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Partial Activation of a Silent Angucycline-type Gene Cluster from a Rubromycin β Producing *Streptomyces* sp. PGA64

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In the course of DNA-fingerprinting our strain collection for antibiotic biosynthesis genes, two different type II polyketide synthase (PKS) gene clusters were observed from *Streptomyces* sp. PGA64. Phylogenetic analysis placed these together with known rubromycin and angucycline biosynthetic gene clusters. The host strain itself has a very clean production profile of secondary metabolites, which composes mainly of rubromycin β under typical fermentation conditions. Sequencing of a 16.5 kb fragment from the putative angucycline cluster revealed eight genes that were homologous to typical type II PKS genes responsible for synthesizing aromatic polyketides. These genes were especially similar to genes from known angucycline biosynthetic gene clusters and also synteny to these clusters was observed. In addition, three genes were recognized that are needed for priming the minimal PKS complex before polyketide synthesis can initiate, but which are not normally found to cluster with antibiotic biosynthesis genes. A putative repressor gene that was dissimilar to repressor genes found from well-characterized antibiotic biosynthesis gene clusters was also discovered. Gene disruption of the repressor resulted in partial activation of the cluster and production of two angucycline metabolites, UWM6 and rabelomycin. The results confirm that the DNA-fingerprinting method we have developed can be used to correctly detect compounds that are not visible in chemical screens.

Streptomyces are a genus of soil-dwelling Gram-positive bacteria, which have been extensively used in the pharmaceutical industry for the past decades as a rich source of bioactive natural products. The genomes of *Streptomyces coelicolor* A3(2)[1] and *Streptomyces avermitilis*[2], which have recently been sequenced, have revealed that these bacteria seem to have even more genes for antibiotic production than what has been previously thought by detecting compounds with traditional screens. For example, sequence analysis of non-ribosomal peptide synthetase homologues showed that a peptide siderophore coelichelin (a compound that has not been detected in *S. coelicolor* A3(2) cultures) could be encoded in the *S. coelicolor* A3(2) genome[3]. With the availability of recombinant DNA technology and the ever-increasing molecular methodology toolbox for the genetic engineering of *Streptomyces*, it is intriguing to examine the possibilities of using these cryptic antibiotic biosynthesis genes for combinatorial biosynthesis[4] and for the production of novel compounds.

In order to take full advantage of this genetic diversity, we have developed a screening method for the classification of bacterial strains based on the phylogenetic differences in their antibiotic biosynthesis genes[5,6]. The method is based on amplification and sequencing of a conserved fragment from a ketosynthase (KS_α) gene, which is an essential gene in the biosynthetic pathway of aromatic (type II) polyketides. The KS_α is responsible, together with a homologous ketosynthase (KS_β) and an acyl carrier protein (ACP), for the production of a linear polyketide chain with a fixed chain length, which is subsequently cyclized, aromatized and modified by other gene products found in type II PKS gene clusters[7]. The biosynthesis of many clinically important aromatic polyketides, like doxorubicin[8] and tetracycline[9], proceed through a type II polyketide pathway.

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Table 1. Bacterial strains and plasmids used in this study.

Strain	Comment	Source/Reference
E. coli XL1/XL2-Blue MRF'	Cloning host	Stratagene
Streptomyces sp. PGA64	Rubromycin β producer	6)
Streptomyces sp. PGA64-min	minimal PKS mutant	This work
Streptomyces sp. PGA64-R4	regulatory gene mutant	This work
pFD666 ATCC 77286	Cosmid vector	ATCC
pUC19	*E. coli* cloning vector	Pharmacia

During the course of screening our strain collection, two different type II PKS gene clusters were found from one of our strains[6]. In our production media the strain designated as PGA64 produced mainly one compound that was active against *Candida albicans* and Gram-positive bacteria. Structural analysis of the active compound revealed that the compound was a known aromatic polyketide, rubromycin *β*. Here we describe the cloning, characterization and partial activation of the other type II PKS gene cluster that was isolated from the genome of *Streptomyces* sp. PGA64.

Materials and Methods

Bacterial Strains and Culture Conditions

The bacterial strain PGA64 was isolated from a soil sample collected at Barcelona, Spain, and classified to belong to the genus *Streptomyces* as previously described[6]. The strain was cultivated in E1-medium[10] for production of metabolites. DNA manipulations were performed in *Escherichia coli* XL1-Blue MRF′ (Stratagene), which was cultivated in LB-medium[11]. The bacterial strains used in this study are shown in Table 1.

Cloning of the Angucycline Gene Cluster

Genomic DNA was isolated from PGA64 using standard procedures[12]. A genomic library was prepared with Gigapack III XL (Stratagene) using DNA partially digested with *Sau*3AI and ligated to BamHI digested pFD666 vector according to supplier's protocols. A 613 bp fragment from the KS_{α} gene was previously PCR-amplified and sequenced with the method devised by us[5] and used as a probe in colony hybridization.

Sequencing and Sequence Analysis

First, ends of *Pst*I and *Bam*HI subclones from cosmid pGA64-11 (Fig. 1) were sequenced to get an overview of the region cloned. A 16.5 kb DNA region containing genes for aglycone biosynthesis was then sequenced by subcloning with suitable restriction enzymes using standard methods[11]. Sequencing was performed using an automatic ABI310 DNA sequencer (Applied Biosystems) according to manufacturer's instructions. Sequence analysis was done with the Vector NTI Suite Version 6.0 software package (Informax) and with the GCG package Version 8.0 (Genetics Computer Group).

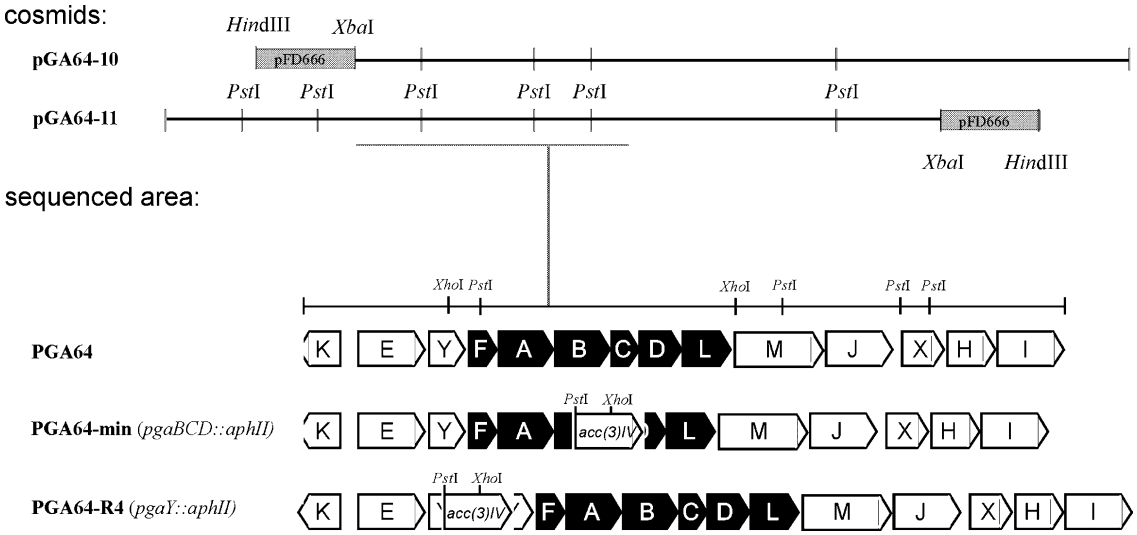
Gene Disruption

For inactivation of the minimal PKS region by homologous recombination a 1.6 kb *Bgl*II-*Not*I fragment from a 5.8 kb *Pst*I fragment in pUC19 (Pharmacia), which contained the minimal PKS region, was first replaced with a 1.5 kb apramycin resistance gene (*aac(3)IV*). The subsequent 5.6 kb insert was further cloned into plasmid pDH5, which contains both ampicillin and thiostrepton resistance genes, to give the final gene disruption construct pDST2ap^r. Protoplasts from strain PGA64 were transformed according to standard methods[12] with approximately 10 μg of single stranded plasmid pDST2ap^r. Six hundred micrograms of apramycin was added to R2YE plates after a 24-hour's regeneration period. Thiostrepton sensitive (Thio^S) and apramycin resistant (Apr^R) colonies were then analyzed with Southern blot hybridization.

For inactivation of the putative repressor gene *pgaY*, the apramycin resistance gene was cloned into a *Xho*I-*Psh*AI digested plasmid pNCO, which contained a 3.8 kb *Nco*I fragment from the strain PGA64 in pUC19 (Pharmacia), to give plasmid pNCOap^r. The insert of pNCOap^r was further cloned into pDH5 as a *Pae*I fragment to give the final gene disruption construct pDNap^r. Transformation and screening of mutants was done similarly as with plasmid pDST2ap^r.

In both cases the phenotype of all of the transformants was Thio^S Apr^R indicating that homologous recombination appears to proceed frequently through a double recombi-

Fig. 1. Organization of the gene cluster isolated from *Streptomyces* PGA64.



The two cosmids are depicted in the upper portion, with *Pst*I restriction sites shown in the inserts. Boxes represent the cloning vector pFD666 that was used. In the lower segment, the sequenced area is shown in detail as well as the genetic organization of mutants PGA64-min and PGA64-R4, which were generated in this study. *Pst*I and *Xho*I restriction sites, which were used in the identification of the mutants in Southern blot hybridization, are also shown. Genes are marked with arrows; black arrows indicate PKS genes (see text for details).

nation event in this strain. The mutants were designated as PGA64-min and PGA64-R4 (transformed with plasmids pDST2ap^r and pDNap^r, respectively).

Southern Blot Hybridization

Chromosomal DNA was isolated from the mutant strains PGA64-min and PGA64-R4 by standard procedures[12]. To investigate the integration of the minimal PKS gene disruption construct, chromosomal DNA from the wild type and from mutant PGA64-min were digested with *Pst*I. In the minimal PKS mutant, the integration of the resistance gene changed the length of the hybridizing *Pst*I-fragment from 5.8 kb to 2 kb (the resistance gene contains an additional *Pst*I restriction site, see Figure 1) when a probe hybridizing to *pgaA* was used, confirming that the resistance gene had integrated into the desired region. In order to see if the repressor gene disruption construct had integrated correctly, chromosomal DNA from the wild type and from mutant PGA64-R4 were digested with *Xho*I. The *Xho*I site within the *pgaY* repressor gene was destroyed during the construction of the gene disruption construct (see above) and subsequently, the length of the hybridizing fragment changed from 5.5 kb to 6.2 kb (the apramycin resistance gene contains an internal *Xho*I restriction site 700 bp from the end of the gene, see Figure 1) when the same probe as with PGA64-min was used, indicating again that the integration had occurred in the right position.

Analytical Methods

Metabolites were detected by HPLC (LaChrom, Merck Hitachi, pump L-7100, detector L-7400 and integrator D-7500) using a Purospher (5 μm, 125×4 mm) column and an acetonitrile - 0.1% HCOOH-gradient. The flow-rate was 1 ml/minute and detection at 254 and 515 nm. MS-FAB+-spectra was taken on a FISONS ZABSPEC-oaTOF spectrometer.

Nucleotide Sequence

The nucleotide sequence data reported in this paper has been deposited in the GenBank database under the accession number AY034378.

Results

Cloning and Sequence Analysis of the 16.5 kb DNA Region

Approximately 10,000 colonies were screened with the

KS_α hybridization probe and from these, two overlapping cosmids, pGA64-10 and pGA64-11, were obtained that spanned about 50 kb of DNA from the region of the probe. A general architecture about the antibiotic gene cluster isolated was obtained through partial shotgun sequencing of the cosmids and it is shown in Figure 1. The sequenced 16.5 kb fragment, which includes the minimal PKS and other genes responsible for aglycone biosynthesis, was located in the left region of the cosmids, so that about 8 kb and 25 kb of cloned DNA extends from the sequenced region to the upstream and downstream directions, respectively.

The left, upstream, region seems to contain more regulatory genes: in addition to the repressor gene found within the sequenced region (see later), partial sequence of a two component regulatory system, homologous to the regulatory genes *jadR1* and *jadR2* from *Streptomyces venezuelae* ISP5230, was observed. The *S. venezuelae* ISP-5230 genes *jadR1* and *jadR2* have been shown to control the biosynthesis of the antibiotic jadomycin by forming an interacting stress-responsive regulatory system[13]. In turn, the right, downstream, region seems to contain genes needed for the biosynthesis of deoxysugar moieties found typically in aromatic polyketides. Genes homologous to various glycosyl transferases, dNTP-glucose synthases and 4′-ketoreductases were found (data not shown).

The sequenced 16.5 kb DNA fragment contains a total of 14 open reading frames with an interesting mixture of both typical and atypical genes when compared to other characterized type II PKS gene clusters (see below). The codon usage of the observed genes was investigated with the CODON-PREFERENCE program and was found to be very similar to the known codon usage of *Streptomyces* with a high GC-bias on the 3rd nucleotide. The overall GC content of the sequenced region was 72% and within the coding regions the average 3rd nucleotide GC content was 94%. From the 14 sequenced genes 13 genes are translated in the same direction and only *pgaK* is translated from the other DNA strand. Moreover, a clear transcriptional stop signal was encountered in the 370 bp non-coding region between *pgaJ* and *pgaX* in the form of a highly stable secondary structure, which we were able to sequence only by subcloning a short 800 bp fragment near the site of the secondary structure. The deduced functions of the pga gene products are shown in Table 2 as well as their closest homologs obtained from BLAST analysis and motifs found to match Pfam documentations.

Table 2. Deduced functions of the gene products found in the *pga* angucycline gene cluster, their closest homologs and protein motifs.

Name	Size	Function	Closest homolog and antibiotic produced	GenBank accession no.	Identity / Similarity	Protein motif	Pfam accession no.
PgaK	incomplete	unknown	*S. fradiae*, Orf12, tylosin	T44591	39 / 50		
PgaE	492	oxygenase	*S. aureofaciens*, Aur1A, unknown	AAK59996	79 / 85	monooxygenase FAD binding domain	01360 01494
PgaY	220	regulator	*S. aureofaciens*, Aur1B, unknown	AAK59997	66 / 74	tetR family	00440
PgaF	109	cyclase	*S. cyanogenus*, LanF, landomycin	AAD13535	82 / 90		
PgaA	423	ketosynthase a	*Kibdelosporangium aridum*, ArdIorf1, unknown	AAA67433	84 / 91	ß-ketoacyl synthase	00109 02801
PgaB	406	ketosynthase ß	*S. cyanogenus*, LanB, landomycin	AAD13537	77 / 86	a Q163H mutation in the active site	
PgaC	90	acyl carrier protein	*S. griseus*, ORF3, griseusin	C55587	77 / 87	phosphopantetheine attachment site	00550
PgaD	262	ketoreductase	*S. venezuelae*, JadE, jadomycin	AAB36565	88 / 94	dehydrogenase	00106
PgaL	315	aromatase	*S. griseus*, ORF4, griseusin	E55587	81 / 85	polyketide cyclase/dehydrase	03364
PgaM	785	oxygenase-reductase	*S. aureofaciens*, Aur1I, unknown	AAK60004	70 / 77	monooxygenase FAD binding domain dehydrogenase	01360 01494 00106
PgaJ	489	transporter	*S. fradiae*, UrdJ2, urdamycin	AAF00207	46 / 54		
PgaX	281	phosphopantheine transferase	*S. aureofaciens*, Aur1L, unknown	AAK60007	62 / 71	4´-phosphopante-theinyl transferase	01648
PgaH	318	malonyl-CoA acyl carrier protein	*S. aureofaciens*, Aur1M, unknown	AAK60008	62 / 70	acyl transferase domain	00698
PgaI	522	decarboxylase, β subunit	*S. cyanogenus*, LanP, landomycin	AAD13544	85 / 92	carboxyl transferase	01039

Deduced Functions of the *pga* Gene Products

PKS Genes

The *pgaABC* gene products were similar to known antibiotic biosynthesis genes that form the minimal PKS complex, which is responsible for the formation of the polyketide chain. *pgaA* is likely to be translationally coupled to the following *pgaB* gene as the termination codon of *pgaA* overlaps the start codon of *pgaB*. Based on sequence similarity, PgaA and PgaB are ketosynthase subunits KS_{α} and KS_{β}, respectively. Translational coupling of these subunits is typical in many aromatic polyketide biosynthesis gene clusters. A surprising finding was that a thorough protein motif scan revealed that the highly conserved glutamine in the putative KS_{β} active site[14] has changed to histidine in PgaB. The exact role of this amino acid in polyketide synthesis has not been unequivocally elucidated, since in the actinorhodin system changing the residue to alanine greatly reduced malonyl-ACP decarboxylation[14], but in another study a change to cysteine did not affect decarboxylation[15]. Nevertheless, the unusual Q163H replacement in PgaB might have an effect in the activity of the gene product. The *pgaC* gene product is highly similar to various acyl carrier proteins and it contains a putative phosphopantetheine attachment site.

pgaD is homologous to ketoreductases that are responsible for the reduction of the C9 carbonyl group to hydroxyl in the biosynthetic pathways of reduced polyketides like anthracyclines and angucyclines. Consistent with the biosynthetic pathways of reduced polyketides, the cloned cluster also contains a gene, *pgaL*, which is homologous to bifunctional cyclase/dehydrogenases (aromatases) that catalyze the closure and aromatization of the first ring. Finally, immediately upstream of *pgaA*, separated by 37 nt, there is an open reading frame that contains a short gene (*pgaF*, 109 amino acids) that is homologous to angucycline specific cyclases like *jadI*[16]. In a previous study we have shown that PgaF is a polyketide cyclase, which is responsible for the cyclization of the fourth angular ring in angucycline biosynthesis[17].

The six genes presented here (shown in black in Figure 1) are highly conserved in the biosynthetic pathways of angucyclines and are responsible for the production of the first stable intermediate compound, UWM6[16] in angucycline biosynthesis. These genes also show synteny to all of the gene clusters that are responsible for the production of known angucycline metabolites, namely to the gene clusters of landomycin[18], urdamycin A[19] and jadomycin B[20].

Post-PKS Genes

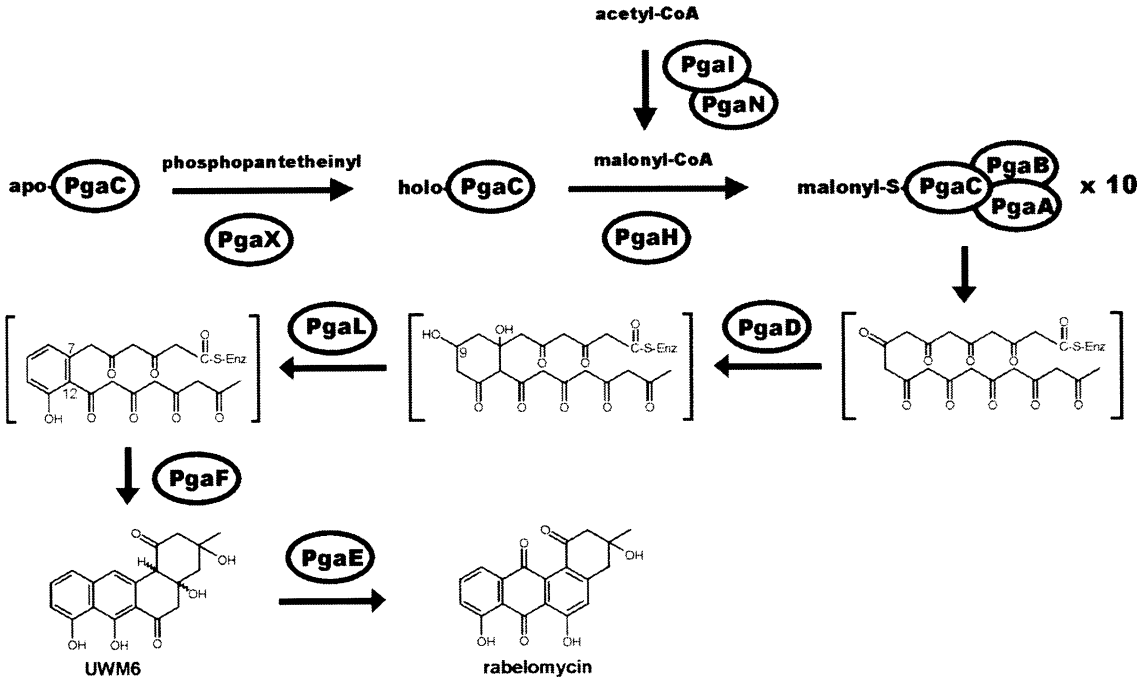
The sequenced area contains two genes, *pgaE* and *pgaM*, that are most likely responsible for modification of the polyketide aglycone. *pgaE* is similar to many oxygenases, for example *urdE* from the urdamycin pathway, which is likely to be responsible for the oxygenation at C12 of UWM6 in the formation of rabelomycin[21]. Correspondingly, PgaE contains motifs for mono-oxygenation and FAD-binding according to Pfam. The other polyketide modification gene *pgaM* resembles oxygenase/reductases (*urdM* from the urdamycin gene cluster that has been shown to be involved in the oxygenation of C12b in urdamycin A biosynthesis[21] and *aur1I* from an unknown angucycline-like gene cluster[22]). *pgaM* contains an oxygenase like *N*-terminal domain that contains the mono-oxygenase and NAD-binding motifs and a reductase like *C*-terminal domain, which contains a motif for dehydrogenation. In addition, the gene contains an unusual internal start codon between the *N*- and *C*-terminal domains similarly to *urdM*.

Genes Involved in Priming of the PKS

Before the minimal PKS complex can condense malonyl-CoA units to form a polyketide chain, a few general requirements have to be met. First, a sufficient cellular pool of malonyl-CoA has to be generated to meet the substrate requirements of the PKS. Strong evidence indicates that, at least in *S. coelicolor* A3(2), this pool is generated by carboxylation of acetyl-CoA[23]. In the cryptic angucycline gene cluster, this function could be performed by PgaI, which is similar to the non-biotinylated β-subunit of acyl-CoA carboxylases. In addition, the biotinylated α-subunit of the two component acyl-CoA carboxylases was found to reside approximately 6 kb upstream of the minimal PKS in the partially sequenced region (data not shown). Second, a flexible phosphopantetheinyl arm has to be attached to the acyl carrier protein to convert it from an inactive apo-protein to a catalytically active holo-form. This could be catalyzed by PgaX that is homologous to 4′-phospho-transferases and contains a motif typical for this enzyme superfamily. And finally, the malonyl group has to be transferred from CoA to the phosphopantetheine arm of the ACP by the action of a malonyl CoA:ACP acyltransferase[24]. Correspondingly, a gene designated as *pgaH* was found within the sequenced region, which is homologous to CoA:ACP acyltransferases.

In general, genes responsible for these enzymatic activities have not been found in most of the characterized type II PKS gene clusters and it is thought that these

Fig. 2. A hypothetical biosynthesis route for the production of UWM6 and rabelomycin by the *pga* gene products.



The possible priming mechanism of the minimal PKS complex is also shown. The gene product PgaN was identified from the partially sequenced region approximately 6 kb upstream of the minimal PKS region. Brackets indicate hypothetical structures.

functions are performed by proteins borrowed from fatty acid biosynthesis. Therefore it is interesting to notice that the *pga* cluster contains all of the enzymatic activities needed for priming the minimal PKS complex (Fig. 2) and that the cluster could, in theory, function totally independently from primary metabolism.

Other Genes

pgaJ is similar to various transmembrane efflux proteins and according to TMHMM analysis[25] it is likely to contain 14 transmembrane helices. *pgaK*, which was only partially sequenced, was found to be similar to ORF12 of unknown function from the tylosin biosynthesis gene cluster. Finally, a gene designated as *pgaY*, which contains a DNA binding motif of the tet^r-repressor family, was found within the sequenced area. *pgaY* was found to have sequence homology only to *aur1B* from a recently sequenced PKS gene cluster of unknown function[22]. Based on the DNA binding motif found in the protein, PgaY is likely to be involved in regulation of the gene cluster.

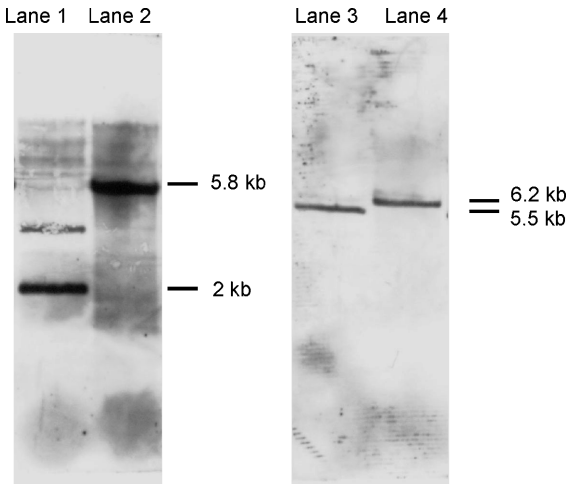
Inactivation of the *pgaBCD* and *pgaY* Genes

In order to confirm that the cloned area is responsible for the production of an angucycline metabolite, like sequence analysis suggested, two different gene disruptions were performed. First, in order to rule out the possibility that the region was responsible for the production of rubromycin, a fragment from the minimal PKS region was replaced with an apramycin resistance gene (Fig. 1). Second, in order to investigate the role of the putative repressor gene (*pgaY*), another gene disruption construct was designed to delete an internal part of this gene (Fig .1).

Apramycin resistant thiostrepton sensitive mutants designated as PGA64-min (for the minimal PKS mutant) and PGA64-R4 (for the repressor gene mutant) were analyzed with Southern blot hybridization (Fig. 3), which confirmed that integration of the gene disruption plasmids had occurred as desired. The appearance of colonies, sporulation and growth characteristics of the mutants were identical to those of the wild type.

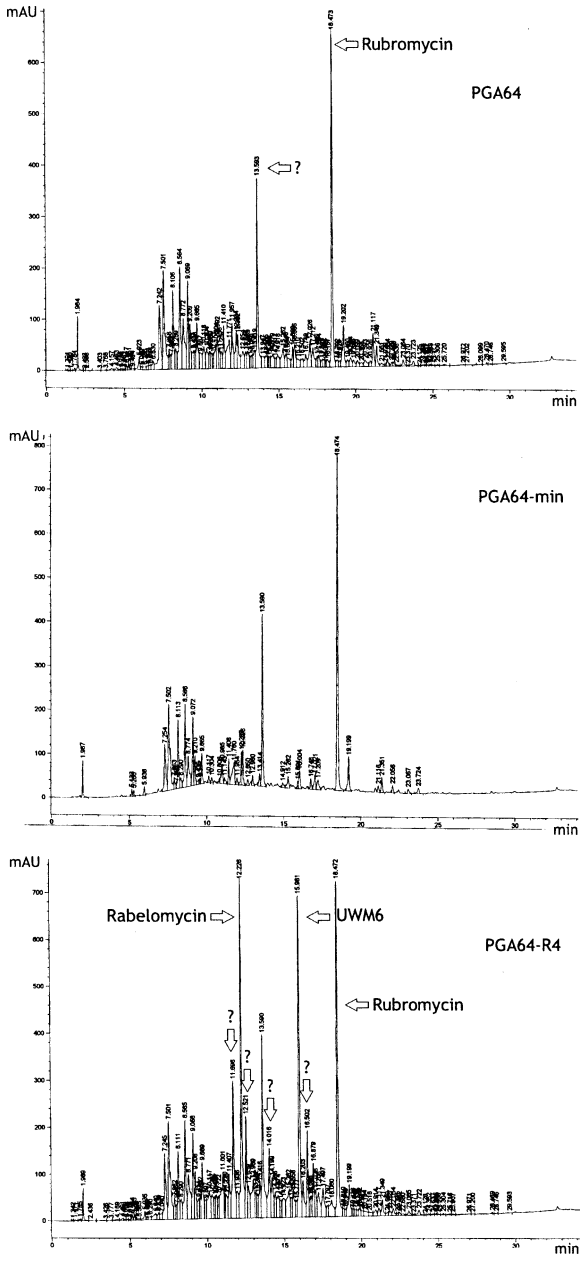
The effect of the disruption of the minimal PKS region

Fig. 3. Results from the Southern blot hybridizations of the mutant strains PGA64-min and PGA64-R4.



The samples are in the following order; (lane 1) digestion of PGA64-min chromosomal DNA with *Pst*I, (lane 2) wild type *Pst*I-digestion, (lane 3) wild type *Xho*I-digestion and (lane 4) PGA64-R4 *Xho*I-digestion. The approximate sizes of the fragments are also shown.

Fig. 4. HPLC-chromatograms of chloroform extacts of different cultures used in this study.



The chromatograms that are shown were recorded by a UV-VIS detector set to 254 nm. The samples taken are from a) PGA64, b) PGA64-min and C) PGA64-R4 cultivations, which were all grown in E1-medium.

and the repressor gene on the production of secondary metabolites was then investigated further. The strains were cultivated in E1-medium[10] supplemented with 20 g/liter of XAD-7 adsorbent for 7 days, after which the metabolites produced were extracted from the XAD-7 resin with methanol and analyzed with HPLC coupled to a UV-VIS detector. The results revealed that the disruption of the minimal PKS region did not have any effect on the production profile of the strain when compared against that of the wild type (Fig. 4), confirming that the cloned region was not responsible for the production of rubromycin. However, the production profile of PGA64-R4 differed markedly from the others (Fig. 4); in addition to the compounds produced by the wild type and strain PGA64-min, various other metabolites could be detected from the culture broth. The major products were identified as UWM6 and rabelomycin by comparing the UV-VIS and mass spectra to authentic samples of UWM6 and rabelomycin obtained from other studies[17]. Because UWM6 and rabelomycin (Fig. 2) are known precursors of angucycline biosynthetic pathways, these results confirmed that the gene cluster is responsible for the production of an angucycline metabolite.

Discussion

Since the discovery of streptomycin in 1944, traditional screening methods have revealed that a variety of microorganisms, especially members of the genus *Streptomyces*, have the capacity to produce a wealth of small bioactive compounds with interesting biological characteristics. However, as a result of decades of screening of bacterial isolates with traditional screening methods, novel compounds are becoming increasingly difficult to obtain; in the 1970s and early 1980s the number of new compounds fluctuated between 70 and 100 per year, after which the number has declined[26]. During the past few years, sequencing of the genomes of two streptomycetes have revealed that these bacteria appear to have the genetic capability to produce even more compounds than what have been thought[1,2]. Set against this background, we thought that it would be interesting to investigate if cryptic antibiotic biosynthesis genes, which are found in abundance in the genomes of streptomycetes, could be used for the production of novel compounds.

In this study, we have cloned and analyzed a silent antibiotic gene cluster that was discovered from one of the strains in our culture collection. Sequence analysis revealed that the cluster has strong sequence similarity to the gene clusters of angucycline antibiotics. Indeed, inactivation of a putative repressor gene *pgaY* resulted in the production of two angucycline metabolites, UWM6 and rabelomycin, by the mutant strain PGA64-R4, which confirmed that the strain has the ability to produce angucyclines. This showed for the first time that with the genetic approach used here it is possible to obtain compounds from *Streptomyces* that cannot be detected from bacterial cultivations with any traditional screens. It also confirmed that the DNA-fingerprinting study[6] correctly predicted that the cluster is responsible for producing an angucycline metabolite, and verified the value of the method in the search of interesting antibiotic biosynthesis gene clusters.

Moreover, the PGA64-R4 mutant strain appears to produce many other minor metabolites in addition to rubromycin, rabelomycin and UWM6, which are not found in the cultivations of the parental strain (Fig. 4). This is interesting in the light that there are two different type II antibiotic PKS gene clusters operational at the same time in this strain. Therefore in the future it would be interesting to identify the metabolites in order to see whether the compounds are shunt products of the individual pathways or whether they are synthesized by the concomitant action of both rubromycin and angucycline gene clusters.

Because the gene cluster isolated in this study contains many putative genes involved in modification reactions as well as in deoxysugar biosynthesis, it is unlikely that UWM6 and rabelomycin are the end products of the gene cluster. Consequently it seems that the disruption of the unusual repressor gene *pgaY* is not solely sufficient to release the whole cluster from its tight regulation. Further evidence for this comes from a separate gene cassette based study in which we have investigated the roles of the modification genes *pgaE* and *pgaM* (Fig. 1); PgaE oxygenase catalyzes the formation of rabelomycin from UWM6, whereas PgaM appears to modify rabelomycin to some other unidentified compound (M. METSÄ-KETELÄ, unpublished data). Therefore the production of UWM6 and rabelomycin, which also forms non-enzymatically from UWM6[16,17], by PGA64-R4 suggests that neither *pgaE* nor *pgaM* that are located upstream and downstream of the *pgaY* repressor gene respectively (Fig. 1) are expressed in this strain. Hence we suggest that the key for the successful activation of the whole cluster resides elsewhere, possibly in the gene homologous to *jadR2*, or in the combination of the *jadR2* homologue and *pgaY*.

The exact role of the PgaY gene product in the regulatory cascade cannot be unequivocally deduced at this point; one possibility is that it controls the regulation of the putative *pgaABCDLF* operon. However, it should be noted that all of the genes needed for the production of UWM6 and rabelomycin[16,17] (*pgaABCDLF*) lie immediately after the integrated apramycin resistance gene *acc(3)IV* (Fig. 1) and therefore the possibility that the production of these compounds results from a polar effect generated by the apramycin resistance gene cannot be excluded.

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