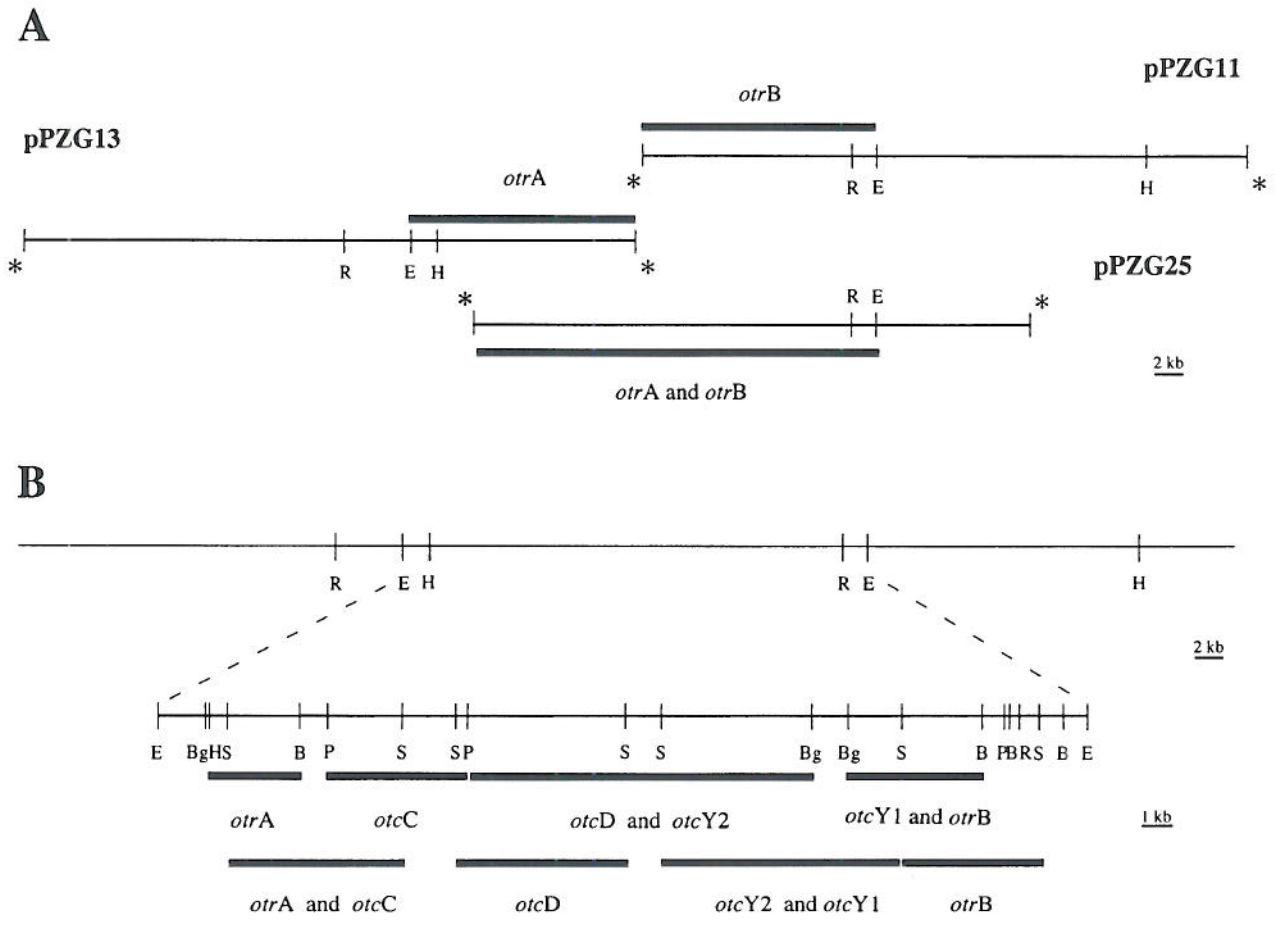

Fig. 1. Restriction map of the *otc* cluster from the strain *S. rimosus* R6.
(A) Shaded boxes denote the *Eco*RI fragments of the cosmids pPZG11, pPZG13 and pPZG25 that hybridized with *otr*A and *otr*B probes, respectively.
(B) Shaded boxes denote restriction fragments of the *S. rimosus* R6 chromosomal DNA digested with different enzymes and hybridized with *otr*A, *otc*C, *otc*D, *otc*Y2, *otc*Y1 and *otr*B probes, respectively.
Abbreviations: B, *Bam*HI; Bg, *Bgl*II, E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RV; S, *Sac*I. In Fig. 1A * denotes an *Eco*RI site present in the recombinant cosmid, but derived from the s-Cos1 vector.

The comparison of the *otc* clusters of *S. rimosus* R6 and M4018 lineages was completed by digestion of their total chromosomal DNAs with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Sac*I and hybridization using *otr*A, *otc*C, *otc*D, *otc*Y2, *otc*Y1 and *otr*B as probes. An example of digestion with *Sac*I is shown in Fig. 3. The fragments that hybridized were 6.1, 6.1, 5.0, 10.6, 10.6 and 4.6 kb in size, respectively (Figs. 3A and B). Therefore, the *otc* gene cluster from the R6 lineage cloned in the Zagreb laboratory has a restriction map that is indistinguishable from that of strain M15883 (4018 lineage). Thus the data obtained with the UK and Croatian strains can be merged.

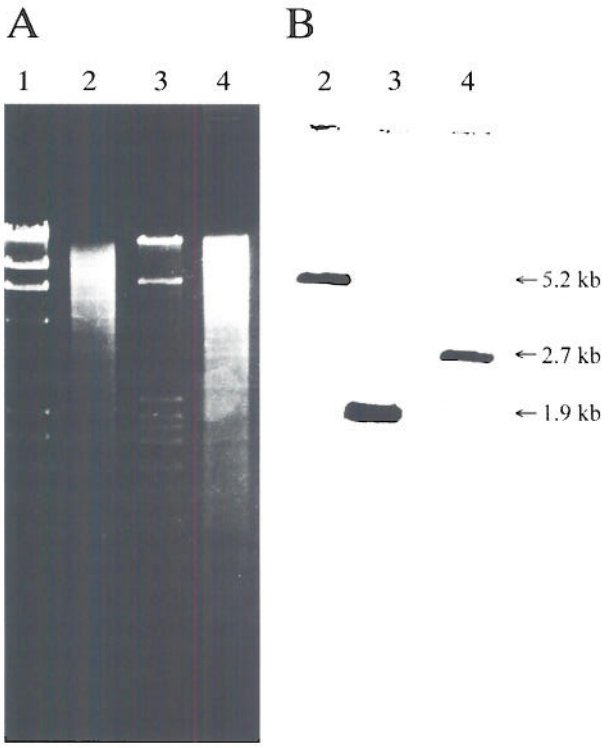

Fig. 2. (A) Agarose-gel electrophoresis of double digests of *S. rimosus* R6 total chromosomal DNA and of the recombinant cosmid pPZG25 hybridized with the *otr*A gene.
Lane 1: λ DNA standard, digested with *Hin*dIII. Lane 2: R6 DNA; and lane 3: pPZG25 DNA; both digested with *Bam*HI and *Eco*RI. Lane 4: R6 DNA digested with *Bam*HI and *Sac*I.
(B) Southern transfer of gel A, hybridized with *otr*A probe.

Disruption of pre-polyketide and post-polyketide genes of the otc gene cluster of S. rimosus R6

Previous work of others (*e.g.* 5) has involved taking individual genes from the same or different Type II polyketide gene clusters, expressing them in the naïve host CH999, elucidating the structures of the (often novel) polyketide metabolites made, and then rationalising what the function of each gene might be.

We have taken a somewhat different strategy. Starting with the entire *otc* gene cluster (Fig. 4) in *S. rimosus* R6, we have undertaken targeted disruption of selected *otc* genes. The approach is to elucidate the structures of the novel polyketides made by these recombinants to deduce the function of the gene that is disrupted. In parallel experiments, we attempted to introduce the following mutations: (*i*) deletion of ketosynthase/acyltransferase (KS_α, *otc*Y1-1) and of the so-called chain length determining factor (KS_β, *otc*Y1-2), (*ii*) inactivation of the cyclase/aromatase gene (*otc*D1), (*iii*) inactivation of the ketoreductase gene (*otc*Y2-1) and (*iv*) inactivation of the 6-hydroxylase (*otc*C) gene (Fig. 4). In this paper we report experiments to disrupt *otc*D1 and *otc*C. The first four genes are all involved in pre-polyketide steps

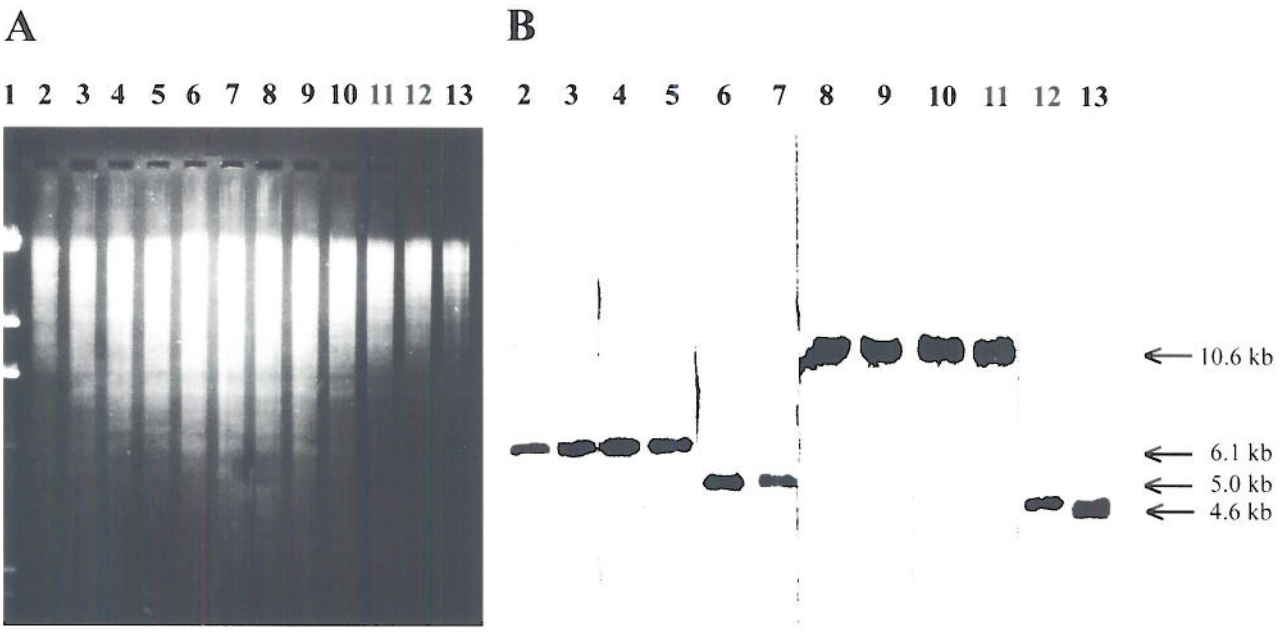

Fig. 3. (A) Agarose-gel electrophoresis of *Sac*I digested total chromosomal DNA of *S. rimosus* strains R6 and M4018 hybridized with various probes from the *otc* cluster. Lane 1: λ DNA digested with *Hin*dIII. Lanes 2, 4, 6, 8 10 and 12: R6 DNA. Lanes 3, 5, 7, 9, 11 and 13: M4018 DNA. (B) Southern transfer of gel A, hybridized with *otr*A (lanes 2, 3), *otc*C (lanes 4, 5), *otc*D (lanes 6, 7), *otc*Y2 (lanes 8, 9), *otc*Y1 (lanes 10, 11) and *otr*B (lanes 12, 13) as probes.

of OTC biosynthesis whereas the hydroxylase adds a hydroxyl group at the C-6 position after biosynthesis of the OTC backbone is completed (*7*).

The strategy to carry out such targeted gene replacements is based on the system used for the analysis of bialaphos production (*15*). An erythromycin (*erm*E) (*16*), or gentamicin (*gmr*) (*17*) resistance cassette was cloned into a plasmid-borne copy of the *otc* gene to be disrupted. The *otc* gene and flanking region were already present either on an unstable bifunctional *E. coli*/*Streptomyces* vector containing the thiostrepton-resistant pIJ487 plasmid (*18*), or on an *E. coli* suicide vector based on pIBI24 (*19*) also embodying the thiostrepton-resistance gene. The disrupted copy was introduced into *S. rimosus* R6 by electrotransformation (*20*), selecting initially for resistance to thiostrepton. After a subsequent period of non-selective growth, during which recombination into the chromosome was presumed to take place, the cells were grown up in the presence of erythromycin or gentamicin. Segregational instability of plasmid pIJ487 in the absence of selective pressure is well documented (*18*). Therefore transformants, which were erythromycin or gentamicin resistant but thiostrepton sensitive, should contain the disrupted copy of the *otc* gene now within the chromosome, and the metabolites made by that recombinant strain were analyzed. It is important to note that many of the *otc* genes lie within polycistronic mRNAs (Fig. 4) (*7*), so disruption of an upstream gene may have a polar effect on genes distal to it on the mRNA, and this could complicate the analysis of data. Therefore, a further step in this overall strategy will be the replacement of the disrupted *otc* gene with a mutant copy of the *otc* gene, designed by PCR, and delivered into the chromosome so that the reading frame was not altered, using the techniques described above.

Gene disruptions of *otc*D1 and *otc*C were undertaken within the chromosome of *S. rimosus*. OtcD1 is thought to be involved in the folding, cyclization and aromatization of the hypothetical nonaketide intermediate in OTC biosynthesis (*i.e.* a pre-polyketide step), whereas OtcC hydroxylates the completed tetracyclic nucleus at C-6 (a post-polyketide step) (*13*). In almost all cases studied so far we were not able to obtain the desired construct, because the transformants isolated carried DNA rearrangements. In only one case a recombinant was isolated that carried a single copy of the disrupted gene within the chromosome (this isolate was one of a number amongst isolated during disruption of *otc*C). Possible reasons for these complications will be described later.

None of the disrupted strains produced OTC. The *otc*C::*gmr* recombinant produced three novel compounds and the *otc*D1::*erm*E isolate also produced a number of compounds, not detectable in the parent strain. Preliminary chemical characterization of isolated polyketide structures showed products containing 9, 15 or 17 C-atoms in their backbones instead of the 19 carbon backbone which is present in oxytetracycline. From their chemical structures, these compounds could plausibly have arisen using the same 3-carbon starter unit as OTC but with 3, 6 or 7 extender units (*i.e.* 3, 6 or 7 iterative cycles of the PKS) instead of the 8 extender units (8 cy-

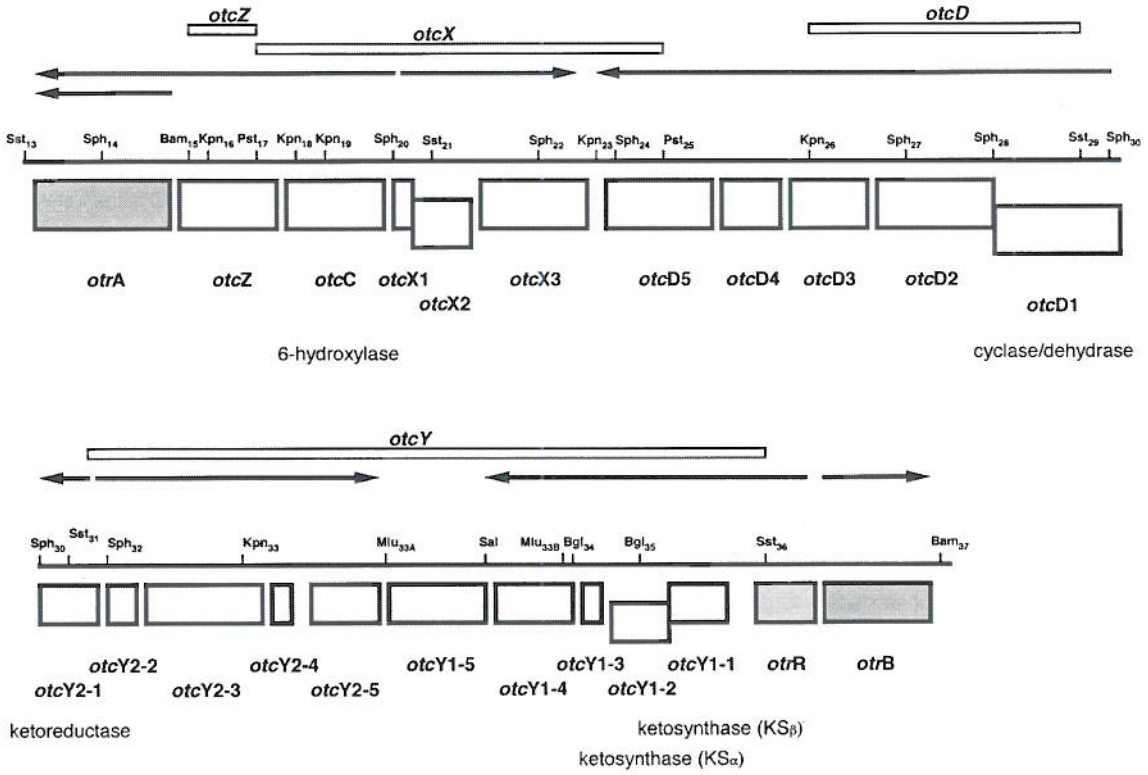

Fig. 4. Architecture of the *otc* cluster. Bars indicate regions where mutants belonging to the same cosynthesis group map, arrows indicate direction of transcription, open boxes are ORFs corresponding to presumed biosynthetic genes and shaded boxes are resistance genes and the *otr*R regulator gene. In cases where translational coupling of two ORFs is believed to occur, one of the ORFs is shown at a lower position.

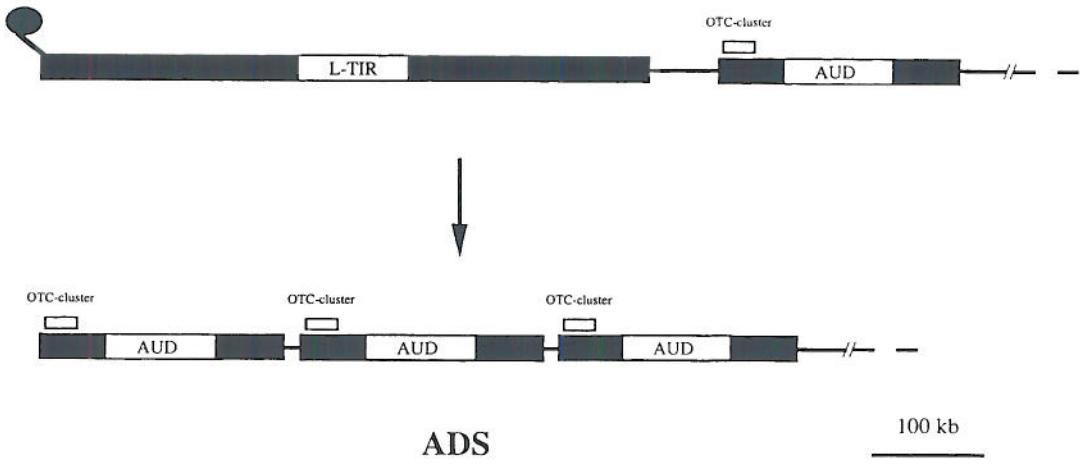

Fig. 5. The replacement of the chromosome end by an amplified DNA sequence (ADS). The left terminal inverted repeat (L-TIR) is shown with the terminal protein. The 250 kb amplifiable unit of DNA (AUD) containing the oxytetracycline biosynthesis cluster (OTC) undergoes tandem amplification and the distal sequences are deleted.

cles) which usually takes place during the synthesis of OTC. Neither *otc*D1 nor *otc*C is within the same cistron as the gene set (*otc*Y1-1, Y1-2, Y1-3) whose products catalyze the biosynthesis of the backbone, including *otc*Y1-2 [also known as *clf* whose product specifies chain length (*21*)]. Thus disruption of *otc*D1 (pre-polyketide) and *otc*C (post-polyketide) have altered the specificity of the PKS so that the same OtcY1-1, 1-2, 1-3 gene products are now producing polyketide structures of markedly different chain length. Our results support the conclusion (*22*) that protein-protein interactions within a PKS complex may exert a profound influence on the nature of end products, and that post-polyketide enzymes may be associated with this complex, in a way that, until now, has been unsuspected.

Structure and stability of S. rimosus R6 genome

None of the recombinants that produced novel chemical products had the predicted DNA structure, but instead they carried a variety of DNA deletions and amplifications. In order to understand the reasons for this it is helpful to consider the spontaneous DNA rearrangements that occur in the region of the chromosome where the *otc* cluster lies. Physical mapping of the chromosome (*23*) showed that it is linear and about 8 Mb in size like those of other *Streptomyces* (*24*). The *otc* cluster lies about 600 kb from one end of the chromosome in a region that undergoes frequent spontaneous DNA rearrangements. One sort of rearrangement (Class II mutants) (*25*) results in loss of at least 750 kb of DNA including the *otc* cluster and the chromosome end. These strains are potentially useful as a 'clean background' for expressing novel polyketide constructs, but there are some problems in this approach which will be considered later. Another rearrangement involves the amplification of a 250 kb amplifiable unit of DNA (AUD) including the *otc* cluster as tandem repeats, accompanied by deletion of distal sequences (Fig. 5).

This probably leads to replacement of the chromosome end with an amplified DNA sequence (ADS) as suggested for amplifications observed in *S. lividans* 66 (*26*). The AUD is a Type II amplification system which means that the same ADS is seen in independent isolates.

Most of the transformants designed to disrupt the *otc* cluster carried DNA amplifications. The amplifications usually included sequences from the introduced vectors, but they have complicated structures which

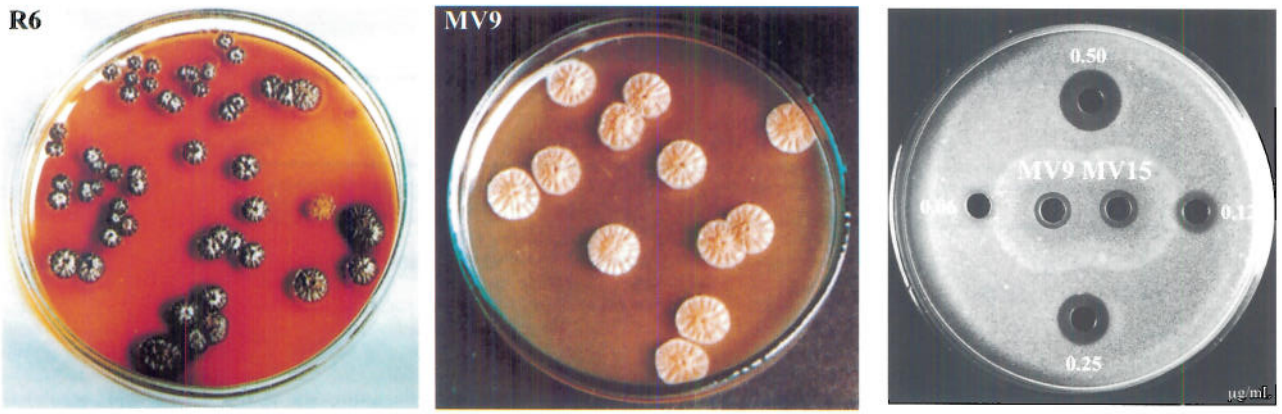

Fig. 6. Colony morphology of *S. rimosus* R6 strains (left) and MV9 (middle). Biological assay (right) of fermentation products of strains MV9 and MV15 using *B. cereus* strain ATCC11778 as test culture. Purified OTC at concentrations of 0.06, 0.12, 0.25 and 0.5 μg/mL were used as controls.

have not yet been elucidated. Hybridisation experiments indicated that the amplified strains had deleted sequences distal to the *otc* cluster. Chromosomal sequences proximal to the cluster were often unchanged in the amplified strains. It is likely that the DNA rearrangements in the transformants are due to the *otc* cluster being in an AUD region. These results indicate that, although the spontaneous amplifications involve specific junction sites outside the cluster, the potential for genetic instability is spread over a region as in the case of Type I amplification systems which amplify more or less random segments of DNA from an AUD region. It is likely that the AUD region is subjected to some sort of hyperrecombination that leads to the constructs being integrated into the chromosome during an amplification event. It is possible that the observed amplification/deletion structures do not represent the primary integration event, but include further rounds of DNA rearrangement to obtain a relatively stable structure. It is interesting to speculate whether the rearrangements caused by the AUD are important in the generation of the range of novel chemical products observed in these experiments. This might be answered if it were possible to transpose the *otc* cluster to a more stable region of the chromosome where the expected disrupted constructs would probably be obtained.

In addition to the linear chromosome, the genome of *S. rimosus* R6 carries the linear plasmid, pPZG101, of 387 kb in size (*10*) and two prophages, RP2 and RP3 (*9, 27*). The plasmid pPZG101 consists of a unique central region of about 30 kb flanked by terminal inverted repeats of about 180 kb. Parts of pPZG101 can integrate into the chromosome. It is also possible for the region of the chromosome, containing the *otc* genes to be translocated to the linear plasmid to form a plasmid prime (pPZG103) (*11*). These interactions occur by a single cross-over between the plasmid and the chromosome to generate a recombinant molecule with one plasmid end and one chromosome end. The linear topology of the molecules means that single cross-overs generate recombinants rather than an even number of cross-overs being necessary as for circular chromosomes and plasmids. If two different polyketide synthesis clusters were present in one strain: one in a linear plasmid and the other in the chromosome, then the homology between the clusters could allow crossing-over to generate plasmid primes carrying recombinant clusters. This might offer an elegant strategy for generating a variety of recombinant polyketides.

There is one more complication that could influence future work considerably. In principle, Class II morphological variants could potentially be excellent hosts for the production of recombinant polyketides. They have lost the entire *otc* cluster, so should be a 'clean' genetic background as they no longer make OTC (discussed above) (*25*). Also, having been isolated from a strain that produces commercial quantities of OTC, they are likely to have a physiology that allows a plentiful supply of metabolic precursors to act as 'fuel' for synthesis of recombinant polyketides. Class II variants are easily recognizable by their pale appearance (Fig. 6). However, spontaneous, but extremely infrequent, pseudo-revertants of the pale Class II mutants were noticed by restoration of the brown (wild-type, R6) phenotype, characteristic of strains that produce OTC. A trivial interpretation, that they were simple contaminants of the original (R6) OTC producer strain, was rejected because all pseudo-revertants tested so far have a different restriction pattern of their genomic DNAs when digested with rare-cutting enzymes and subjected to pulsed field gel electrophoresis. They were named 'Class IIR' (Fig. 7) to designate them as 'pseudo-revertants of the Class II phenotype'. Of nine independent Class IIR isolates (seven from Class II strain MV9 and two from Class II strain MV15), four have been studied in greater detail (MV9R-1 to MV9R-4). The antibiotic that each produces was identified as OTC in three different TLC systems (Fig. 7).

When more sensitive analytical detection (biological assay, Fig. 6; TLC, Fig. 7; and HPLC, Fig. 8) of the progenitor Class II strains (MV4, MV7, MV8, MV9, MV11 and MV15) was undertaken, it was discovered that they actually synthesize an extremely small amount of OTC (about 0.1 μg/mL), despite being deleted for the *otc* cluster when tested on Southern blots.

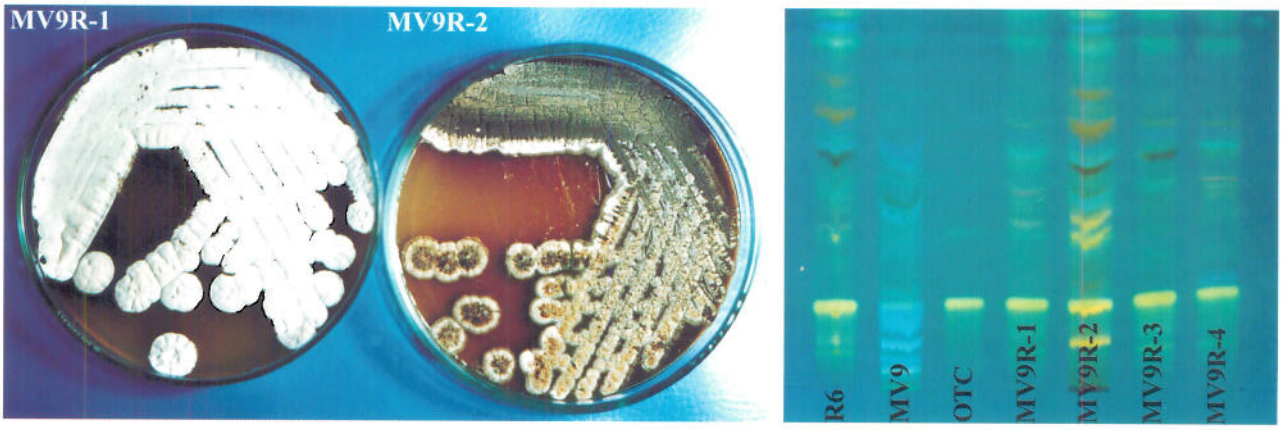

Fig. 7. Colony morphology (left) of *S. rimosus* R6 strains MV9R-1 and MV9R-2. Thin layer chromatography of fermentation products (right) of strains R6, MV9, MV9R-1, MV9R-2, MV9R-3 and MV9R-4 using dichlormethane:methanol:water (59:36:6) system. Lane 1, R6; lane 2, MV9; lane 3, purified OTC; lane 4, MV9R-1; lane 5, MV9R-2; lane 6, MV9R-3; lane 7, MV9R-4.

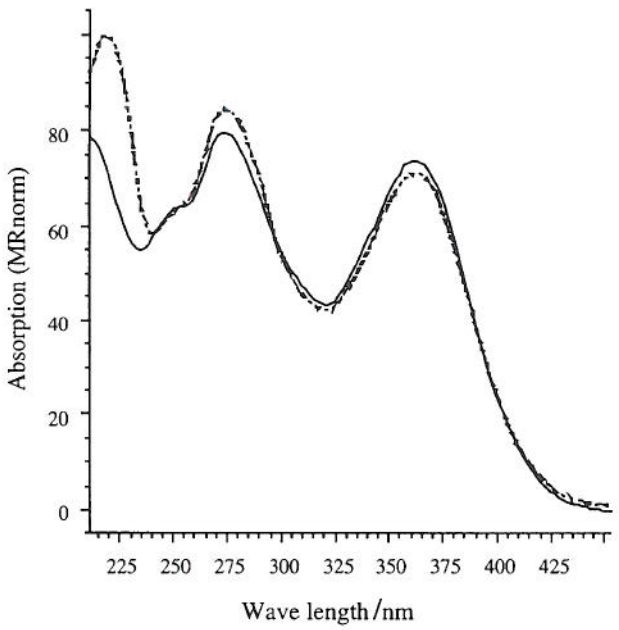

Fig. 8. UV-VIS absorption spectral analysis of the partially purified biologically active fermentation product of the strain MV9 (------). The spectrum of purified OTC is indicated (——) [HPLC was run using t-butanol:phosphate buffer + Na_2EDTA:water (pH = 7.5) step gradient (1:3:22 to 4:3:19) on a reverse-phase (PLRP-S 100Å) column (Polymer Laboratories) at 254 nm].

At present, we have two possible explanations for this phenomenon. Firstly, there is a possibility that a second divergent gene set, presumably silent, and capable of directing synthesis of OTC is present in the chromosome of *S. rimosus* R6. Secondly, since streptomycetes are filamentous bacteria it is possible that an extremely small number of parental genomes is present in hyphae, segregating occasionally to form Class IIR derivatives. Whatever the case, this finding has implications for the production of hybrid polyketides by genetic engineering. This may complicate the construction and use of such strains for the production of hybrid polyketides.

In conclusion, our investigation of *S. rimosus* R6 has revealed that the *otc* gene cluster lies in a somewhat unstable region of the linear chromosome. However, it may still be manipulated to result in recombinants that produce novel chemical structures of potential therapeutic value. This approach is providing a valuable insight into the fundamental biology of polyketide biosynthesis that should, in turn, lead to the generation of further new structures. It is also providing information that will result in engineering of the parent strain R6 to decrease its genetic instability and improve the economics of the commercial production of OTC.

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Nove hibridne poliketidne supstancije, dobivene genetičkim inženjerstvom metaboličkog puta, za biosintezu oksitetraciklina

Sažetak

Iz kromosomske DNA soja S. rimosus R6 klonirana je cjelovita skupina gena čiji produkti sudjeluju u biosintezi antibiotika oksitetraciklina (OTC). Restrikcijska se mapa te skupine ne razlikuje od one klonirane iz soja S. rimosus M15883, što znači da se rezultati dobiveni s ta dva soja mogu spojiti. Geni otcD1 i otcC inaktivirani su unutar kromosomske DNA soja S. rimosus R6 umetanjem genetičkoga biljega. Vjeruje se da je OtcD1 uključen u savijanje, ciklizaciju i aromatizaciju pretpostavljenog nona-ketidnog međuprodukta u biosintezi OTC-a (prije-poliketidna biosinteza), dok OtcC hidroksilira cjelovitu tetracikličku jezgru na poziciji C-6 (poslije-poliketidna biosinteza). Sojevi s inaktiviranim otcD1 i otcC genima više ne sintetiziraju OTC. OtcC::gmr sintetizira tri nove supstancije, dok je proizvod biosinteze otcD1::ermE veći broj supstancija koje roditeljski soj ne sintetizira. Te nove supstancije sadržavaju 9, 15 i 17 C-atoma u svojoj strukturi umjesto 19 C-atoma u OTC-u. Inaktivirani sojevi, međutim, ne sadržavaju očekivanu strukturu kromosomske DNA već pokazuju amplifikacije i delecije. Razlog tomu je što se skupina otc gena nalazi unutar dijela DNA podložnog amplifikaciji (AUD) koji je izložen čestim spontanim preraspodjelama DNA. Spontani su mutanti (II. skupine), u kojih je skupina otc gena izgubljena delecijom, potencijalni domaćini za ekspresiju novih »hibridnih« poliketidnih genskih skupina. Iz tih je mutanata, međutim, moguće izolirati derivate (skupina IIR) koji imaju antibiotičku aktivnost. Pokazalo se da je za tu antibiotičku aktivnost odgovoran OTC, te da mutanti II. skupine i sami sintetiziraju vrlo malu količinu tog antibiotika.