Transcriptional regulation of mithramycin biosynthesis in *Streptomyces argillaceus*: dual role as activator and repressor of the PadR-like regulator MtrY

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The mithramycin biosynthesis gene cluster of *Streptomyces argillaceus* ATCC 12956 contains 34 ORFs and includes two putative regulatory genes (*mtmR* and *mtrY*), which encode proteins of the SARP (*Streptomyces* antibiotic regulatory protein) and PadR transcriptional regulator families, respectively. MtmR was proposed to behave as a positive regulator of mithramycin biosynthesis. Inactivation and overexpression of *mtrY* indicated that it is also a positive regulator of mithramycin biosynthesis, being non-essential but required to maintain high levels of mithramycin production in the producer strain. Transcriptional analyses by reverse transcription PCR and quantitative real-time PCR of mithramycin genes, and promoter-probe assays in *S. argillaceus* polyketide synthase and regulatory mutants and the WT strain, and in the heterologous host *Streptomyces albus*, were carried out to analyse the role of MtmR and MtrY in the regulation of the mithramycin gene cluster. These experiments revealed that MtmR had a positive role, activating expression of at least six polycistronic units (*mtmR–mtmE, mtmQ–mtmTII, mtmX–mtrY, mtnV–mtmTIII, mtnW–mtmMI* and *mtmGII–mtrB*) and one monocistronic unit (*mtmGII*) in the mithramycin gene cluster. However, MtrY played a dual role in the mithramycin gene cluster: (i) repressing the expression of resistance genes and its coding gene itself by controlling the activity of the *mtmRp* promoter that directs expression of the regulator *mtrY* and resistance genes, with this repression being released in the presence of mithramycin; and (ii) enhancing the expression of mithramycin biosynthesis genes when mithramycin is present, by interacting with the *mtmRp* promoter that controls expression of the *mtmR* regulator, amongst others.

INTRODUCTION

*Streptomyces* is a genus of Gram-positive bacteria belonging to the phylum *Actinobacteria*, which is characterized by a cycle of morphological differentiation and is an important source of bioactive compounds, such as antibiotics, antitumour compounds and immunosuppressant agents, amongst others (Bérdy, 2012; Chater, 1993; Flárdh & Buttnner, 2009; Newman & Cragg, 2012). In general, biosynthesis of these compounds takes place in parallel with the development of aerial mycelium in surface-grown cultures or at the end of the exponential growth phase in submerged cultures, when starvation and other environmental factors appear (Bibb, 2005; Liu et al., 2013; Martin et al., 2011; Méndez et al., 1985; van Wezel & McDowall, 2011). Therefore, biosynthesis genes of bioactive compounds are not usually expressed throughout the entire life cycle, but rather are under the control of a complex network of regulatory elements (Bibb, 2005; Cundliffe, 2006; Liu et al., 2013; Martin & Liras, 2010; van Wezel & McDowall, 2011). This regulation is exerted at different levels and usually involves one or more cluster-situated regulators (CSRs; Huang et al., 2005), which are normally considered as 'low-level' or pathway-specific regulators, although it has been found that some of them can control 'high-level' regulators and also the expression of other biosynthesis pathways (Huang et al., 2005; Rodriguez et al., 2008). Pathway-specific regulation can involve a single regulator, as in the case of the actinorhodin and streptomyacin biosynthesis pathways (Fernández-Moreno et al., 1991; Retzlaff & Distler, 1995); in other cases, several pathway-specific regulators are involved, such as in the daunorubicin and tyllosin biosynthesis pathways.

Abbreviations: CSR, cluster-situated regulator; DW, dry weight; qRT, quantitative real-time; RLU, relative luminescence units; RT, reverse transcription; SARP, *Streptomyces* antibiotic regulatory protein.

One supplementary table and two supplementary figures are available with the online Supplementary Material.
Mithramycin is a member of the aureolic acid group that was approved for use as an anticancer drug in 1970 (Newman & Cragg, 2012). The mithramycin gene cluster of Streptomyces argillaceus has been cloned and characterized (Lombo et al., 2006). In addition, using combinatorial biosynthesis strategies, several new derivatives have been generated, some of which showed higher antitumour activity and/or less toxicity than the parental compound (Albertini et al., 2006; García et al., 2011; Núñez et al., 2012; Pérez et al., 2008; Remsing et al., 2003). Moreover, in recent years new uses and activities have been described for mithramycin, such as inhibition of apoptosis or antiangiogenic activity, which has renewed interest in this drug (Chatterjee et al., 2001; Jia et al., 2007, 2010), and in enhancing the production yields of mithramycin and its derivatives. Recently, several metabolic engineering strategies have been successfully applied to increase the production of mithramycin and derivatives (Zabala et al., 2013). A complementary approach could be to manipulate regulatory genes involved in its biosynthesis, which will require an understanding of the regulation of mithramycin biosynthesis. The mithramycin gene cluster contains two CSRs, mttmR and mtrY, which have been proposed to be involved in the regulation of mithramycin biosynthesis (Garcia-Bernardo et al., 2000; Lombo et al., 1999). mtmR codes for a SARP (Streptomyces antibiotic regulatory protein)-like regulator that is essential for mithramycin biosynthesis (Lombo et al., 1999). Previous information on mtrY did not show any similarity to known regulatory proteins, but indicated the existence of a putative helix–turn–helix motif that suggested its possible regulatory role (Garcia-Bernardo et al., 2000). In this paper, we addressed the study of the transcriptional regulation of mithramycin biosynthesis by studying the expression pattern of the mithramycin genes in the WT in comparison with mutants in specific regulatory genes, and by analysing the effect of these regulatory elements on the activity of their promoters, in the presence and absence of mithramycin.

**METHODS**

**Bacterial strains and culture conditions.** Streptomyces argillaceus ATCC12596, a mithramycin producer, was used as the donor of chromosomal DNA, and for gene replacement and expression experiments. *S. argillaceus* M13R1 is a mtmR-minus mutant (Lombo et al., 1999) and *S. argillaceus* APK is a mithramycin non-producer mutant with a deletion in the polyketide synthase genes (Lombo et al., 1996). Streptomyces albus J1074 (Kieser et al., 2000) was used as the host for promoter-probe assays. For mithramycin production and luciferase tests, an overnight culture of the corresponding strain in tryptic soy broth medium was used as a seed culture to inoculate three 250 ml flasks containing 50 ml RSA liquid medium (Fernández et al., 1998), until OD<sub>600</sub> 0.2 was achieved. Cultures were then incubated at 30 °C and 250 r.p.m. for several days. Escherichia coli ET12567/pUB307 (Kieser et al., 2000) was used for conjugation experiments. When required, antibiotics were added at the following final concentrations: ampicillin (100 μg ml<sup>−1</sup>), kanamycin (50 μg ml<sup>−1</sup>), chloramphenicol (25 μg ml<sup>−1</sup>), apramycin (25 μg ml<sup>−1</sup>), thiostrepton (50 μg ml<sup>−1</sup>) and hygromycin (200 μg ml<sup>−1</sup>).

**DNA manipulation and plasmids.** DNA manipulation was performed according to standard procedures for *E. coli* (Sambrook et al., 1989) and for *Streptomyces* (Kieser et al., 2000). Platinum *Pfx* DNA polymerase (Invitrogen) and 2.5 % DMSO were used for all PCR amplifications. Purified amplicons were sequenced to confirm their identities. pBSKT (Lombo et al., 1999) was used for gene replacement. pIAGO (Aguirrezabalaga et al., 2000) is a bifunctional and multicopy plasmid, and was used for gene expression experiments. pLUXAR+ (Rodríguez-Garcia et al., 2007) is an integrative promoter-probe vector. pUK21 (Vieira & Messing, 1991) was used for subcloning. pFL3R1 and pFL3R (Lombo et al., 1999), and pJG144 (Garcia-Bernardo et al., 2000) are pIAGO derivatives containing mtmR and mtrY genes, respectively, and were used for complementation and/or expression experiments.

**Plasmid constructs.** Several plasmid constructs were made in order to assay the promoter activity located upstream of the mtmR, mtrX and mtrY genes. DNA regions were amplified by PCR using oligonucleotides listed in Table S1 (available in the online Supplementary Material) and cloned into the BamHI, EcoRI or NotI sites of the promoter-probe plasmid pLUXAR+, generating pR, pX and pY, respectively. To assay these promoter activities in *S. argillaceus* mutant strains, a hygromycin resistance cassette was subcloned as a 1.7 kb NotI/SpeI fragment from pLYyg (Olano et al., 2004) into the unique XbaI site of pLUXAR +, pR and pY, divergently from luxAB genes, generating pLUX-H, pR-H and pY-H respectively.

To test the effect of regulatory proteins on mtmR and mtrY promoters, several plasmids were constructed as follows. To generate pLUX-E, pR-E and pY-E, the ermEP promoter was first subcloned as a 300 bp EcoRI/BamHI fragment from pIAGO into the EcoRI/BamHI unique sites of pUK21, generating pUK21-E, and then it was rescued as a SpeI fragment and subcloned into the XbaI site of pLUXAR +, pR and pY, respectively (see Fig. 5). pR-R, pY-R, pR-Y and pY-Y were generated by PCR amplifying mtmR and mtrY genes using the primers listed in Table S1, and cloned under the control of the erythromycin resistance promoter (ermEP) in the XbaI site of pUK21-E. Then, each gene plus ermEP was rescued as a SpeI fragment and cloned into the XbaI site of pR and pY, divergently from luxAB genes, generating the different plasmid constructs shown in Fig. 5.

**Generation of mutant *S. argillaceus* ΔY.** For the generation of this mutant, plasmid pABF2 was constructed as follows. A BgII DNA fragment (7.5 kb) containing mtmOIV, mtrX, mtrY, mtrA, mtrB and the 3’ end of mtmGI was cloned into the BamHI site of pBSKT. Then, an apramycin resistance cassette was subcloned as a PstI fragment from pEFBA (Lozano et al., 2000) into the NsiI site located in the mtrY gene, in the same direction of transcription. This construct (pABF2) was used to transform *S. argillaceus* protoplasts. Transformants were selected with apramycin and were tested for their susceptibility to thiostrepton to select mutant strains.

**Gene expression analysis.** Total RNA was obtained from *S. argillaceus* WT and *S. argillaceus* mutants M13R1 and ΔY. Samples (20 ml) were harvested from cultures of these strains after growth for 48 h (i.e. during mithramycin production; see Fig. 1), were mixed with 30 ml RNA Protect Bacteria Reagent (Qiagen) and maintained at...
room temperature for 5 min. Mycelia were then harvested by centrifugation and stored at −20 °C until use. Total RNA was isolated from frozen pellets using a standard lysis method (Kieser et al., 2000) and purified using a RNeasy Midi kit (Qiagen) according to the manufacturer’s instructions. Isolated RNA was subjected to an additional DNase I treatment (TURBO DNA-free; Ambion) to eliminate DNA contamination. This RNA was used as template for gene expression analysis by reverse transcription (RT)-PCR.

Qualitative gene expression was studied using the SuperScript One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen). cDNA synthesis was performed using 20–150 ng total RNA as template (depending of the primer pairs) at 50 °C for 30 min, followed by heating at 94 °C for 2 min. Sample mixtures included 32.2 U RNaseguard RNase inhibitor (Amersham Biosciences). Amplification conditions were: 33 cycles at 95 °C for 1 min; 45–60 °C (depending of primers) for 1 min; 72 °C for 1 min; and a final extension step at 72 °C for 10 min. Oligonucleotides (Table S1) were designed within structural, resistance and regulatory genes of the mithramycin biosynthesis gene cluster. Oligos were designed to produce cDNAs of ~400–600 bp and the identity of these primers was verified by direct sequencing. Negative controls for each pair of primers were carried out with Platinum Taq DNA polymerase (Invitrogen) in the absence of reverse transcriptase. To elucidate the transcriptional organization at the 5′ and 3′ ends of the mithramycin biosynthesis gene cluster, primers were designed to amplify some intergenic regions (Table S1). hrdB gene expression levels were used to normalize RNA concentration of the tested strains, as described previously (Rodríguez et al., 2008). The RT-PCR products were resolved by 1.4% agarose gel electrophoresis, stained with ethidium bromide and visualized using a Gel Doc system (Bio-Rad).

Quantitative real-time (qRT)-PCR was used to quantify gene expression of selected genes. A total amount of 0.5 µg RNA was used to synthesize cDNA using an iScript cDNA synthesis kit (Bio-Rad). qRT-PCRs were carried out on an ABI PRISM 7500 (Applied Biosystems), with SYBR Green PCR Master Mix (Applied Biosystems). Triplicate PCRs were carried out for each sample analysed and three independent experiments were performed. hrdB was used as a housekeeping gene in each sample in order to standardize the results by eliminating variation in RNA and cDNA quantity and quality. Absence of chromosomal DNA contamination was checked by qRT-PCR. Primers (Table S1) were designed using the algorithms provided by Primer Express software 2.0 (Applied Biosystems) and their efficiency was calculated based on the slope of a standard curve. To determine amplification specificity, an additional dissociation curve analysis was performed after the last cycle, showing one single peak in all cases. PCR results were given as the increase in the fluorescence signal of the reporter dye detected and visualized by

![Graph](image-url) **Fig. 1.** Mithramycin production of (a) S. argillaceus WT and regulatory mutant strains, and (b) S. argillaceus WT overexpressing regulatory genes. Lines and bars correspond to the growth of cultures (DNA concentration) and mithramycin production, respectively. S. argillaceus WT and S. argillaceus WT expressing pIAGO (triangles and white bars); S. argillaceus ΔY and S. argillaceus WT expressing mtrY (diamonds and grey bars); and S. argillaceus M13R1 and S. argillaceus WT expressing mtrR (circles and black bars). Values represent the mean±SD of three independent experiments.
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the 7500 System SDS software version 1.4 (Applied Biosystems). Changes in gene expression are represented with respect to the control sample (WT strain).

**Luciferase assay, mithramycin production and growth determination.** The luciferase activity coded by luxAB was measured in a Luminoskan luminometer (Labsystems) as described previously (Rodríguez-García et al., 2007; Santos-Beneit et al., 2008). Cell samples from 1 ml RSA cultures were frozen until use for luminescence assays [in relative luminescence units (RLU)]. Mithramycin production was determined by HPLC using a B condomack C18 column (Waters). Samples (0.5 ml) from cultures of the different strains were extracted with ethylacetate supplemented with 5 % formic acid. Mithramycin concentrations were estimated by measuring peak areas and comparing them with known concentrations of mithramycin. In parallel, samples (1 ml) were harvested for growth determination (as DNA concentration) by the colorimetric diphenylamine method of Burton (1956). For dry weight (DW) determination, samples were washed twice with Milli-Q water, dried for 4 days at 80 °C and then weighed. Measurements were always obtained from three independent cultures.

**RESULTS**

**Role of mtmR and mtrY in mithramycin production**

Two putative regulatory genes have been identified in the mithramycin gene cluster: mtmR, which encodes a SARP-type activator protein (Wietzorrek & Bibb, 1997) that was previously proposed to be an activator of mithramycin biosynthesis (Lombó et al., 1999), and mtrY, which is ~40 kb away from mtmR and codes for a putative regulatory protein (Garcia-Bernardo et al., 2000). Comparisons of MtrY with proteins in databases showed similarities with several PadR-like proteins of unknown function from different actinomycetes, such as GenBank accession number AFO53534 from Streptomyces sp. WAC1438 (59 % identical amino acids), GenBank accession number WP_018681227 from Actinokineospora enzananesis (62 %) or GenBank accession number WP_020644137 from Amycolatopsis balhimycina (59 %). In order to confirm the role of mtrY in mithramycin biosynthesis, a novel null mutant strain (S. arcellaceus ΔY) was generated by inserting the apramycin resistance cassette aac(3)IV in mtrY, in the same direction of transcription to avoid a possible polar effect on downstream genes. PCR and Southern blot analysis were performed to confirm the replacement of the WT copy of mtrY by the mutated version (Fig. S1). Mithramycin production of S. arcellaceus ΔY was monitored in liquid cultures, and was compared with S. arcellaceus M13R1 (mtmR null mutant) and with the WT strain (Fig. 1a). The maximum level of mithramycin production in the WT strain was reached after 120 h, at the stationary phase. Neither mithramycin nor any biosynthesis intermediates were detected in cultures of M13R1, whilst mutant ΔY produced mithramycin, although production levels were ~70 % lower than in the parental strain. Mithramycin production was recovered/increased in S. arcellaceus M13R1 and ΔY expressing WT copies of mtmR and mtrY, respectively, indicating that genes located downstream of the targeted regulatory genes were expressed in the mutant strains (Fig. S2). In addition, expression of any of these genes in the WT strain had a positive effect on mithramycin production: mtmR (pFL3R) led to a high increase in mithramycin production throughout the growth period tested, with final yields ~155 % higher, whilst in the case of S. arcellaceus expressing mtrY (pJG144), mithramycin production was slightly higher than in the control strain (final yields ~13 % higher) (Fig. 1b). These results were in agreement with previous data obtained under other cultivation conditions (Garcia-Bernardo et al., 2000; Lombó et al., 1999). All these results suggested that both mtmR and mtrY coded for positive transcriptional regulators of mithramycin biosynthesis, mtmR but not mtrY being essential for mithramycin production, and the latter being required to maintain high levels of mithramycin production.

**Transcriptional regulation of the mithramycin gene cluster**

To investigate the role of mtmR and mtrY in the transcriptional regulation of mtm genes, gene expression analysis of selected mtm genes was evaluated by RT-PCR. Total RNA was isolated from cultures grown for 48 h (corresponding to the beginning of the production stage in the WT strain) from S. arcellaceus WT and mutant strains S. arcellaceus ΔY (mtrY-minus) and S. arcellaceus M13R1 (mtmR-minus). As shown in Fig. 2, all genes were expressed in the WT strain, including regulatory (mtmR and mtrY), resistance (mtmX, mtrA and mtrB) and structural genes. On the contrary, transcription was absent for most of the genes in the mtmR-minus mutant, with the sole exception of two sets of genes located at the ends of the mtm gene cluster, which were transcribed, but at a significantly lower level of expression than in the WT strain: mtmA–mtmD (at the left end of the cluster) and mtrX–mtrB (at the right end of the cluster). Mutant M13R1 contains an apramycin resistance cassette that replaced mtmR and its upstream promoter region, and the upstream region of mtmZ. This resistance cassette had its own promoter and lacked a transcriptional terminator. Therefore, transcription of genes located downstream of the apramycin resistance cassette could be directed by the apramycin resistance promoter. This would imply that this group of genes is transcribed as a polycistronic transcriptional unit from mtmR to mtmE. To confirm this fact, the possible co-transcription of these genes in the WT strain was analysed. Primers were designed to synthesize cDNAs from the different intergenic regions (Table S1). As shown in Fig. 3(a), transcripts between upstream and downstream genes were detected in all the cases, confirming that mtmR–mtmE could be co-transcribed as a polycistronic unit. However, transcription of genes located at the right end of the cluster (mtrY–mtrB) in the mtmR-minus mutant could be explained if transcription of these genes was partially MtmR-independent (see below).

In the case of the mtrY-minus mutant, transcription of most genes was lower than in the WT strain, including...
mtrmR and those genes involved in polyketide and sugar biosynthesis. Exceptions were mtrX–mtrB genes, which were apparently transcribed at the same or even higher levels in the mtrY-minus mutant than in the WT strain. The mtrY-minus mutant was generated by inserting the apramycin resistance cassette in mtrY and therefore some transcription of genes downstream of this cassette could be driven by the apramycin resistance promoter.

These results were consistent with the idea that MtmR was the main and essential activator of the mithramycin gene cluster, and that MtrY was a positive regulator of mtrmR. Moreover, these results indicated that transcription of mtrX–mtrB could be differentially regulated from the rest of the genes and suggested the existence of additional promoters within this region that could be MtmR-independent.

Transcriptional organization of the right end of the cluster

At the right end of the mtm gene cluster there is a set of genes transcribed in the same direction (mtmGI–mtrB) that could constitute a polycistronic unit. However, results described above suggested that some of these genes (mtrX–mtrB) were differentially expressed. In order to further investigate the transcriptional organization of this region we decided to analyse expression of intergenic regions from mtmGI to mtrB by RT-PCR, in the WT and in the regulatory null mutants (Fig. 3b). Expression was detected for all intergenic regions both in the WT strain and in the ΔY mutant, although the amount of transcripts was significantly different in both strains: whilst in the ΔY mutant the transcript levels for the mtmOIV–mtrX intergenic region were lower, those corresponding to intergenic regions mtrX–mtrY, mtrY–mtrA and mtrA–mtrB were equal or even higher. Moreover, in the mtrR-minus mutant (M13R1), transcripts corresponding to intergenic regions mtmGI–mtmOIV and mtmOIV–mtrX were not detected and those for other intergenic regions were detected, but at a lower level.

To further support these results, qRT-PCR analysis was carried out (Fig. 3c). RNA from the WT strain was used as a calibration sample. Analysis of transcripts from the ΔY mutant confirmed that expression levels of mtmGI and mtmOIV were lower in the mutant in relation to the WT strain; however, on the contrary, expression levels of mtrX, mtrY, mtrA and mtrB were higher, with mtrA and mtrB showing the highest expression levels. Although some expression from mtrY to mtrB could be attributed to the inserted apramycin resistance cassette within mtrY in this mutant, this could not explain the amount of transcripts corresponding to mtrX. However, expression of mtmGI and mtmOIV was not detected in the M13R1 mutant, whilst mtrX, mtrY, mtrA, and mtrB genes were poorly expressed and clearly at a much lower level than in the WT strain, with the amount of the mtrX transcript being the lowest. All these results indicated that in the WT strain mtmGI–mtrB were co-transcribed from a promoter located upstream of mtmGI that was MtmR-dependent; however, in the absence of MtmR, mtrX–mtrB could be co-transcribed from other promoter regions.

In order to confirm the existence of additional promoter regions, DNA regions upstream of mtrX and mtrY were amplified by PCR and were subcloned into the promoter-probe vector pLUXAR + upstream of reporter genes luxAB, generating pX (mtrXp) and pY (mtrYp). These plasmids were introduced into the S. albus J1074 strain, and the activities of promoters were measured by the luciferase assay and using S. albus containing the empty vector pLUXAR + as control (Table 1). The luciferase activities observed with pX and pY compared with the control confirmed the existence of promoter regions in these DNA fragments, but with important differences in promoter strength: whilst pY showed a luciferase activity ~99-fold higher than the control at 24 h, the activity of pX was much lower (~13-fold higher than the control). In all cases, the maximum promoter activity was achieved after 24 h and then gradually decreased (data not shown). These results indicated that in the absence of MtmR, mtrYp was the main promoter activity controlling expression of mtrY–mtrB and that mtrXp could be
responsible for the low transcription level detected for mtrX under these conditions.

Regulation of mtmRp and mtrYp promoters by MtmR and MtrY

The results described above showed that expression of mtm genes was MtmR-dependent, with the exception of resistance genes that could be expressed in its absence, but at a much lower extent. In addition, they indicated that MtrY positively affected expression of mtmR, thus favouring expression of all MtmR-dependent genes. Also, these results suggested that MtrY would act as a repressor of resistance genes and of its coding gene. To further clarify the role of regulatory proteins MtmR and MtrY, it was decided to assay promoter activities of mtmRp (promoter located upstream of mtmR) and mtrYp in the presence and absence of MtmR and MtrY.

Fig. 3. Transcriptional analysis of mtm genes located at the ends of the mithramycin gene cluster. (a, b) Gene organization and transcription analysis by RT-PCR of the intergenic regions of mtm genes located at the left (a) and right (b) end of the cluster. Genes are represented by black (regulatory), dark grey (structural), light grey (resistance) and white (unassigned function) arrows. Deduced transcriptional units are indicated by solid arrows. (c) qRT-PCR quantification of the expression levels of mtm genes located at the right end of the cluster (mtmGI–mtrB). The graph shows the relative expression of these genes in M13R1 (mtmR-minus) and ΔY (mtrY-minus) mutants in relation to the WT strain (WT). The hrdB gene was used as an internal control. Results represent the mean ± SD of three independent experiments.
First, promoter activities of \textit{mtmRp} (pR-H) and \textit{mtrYp} (pY-H) were tested in \textit{S. argillaceus} strains either blocked in \textit{mtmR} (\textit{S. argillaceus} M13R1) or in \textit{mtrY} (\textit{S. argillaceus} \textit{ΔY}). As shown in Fig. 4, both promoters showed the highest activity between 12 and 24 h of incubation, and then their activities gradually decreased. In the case of \textit{mtrYp}, the activity increased again after 72 h. This pattern was not observed when this promoter was tested in the \textit{S. albus} J1074 strain (data not shown), which suggested the existence of additional factors in \textit{S. argillaceus}, such as regulatory proteins or metabolites (i.e. mithramycin and/or biosynthesis intermediates) that could modulate \textit{mtrYp} expression. As can be observed in Fig. 4(a), the lack of MtM caused a decrease in the activity of \textit{mtmRp}, which was much more noticeable at later incubation times, and also of \textit{mtrYp} (Fig. 4b). However, the absence of MrT caused a consistent increase in promoter activity of \textit{mtrYp}, being ~24- and 22-fold higher at 24 and 72 h, respectively (Fig. 4b). In the absence of MrT, the \textit{mtmRp} activity increased at 12 and 24 h of incubation, and then showed a sharp decrease in activity at later incubation times. These results suggested that \textit{mtrYp} and \textit{mtmRp} were positively regulated by MtM, and negatively regulated by MrT, although this negative effect on \textit{mtmRp} was dependent on the incubation time. The positive role of MtM on \textit{mtrYp} activity suggested by these experiments was apparently in contradiction to the fact that \textit{mtrY} was expressed from \textit{mtmRp} in the \textit{mtmR}-minus mutant. To try to clarify this, \textit{mtrYp} activity was also assayed in a mutant containing \textit{mtmR} and \textit{mtrY}, but blocked at the earliest step of mithramycin biosynthesis (\textit{S. argillaceus} \textit{ΔPK}). \textit{mtrYp} showed a decrease in promoter activity in this mutant strain compared with the WT strain, suggesting that it is the absence of mithramycin that affects \textit{mtrYp} activity rather than the presence/absence of MtM.

To clarify these results, lessening the influence of the \textit{S. argillaceus} background and of mithramycin (and biosynthesis intermediates), we tested the effect of each regulatory protein on promoter activities in \textit{S. albus}. The \textit{mtmRp} and \textit{mtrYp} promoters were cloned upstream of the \textit{lux} operon in a pLUXAR derivative (pLUX-E), in which the erythromycin resistance promoter (\textit{ermEp}) was cloned divergently from the \textit{lux} operon (Fig. 5). To test the effect of MtM and MrT on the activity of these promoters, their coding genes were independently cloned downstream of \textit{ermEp}, generating the constructs shown in Fig. 5. These plasmid constructs were introduced into \textit{S. albus}, and the effect of the MtM and MrT proteins on promoter regions was evaluated by measuring luciferase activity over time (Fig. 6a). Comparisons of luciferase activities driven by \textit{mtmRp} in the absence of regulatory proteins (pR-E) with those obtained in the presence of either \textit{mtmR} (pR-R) or \textit{mtrY} (pY-E) genes confirmed that this promoter was positively regulated by MtM and that MrT caused some repression, which was more noticeable at earlier incubation times (31 and 26% lower luciferase activities at 24 and 48 h, respectively). On the contrary, comparison of the promoter activity of \textit{mtrYp} in the absence of regulatory proteins (pY-E) and in the presence of \textit{mtrY} (pY-Y) also showed that MrT exerted some repressor effect on its own promoter, which was very obvious at 24 h of incubation.

**Table 1. Luciferase activity of \textit{mtrXp} and \textit{mtrYp} promoters in \textit{S. albus}**

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<th>Strain</th>
<th>Luciferase activity* [RLU (mg DW)(^{-1})]</th>
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<td>24 h</td>
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<td>48 h</td>
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<tr>
<td>\textit{S. albus} pLUXAR +</td>
<td>1294 ± 310</td>
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<tr>
<td>\textit{S. albus} pX</td>
<td>17 377 ± 380</td>
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<td>\textit{S. albus} pY</td>
<td>127 493 ± 9036</td>
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*The results are the mean ± SD of three independent determinations.

**Fig. 4.** Promoter activities of (a) \textit{mtmRp} and (b) \textit{mtrYp} in \textit{S. argillaceus} WT, and mutant strains M13R1 (\textit{mtmR}-minus), \textit{ΔY} (\textit{mtrY}-minus) and \textit{ΔPK} (mithramycin non-producer mutant). Cultures were grown in R5A supplemented with hygromycin, and samples were taken at 0, 12, 24, 48 and 72 h post-incubation for luciferase assays. Results represent the mean ± SD of three independent experiments. pR-H and pY-H are pLUX-H derivatives with \textit{mtmRp} and \textit{mtrYp} cloned upstream of the \textit{lux} operon, respectively.
(50% less luciferase activity). However, the presence of mtmR (pY-R) did not increase the activity of mtrYp. This result was in accordance with those results shown in Figs 2 and 3 that indicated that mtrYp activity was MtmR-independent and supports the assertion outlined above.

Results described above demonstrated the positive regulatory role of MtmR on its own promoter mtmRp, but not on mtrYp, and suggested that MtrY acts as a repressor of mtrYp and mtmRp. This result contradicts the initial data both at the production and transcriptional level in S. argillaceus that indicated that MtrY acts as an activator of mithramycin biosynthesis. In concordance with the results shown above, we considered that some of the differences observed could be caused by the absence of mithramycin in cultures of S. albus. To test the influence of mithramycin on promoter activities, we incubated S. albus strains in R5A medium supplemented with 1 μg mithramycin ml⁻¹ (a concentration below the MIC of mithramycin for S. albus) and evaluated the activity of promoters by measuring luciferase activity (Fig. 6b). When mithramycin was added to the cultures, MtrY did not repress mtmRp, but rather increased its activity (49 and 53% higher luciferase activities at 24 and 48 h, respectively; see pR-Y versus pR-E in Fig. 6b). Under these conditions, MtrY also did not repress mtrYp after 24 h of incubation. The positive effect of MtrY in the presence of mithramycin on mtrYp was not so obvious, only being clearly detected in cultures at 24 h of incubation. This and the above-mentioned results would explain the apparent inconsistency of why mtrYp activity was lower in the mtmR-minus and ΔPK mutants (Fig. 4b): production of mithramycin is blocked in

**Fig. 5.** Plasmid constructs used in this work to test the effect of expressing mtmR and mtrY on mtrYp and mtmRp promoter activities. luxA and luxB code for the components of luciferase; luxC codes for the enzyme acyl-reductase; mmm, mmr-1 and mmr-2, terminator sites; mtmRp, promoter region upstream of mtmR; mtrYp, promoter region upstream of mtrY; ermEp, erythromycin resistance promoter. The length of the different plasmids is shown in parentheses.
 Regulation of the mithramycin gene cluster is also under the control of the second CSR, mtrY, which codes for a protein similar to PadR-like proteins of unknown function identified in different actinomycetes strains. This is a quite recently identified family of regulatory proteins, named after the phenolic acid decarboxylation repressor of Bacillus subtilis, Pediococcus pentosaceus and Lactobacillus plantarum (Barthelmès et al., 2000; Gury et al., 2004; Tran et al., 2008). To date, only a few members of this family have been characterized and these were shown to regulate diverse processes, including multidrug resistance, virulence, circadian rhythms and detoxification (Agustiandari et al., 2011; Barthelmès et al., 2000; Gury et al., 2004; Huillet et al., 2006; Kovackova et al., 2003). None of these characterized regulators belong to the actinomycetes group nor are they involved in antibiotic biosynthesis. This family of proteins typically possesses a conserved N-terminal winged helix–turn–helix DNA-binding domain and a variable C-terminal helical domain involved in dimerization (Arita et al., 2007; De Silva et al., 2005; Fibrianaš et al., 2012; Madoori et al., 2009). In LmrR, a flat-shaped hydrophobic pore at the dimer centre serves as a multidrug-binding site, existing in an allosteric coupling between the multidrug- and DNA-binding sites, which is proposed to be involved in the induction mechanism (Madoori et al., 2009). Results shown in the current paper indicate that MtrY could play a dual role as a regulatory protein in the mithramycin gene cluster. In a similar way to other PadR-like regulators, various results indicated that MtrY is a repressor of resistance genes and of its coding gene itself, and that this effect is inhibited by the presence of mtrYp (Fig. 6a).

**DISCUSSION**

In this study we carried out a transcriptional analysis of the mithramycin gene cluster in *S. argillaceus* and analysed the role of two CSRs in the regulation of the cluster. The biosynthesis gene cluster of antitumour mithramycin in *S. argillaceus* comprises 34 ORFs, which are organized in at least seven transcriptional units. It includes two CSRs (*mtmR* and *mtrY*) that are located at each end of the cluster (Garcia-Bernardo et al., 2000; Lombó et al., 1999). MtmR is highly similar to transcriptional activators of the SARP family that are involved in the biosynthesis of different antibiotics. Members of this family have been shown to activate the transcription of biosynthesis genes by binding to operator regions located at target promoters (Arias et al., 1999; Sheldon et al., 2002). Similar to other SARPs, MtmR is also a positive regulator that is essential for mithramycin biosynthesis. Transcriptional analysis of the *mtmR*-minus mutant in comparison with the WT strain supports the existence of four polycistronic units (*mtmQ–mtmTII, mtmX–mtmY, mtmV–mtmTIII* and *mtmW–mtmMI*) and one monocistronic unit (*mtmGI*) that are positively regulated by MtmR, as no transcription of these genes was detected in the *mtmR*-minus mutant. MtmR also regulates transcription of a polycistronic unit (*mtmGI–mtrB*) from a promoter located upstream of *mtmGI*, although some transcription of *mtrX–mtrB* still occurs in the absence of MtmR. This could be explained by the existence of additional promoters within this region (mainly *mtrYp*). Moreover, we propose that MtmR also regulates expression of a polycistronic unit (*mtmR–mtmD*) by acting on the *mtmRp* promoter. Although transcripts from this region are still detected in the *mtmR*-minus mutant, we have demonstrated by promoter-probe assays that the activity of the *mtmRp* promoter was lower in the absence of MtmR (*S. argillaceus* AR pR-H versus *S. argillaceus* WT pR-H; Fig. 4a) and that this activity is enhanced by its presence (*S. albus* pR-E versus *S. albus* pR-E; Fig. 6a). The detected residual transcription in this region could be explained by the promoter of the apramycin resistance cassette replacing the *mtmR* gene in this mutant, which directs transcription of downstream genes.

Fig. 6. Effect of expression of *mtmR* and *mtrY* on *mtmRp* and *mtrYp* promoter activities tested in *S. albus* in the (a) absence and (b) presence of mithramycin. Cultures were grown in R5A supplemented with apramycin without (a) or with 1 mg mithramycin ml⁻¹ (b). Samples were taken at 0, 12, 24, 48 and 72 h post-incubation for luciferase assays. Results represent the mean±SD of three independent experiments.

Both mutants, but *mtrY* is expressed. Therefore, in the absence of mithramycin, MtrY negatively affects the activity of *mtrYp* (Fig. 6a).
of mithramycin. In accordance with this, transcription analysis of mtrY and resistance genes comparing the mtrY-minus mutant with the WT strain showed that all these genes were expressed at a higher level in the former than in the latter. We propose that MtrY could play this repressor role by mainly regulating mtmRp promoter activity. Accordingly, promoter-probe assays of mtmRp carried out in S. argillaceus indicated that the activity of this promoter was higher in the absence of MtrY (S. argillaceus ΔY pY-H versus S. argillaceus WT pY-H; Fig. 4b). In addition, promoter-probe assays of mtmRp using S. albus as host in the presence or absence of MtrY showed that expression of mtrY has a negative effect on mtmRp activity (S. albus pY-Y versus S. albus pY-E; Fig. 6a), reinforcing the role of MtrY as a repressor of its own promoter and consequently of genes expressed under its control. Moreover, we showed that mithramycin plays an important role in this regulation, as this repressor effect of MtrY was not observed in a mithramycin non-producer mutant (S. argillaceus ΔPK) or when mithramycin was added to cultures (S. albus pY-Y versus S. albus pY-E; Fig. 6b), which suggests that mithramycin could interact with MtrY by attenuating its putative binding activity to target DNA and thereby modulating mtmRp activity. Some characterized PadR-like proteins play similar roles in the expression of resistance genes. Such is the case of LadR and LmrR that negatively regulate expression of multidrug efflux pumps in Listeria monocytogenes and Lactococcus lactis, respectively; this repression being released upon their interaction with some drugs (Agustiandari et al., 2011; Huillet et al., 2006). In Streptomyces, there are examples of repressors that inhibit expression of antibiotic resistance and export in antibiotic biosynthesis pathways; this repression being diminished by the antibiotic itself and/or some biosynthesis intermediates. Such is the case of TetR-like repressors ActR and SimR from the actinorhodin and simocyclinone gene clusters, respectively (Le et al., 2009; Tahlan et al., 2007; Xu et al., 2012). However, as far as we know this is the first example showing that a PadR-like regulator acts as a repressor of expression of resistance genes in antibiotic gene clusters in Streptomyces.

MtrY also plays a positive regulatory role in mithramycin biosynthesis, as mutants affected in this gene produced lower amounts of mithramycin and its overexpression in the WT strain enhanced mithramycin production. In addition, transcriptional analysis of the mtrY-minus mutant in comparison with the WT strain supports this assertion as all biosynthesis genes were clearly less transcribed in the mutant than in the WT strain, the activator mtmR-coding gene included. We have hypothesized that this positive effect of MtrY on mithramycin biosynthesis is the result of increasing expression of mtmR, either directly or indirectly. However, promoter-probe assays demonstrated that the activity of mtmRp was higher in the absence of MtrY (S. argillaceus ΔY pR-H versus S. argillaceus WT pR-H; Fig. 4a) and was lower when MtrY was present (S. albus pR-Y versus S. albus pR-E; Fig. 6a) at early incubation times, which suggests that MtrY could also be acting as a repressor of mtmRp activity, but was somehow required at later times to sustain its high activity. Curiously, addition of mithramycin to cultures of S. albus expressing mtrY (S. albus pR-Y) not only released the repression, but rather had the opposite effect, as mtmRp showed higher promoter activity in the presence of MtrY and mithramycin (pR-Y versus pR-E; Fig. 6b). These results support the idea that MtrY plays a dual role on mtmRp depending on the conditions tested (i.e. acting as a repressor or positive regulator in the absence or presence of mithramycin, respectively). Although we cannot discard indirect regulatory effects of MtrY on still unknown regulatory signals, the hypothesis drawn here would explain results shown in this paper and would be in agreement with the fact that normal mithramycin yields depend on mtrY expression. A dual role has also been proposed for the TetR-like DnrO

**Fig. 7.** Model for transcriptional regulation of the mithramycin gene cluster. Genes are represented by black (regulatory), dark grey (structural), light grey (resistance) and white (unassigned function) arrows. Dotted arrows indicate deduced transcriptional units. Solid sharp-ended and round-ended arrows indicate activation and repression, respectively.
regulatory protein in the biosynthesis pathway of doxorubicin of Streptomyces peucetius. It was shown that this protein represses expression of its coding gene (this repression being released by the binding of glycosylated biosynthesis intermediates to DnrO) and that the modified protein triggers expression of the regulator dnrN that is essential for doxorubicin biosynthesis (Ajithkumar & Prasad, 2010; Jiang & Hutchinson, 2006; Otten et al., 2000).

Based on the results in this paper, we propose a model for the regulation of mithramycin biosynthesis (Fig. 7). According to this model, at early stages of growth mtrY would be transcribed from mttrYp, and the product MtrY would repress mtmrRp and mttrYp promoters, keeping expression of mithramycin biosynthesis and resistance genes at a low level. With increasing cell growth and in response to still unknown regulatory signals acting on mttrRp, transcription of mttrR would start. MtrR would activate expression of mithramycin biosynthesis genes by acting on at least seven promoters that control expression of one monocistronic and six polycistronic units, which code for all mithramycin biosynthesis enzymes (and resistance genes). This would lead to the beginning of mithramycin production. When the intracellular concentration of mithramycin (or some biosynthesis intermediates) increases, this would bind to MtrY releasing it from mttrYp and allowing stronger expression of resistance genes, and consequently avoiding self-suicide of the producer strain during the production period. In addition, MtrY combined with its ligand mithramycin would interact with mttrRp, enhancing its activity and so transcription of mttrR, which would lead to an increase of production levels of mithramycin at later times.

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