Involvement of a chromomycin ABC transporter system in secretion of a deacetylated precursor during chromomycin biosynthesis

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Chromomycin A₃ is an antitumour antibiotic that acts by inhibiting transcription and replication of DNA. The producer micro-organism *Streptomyces griseus* subsp. *griseus* is highly resistant to chromomycin A₃ and to the structurally related compound mithramycin upon induction with chromomycin A₃. The biosynthetic gene cluster of chromomycin contains three genes involved in self-resistance to chromomycin in *S. griseus*: *cmrA* and *cmrB* encode a type I ATP-binding cassette (ABC) transporter, and *cmrX* encodes a UvrA-like protein of ABC excision nuclease systems. These genes are linked in the chromosome, together with a gene encoding a transcriptional repressor (*cmrR*). Involvement of these genes in chromomycin resistance was determined through gene inactivation, and heterologous expression in *Streptomyces albus*. Inactivation of *cmrX* produced a chromomycin-sensitive low-producer strain, while inactivation of *cmrR* generated a high-chromomycin-producer strain, which was resistant to chromomycin, and also to mithramycin. Expression of either *cmrA* and *cmrB*, or *cmrX*, in *S. albus* generated strains with low chromomycin resistance; it was therefore necessary to co-express the three genes to achieve high levels of resistance. However, the CmrAB ABC transporter conferred a high level of resistance to the biosynthesis intermediate 4A,4E-O-dideacetyl-chromomycin A₃. A model is proposed for the biosynthesis of, and self-resistance to, chromomycin A₃ in *S. griseus* subsp. *griseus*.

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INTRODUCTION

The aureolic acid group of antitumour agents comprises mithramycin, chromomycins, olivomycins, chromocyclomycin, UCH9 and durhamycin (Rohr *et al.*, 1999). The agents inhibit growth and multiplication of several tumour cell lines, and they also act on Gram-positive bacteria. Some of the members of this family have clinical applications, and they are used for the treatment of certain tumours, such as disseminated embryonal cell carcinoma and Paget's bone disease, and also for the control of hypercalcaemia in patients with malignant diseases (Remers, 1979; Skarbek & Speedie, 1981). More recently, chromomycin and mithramycin have been found to stimulate K562 cell erythroid differentiation (Bianchi *et al.*, 1999), and both drugs have been suggested as potential neurological therapeutics (Chatterjee *et al.*, 2001), and for use in the treatment of HIV-1 (Bianchi *et al.*, 1997). The antitumour properties of these compounds are ascribed to their inhibitory effects on replication and transcription processes during macromolecular biosynthesis by interacting, in the presence of Mg²⁺, with

GC-rich nucleotide sequences located in the minor groove of DNA (Sastry & Patel, 1993; Sastry *et al.*, 1995; Barcelo *et al.*, 2007).

Structurally, the aureolic acid compounds belong to the large and important family of the aromatic polyketides. All members of the group (with the exception of chromocyclomycin) consist of a tricyclic chromophore that has one or two aliphatic side chains (aglycone), and which is glycosylated at two different positions with saccharide chains of variable length. Mithramycin and chromomycin A₃ (Fig. 1) share the same aglycone, and they differ only in some of the sugars attached to it (Wohlert *et al.*, 1999). The gene clusters involved in the biosynthesis of mithramycin (Blanco *et al.*, 1996, 2000; Fernández *et al.*, 1998; Lozano *et al.*, 2000; González *et al.*, 2001; Lombó *et al.*, 1996, 1997; Prado *et al.*, 1999) and chromomycin A₃ have been cloned and characterized (Menéndez *et al.*, 2004a, b, 2006). Interestingly, although there is high similarity between most individual mithramycin and chromomycin genes, the arrangement of genes in both clusters is organized in a substantially different way (Menéndez *et al.*, 2004a). It has also been reported that the producer organisms of these two compounds, *Streptomyces argillaceus* ATCC 12956 (mithramycin producer) and *Streptomyces griseus* subsp.

Abbreviations: ABC, ATP-binding cassette; DDACA₃, 4A,4E-O-dideacetyl-chromomycin A₃.

Fig. 1. Chemical structures of mithramycin, chromomycin A₃ and DDACA3.

griseus ATCC 13273 (chromomycin A₃ producer), are naturally highly resistant to their own produced antibiotic, but, despite the close structural similarity between these two drugs, they do not show cross-resistance (Fernández *et al.*, 1996). In the mithramycin producer, the main resistant determinant is a type I ABC (ATP-binding cassette) transporter system (Méndez & Salas, 2001) encoded by the *mtrA* and *mtrB* genes; the system is able to confer high level of resistance to mithramycin, but not to chromomycin (Fernández *et al.*, 1996). Analysis of the chromomycin gene cluster revealed, as a putative self-resistance mechanism, two genes encoding an ABC transporter system, and a gene encoding an UvrA-like protein of ABC excision nuclease systems that are responsible for DNA repair (Menéndez *et al.*, 2004a).

Here, we report the characterization of the chromomycin-A₃-resistance determinants CmrAB and CmrX. We propose a model for chromomycin secretion that involves the transport by the CmrAB ABC transporter of one of the last biosynthetic intermediates in chromomycin A₃ biosynthesis, 4A,4E-O-dideacetyl-chromomycin A₃ (DDACA3),

and its subsequent acetylation by the membrane-bound acetyltransferase CmmA, to finally render the fully active chromomycin A₃. Implications of this secretion model in the biosynthesis and self-resistance to chromomycin in the producer organism are discussed.

METHODS

Micro-organisms, culture conditions and plasmids. *S. griseus* subsp. *griseus* ATCC 13273, a chromomycin A₃ producer, was used as donor of chromosomal DNA, and for gene-disruption experiments. The mutant strain *S. griseus* C10A was used for production of DDACA3, as previously described (Menéndez *et al.*, 2004b). For sporulation on solid medium, the organisms were grown at 30 °C on plates containing A medium (Fernández *et al.*, 1998). For growth in liquid medium, the organisms were grown either in TSB medium (trypticase soya broth; Oxoid) or in R5A medium (Fernández *et al.*, 1998). *Streptomyces albus* J1074 (*ilv-1*, *sal-2*; Kieser *et al.*, 2000) was used as host for expression of chromomycin genes. *Escherichia coli* DH10B (Invitrogen) was used as a host for subcloning. *E. coli* ET12567 (pUB307; Kieser *et al.*, 2000) was used as donor for intergeneric conjugation. When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 5 or 25 µg thiostrepton ml⁻¹ for liquid or solid cultures,

respectively; 100 µg ampicillin ml⁻¹; 25 µg apramycin ml⁻¹; 20 µg tobramycin ml⁻¹, 25 µg kanamycin ml⁻¹; and 25 µg chloramphenicol ml⁻¹. pUC18, pHZ1358 (Kieser *et al.*, 2000), pUK21 (Vieira & Messing, 1991) and pIJ2925 (Kieser *et al.*, 2000) were used for subcloning. pWHM3 (Kieser *et al.*, 2000) and pEM4 (Quirós *et al.*, 1998) were used for expression in *S. albus*. cosGR10 was used as a source of DNA (Menéndez *et al.*, 2004a).

DNA manipulation. Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, DNA ligations, Southern hybridization, and other DNA manipulations, were performed according to standard techniques for *E. coli* (Sambrook *et al.*, 1989) and *Streptomyces* (Kieser *et al.*, 2000). Preparation of *S. albus* protoplasts, and transformation and selection of transformants, were carried out as described (Kieser *et al.*, 2000). Intergeneric conjugation from *E. coli* ET12567 (pUB307) to *S. griseus* subsp. *griseus* was performed as described (Kieser *et al.*, 2000). Computer-aided database searching and sequence analysis were carried out using the University of Wisconsin Genetics Computer Group program package and the BLAST program.

Plasmid constructs

Several constructs (Fig. 2a) were generated in order to localize the chromomycin-resistance determinant, as described below. All

the constructs were introduced by protoplast transformation into *S. albus*, and transformants were selected for thiostrepton resistance.

pNM28 (*cmrA* and *cmrB* genes): a 1.9 kb *EagI* fragment containing *cmrA* and *cmrB*, and the 3' end of *cmrR*, was subcloned into the *NotI* site of pUK21; a *PstI*-*XbaI* fragment was then rescued (using these sites from the polylinker), and subcloned into the same sites of pEM4, downstream of the *ermEp* promoter.

pNM30 (*cmrR*, *cmrA* and *cmrB* genes): a 4.6 kb *PstI* fragment from cosGR10 (one of the restriction sites derived from the vector), including *cmrR*, *cmrA*, *cmrB* and the 5' end of *cmrX*, was subcloned into the *PstI* site of pEM4; in this construct, the *ermEp* promoter controls expression of *cmrR*, *cmrA* and *cmrB*.

pNM34 (*cmrX*, *cmrR*, *cmrA* and *cmrB* genes): a 4.2 kb *BamHI* fragment containing the 5' end of *cmrR*, *cmrX* and *cmrC* was subcloned into the same site of pUC18; this construct, pNC60A, was then digested with *PstI* (there is a *PstI* site within *cmrX*, and another in the polylinker), and a 4.6 kb *PstI* fragment from cosGR10 (one site derived from the vector), containing *cmrR*, *cmrA*, *cmrB* and the 5' end of *cmrX*, was inserted in the correct orientation; the whole insert in pUC18 was finally rescued as a 7.3 kb *HindIII*-*EcoRI* fragment (using these sites from the polylinker), and subcloned into the same sites of pWHM3, generating pNM34.

Fig. 2. (a) Genetic organization of the chromomycin biosynthesis gene cluster, and overlapping cosmids isolated from this region. Black and white bars represent cosmids conferring resistance and sensitivity to chromomycin A₃, respectively. Genes studied in this paper are in dark grey. B, *BamHI* sites. (b) Map of the DNA region surrounding the chromomycin-resistance genes, and constructs generated to localize the resistance determinants. MICs of chromomycin A₃ (C-A3) and DDACA3 for *S. albus* strains expressing the different constructs are shown. Triangles indicate the position and orientation of the *ermEp* erythromycin-resistance promoter. B, *BamHI*; E, *EagI*; Nc, *NcoI*; Nd, *NdeI*; Nt, *NotI*; P, *PstI*.

pNM39 (*cmrR* and *cmrX* genes): a 4.2 kb *Nco*I–*Nde*I fragment, including *cmrR*, *cmrX* and the 5' end of *cmrA*, was subcloned into the same sites of pUK21, generating pUK39; a *Spe*I fragment was rescued (using these sites from the polylinker), and subcloned into the *Xba*I site of pWHM3.

pNM42 (*cmrX* gene): a 4.2 kb *Bam*HI fragment, containing *cmrX* and the 5' end of *cmrR*, was subcloned from pNC60A into the same site of pEM4, downstream of the *ermEp* promoter.

pNM46 (*cmrA*, *cmrB* and *cmrX* genes): a 2.1 kb *Xba*I–*Hind*III (both sites blunt-ended) fragment from pNM28, containing *cmrA* and *cmrB* under the control of *ermEp*, was subcloned into the *Hind*III site (blunt-ended) of pNM42; in this construct, *cmrX* is divergently transcribed to *cmrA* and *cmrB* genes.

Generation of mutants

For the generation of mutant C60RII, plasmid pC60RII was constructed as follows (Fig. 3a). A 2.65 kb *Pst*I–*Bgl*II fragment (this last site was derived from the polylinker) of pUK39, containing *cmrR* and the 5' ends of *cmrA* and *cmrX*, was subcloned into the *Pst*I and *Bam*HI sites of pUC18, generating pUR2. Then, a *Bam*HI–*Hind*III fragment (blunt-ended), containing an apramycin-resistance cassette, was inserted into the unique *Bam*HI site (blunt-ended) of pUR2 located within *cmrR*, and in the same direction of transcription, generating pUR2A. Afterwards, the whole insert was rescued from pUR2A as an *Eco*RI–*Hind*III fragment (using these sites from the polylinker), and subcloned into the same sites of pIJ2925, generating pJR2A. Finally, the complete insert was subcloned as a *Bgl*II fragment (using these sites from the polylinker) into the *Bam*HI site of pHZ1358, generating pC60RII. This construct was introduced by intergeneric conjugation into *S. griseus*, and apramycin-resistant thiostrepton-sensitive transconjugants were selected for further characterization.

For the generation of mutant C60X, plasmid pC60X was constructed as follows (Fig. 4a). pNC60A was digested with *Not*I, which cuts twice within *cmrX*, then treated with Klenow polymerase, and ligated to a

*Hind*III–*Bam*HI fragment (blunt-ended) containing the apramycin-resistance cassette, generating pXA. The whole fragment was rescued from pXA as a *Bam*HI fragment, and subcloned into the same site of pHZ1358, generating pC60X. This construct was introduced by intergeneric conjugation into *S. griseus*, and apramycin-resistant thiostrepton-sensitive transconjugants were selected for further characterization.

Determination of antibiotic resistance. Determination of the MIC was carried out by replica plating the different strains on A medium agar plates containing different concentrations of the compounds. Susceptibility of *S. albus* recombinant strains to chromomycin A₃ and DDACA3 was tested by an agar-diffusion assay. Paper disks, containing 10 µl of each serially diluted compound, were put on A medium that had been seeded with spores of the strains. After incubation at 30 °C for 24 h, the radius of the inhibitory zone was measured, and the radius of the paper disk was subtracted. Data were obtained from three independent experiments.

Determination of chromomycin A₃ production. Chromomycin A₃ production by *S. griseus* wild-type (WT) and mutant strains was determined as follows. A seed culture was prepared in TSB medium, and inoculated with a spore suspension. After incubation for 24 h at 30 °C and 250 r.p.m., the culture was used to inoculate (at 2%, v/v) 250 ml flasks containing 50 ml R5A medium. Production of chromomycin A₃ was monitored daily for 7 days, as follows: 1 ml samples were centrifuged at 14 000 r.p.m. in an Eppendorf centrifuge (model no. 5417) for 2 min, and 200 µl of the supernatant was used to test for chromomycin A₃ production by using HPLC. Samples (10 µl) were analysed for chromomycin A₃ using a Symmetry C18 column (Waters), and isocratic elution with a mixture of 40% acetonitrile and 60% water, acidified with 0.1% TFA, at a flow rate of 1 ml min⁻¹. Detection and spectral characterization of peaks were performed with a photodiode array detector (Waters). Measurement of peak areas, and comparison with known concentrations of chromomycin, was used to estimate chromomycin A₃ concentrations. For each experiment, two independent cultures were run and analysed.

Fig. 3. Insertional inactivation of the *cmrR* gene. (a) Scheme representing the replacement in the chromosome of the WT *cmrR* gene with the gene mutated *in vitro*. *aac*(3)/IV, apramycin-resistance gene; *tsr*, thiostrepton-resistance gene; *bla*, ampicillin-resistance gene. (b) Southern hybridization using the 2.7 kb *Nco*I–*Pst*I fragment as a probe. Chromosomal DNA from the WT and the mutant C60RII was digested with *Nco*I and *Pst*I.

Fig. 4. Insertional inactivation of the *cmrX* gene. (a) Scheme representing the replacement in the chromosome of the WT *cmrX* gene with the gene mutated *in vitro*. *aac3(IV)*, apramycin-resistance gene; *tsr*, thiostrepton-resistance gene; *bla*, ampicillin-resistance gene. (b) Southern hybridization using the 4.2 kb *Bam*HI fragment as a probe. Chromosomal DNA from the WT and the mutant C60X was digested with *Bam*HI and *Pst*II.

RESULTS

Role of the CmrAB transporter system in chromomycin A₃ resistance

The chromomycin gene cluster of *S. griseus* contains two genes, *cmrA* and *cmrB*, that constitute a type I ABC transporter (Menéndez *et al.*, 2004a). An indication of the involvement of this ABC transporter system in chromomycin resistance was first obtained when several cosmid constructs coming from a gene library of *S. griseus*, and harbouring the *cmrA* and *cmrB* genes, were expressed in the chromomycin-sensitive strain *S. albus*, rendering recombinant strains resistant to high levels of chromomycin A₃ (MIC >100 µg ml⁻¹) (Fig. 2a). To confirm that the ABC transporter genes were responsible for this phenotype, we expressed the *cmrA* and *cmrB* genes, under the control of the constitutive erythromycin-resistance promoter *ermE**p (pNM28) (Fig. 2b), in *S. albus*, and we compared the susceptibility to chromomycin A₃ of this recombinant strain (strain NM28) with that of *S. albus* harbouring the vector only (strain EM4). We found that the chromomycin ABC transporter conferred a low level of resistance to chromomycin A₃ (MIC 20 µg ml⁻¹) in comparison with that conferred by the vector (MIC 2.5 µg ml⁻¹). These experiments indicate that the ABC transporter genes alone do not account for the high level of resistance shown by the chromomycin producer. Therefore, other genes must participate in self-resistance to chromomycin in *S. griseus*, and these could be harboured in cosmids, thus conferring a high level of resistance to chromomycin A₃ to other hosts.

Strain NM28 was also tested for mithramycin resistance. Surprisingly, it was found to show a high level of resistance

to mithramycin (MIC >100 µg ml⁻¹). This result was an apparent contradiction to earlier reports that have indicated that the chromomycin producer is sensitive to mithramycin, i.e. *S. griseus* (chromomycin producer) was unable to grow in the presence of mithramycin (Fernández *et al.*, 1996). Since, in the present experiment, the *cmrA* and *cmrB* genes were under the control of a constitutive promoter, we wondered whether the lack of resistance to mithramycin previously observed in the chromomycin producer had been due to the absence of expression of the ABC transporter genes in the conditions tested. We hypothesized that, perhaps, in the chromomycin producer, expression of the chromomycin-resistance genes could be induced during chromomycin production, as this has been shown to occur for other resistance determinants in producer organisms (Pernodet *et al.*, 1993; Vilches *et al.*, 1990). To test this hypothesis, we assayed mithramycin resistance in *S. griseus* after induction with low amounts of chromomycin A₃. On plates inoculated with spores of *S. griseus*, 20 µg chromomycin A₃ was loaded onto a disc, and this was placed in the proximity of another disc containing 100 µg mithramycin. The growth inhibition halo that formed around the mithramycin disc was sharply reduced in the vicinity of the chromomycin disc, suggesting a phenomenon of induction of resistance (Fig. 5). This was verified by evaluating the growth of *S. griseus* on agar plates containing 100 µg ml⁻¹ mithramycin, with and without the addition of 2 µg ml⁻¹ chromomycin A₃ as the inducer agent: the presence of chromomycin allowed normal development of the strain on mithramycin-containing agar plates, while there was no growth in the absence of chromomycin. These experiments clearly indicate that expression of the chromomycin ABC transporter in the

Fig. 5. Induction of mithramycin resistance in *S. griseus* subsp. *griseus* by chromomycin A₃. CH, 20 µg chromomycin A₃; M, 100 µg mithramycin.

producer organism is inducible, and that chromomycin A₃ can be used as an inducer. These results also confirm that the chromomycin ABC transporter confers resistance to mithramycin. In light of these results, the apparent contradiction reported above, regarding the results reported in this paper and those of Fernández *et al.* (1996), is not such a great contradiction, since, in those experiments, *S. griseus* was exposed to mithramycin before the onset of chromomycin production. Consequently, in those conditions, the CmrAB transporter was not expressed, and therefore *S. griseus* could not grow.

Coexpression of the ABC transporter genes with other closely linked genes

In the chromomycin gene cluster, functions have been assigned to most of the gene products and, for many of products, these functions have been proved by insertional inactivation and analysis of the accumulated compounds (Menéndez *et al.*, 2004a, b, 2006). Two genes of the chromomycin cluster, *cmrX* and *cmmRII*, were candidates to participate in chromomycin resistance based on: (i) their location in the vicinity of *cmrA* and *cmrB*, (ii) their presence in cosmids conferring a high level of resistance to chromomycin A₃, and (iii) the putative roles of the deduced gene products by database comparisons. CmrX shows high similarity to several UvrA-like proteins of ABC excision nuclease systems involved in DNA repair. It also showed similarity to MtrX, which is a protein involved in mithramycin resistance in *S. argillaceus* (52% identity) (García-Bernardo *et al.*, 2000), and to DrrC, which is involved in daunorubicin resistance in *Streptomyces peucetius* (38.2% identity) (Lomovskaya *et al.*, 1996). DrrC has been shown to behave like an ATP-dependent DNA-binding protein *in vitro* (Furuya & Hutchinson, 1998). CmmRII showed the highest similarities to putative transcriptional regulators present in the genomes of

Nocardia farcinica (accession nos YP_118862 and YP_121769), *Gloeobacter violaceus* (accession no. NP_924384) and *Streptomyces avermitilis* (accession no. BAC74016); the similarities ranged from 23 to 31% identical amino acids, and CmmRII was shown to contain the conserved region COG1695 (predicted transcriptional regulators). CmmRII also showed similarity (25.4% identity) to MtrY from the mithramycin cluster (Fernández *et al.*, 1996). To test the possible requirement of one or both of these genes in conferring a high level of resistance to chromomycin A₃, we subcloned a 6.8 kb fragment (Fig. 2b), containing the four genes *cmrB*, *cmrA*, *cmmRII* and *cmrX*, into pWHM3. When the corresponding construct (pNM34) was introduced into *S. albus*, the recombinant strain became highly resistant to chromomycin A₃ (MIC >100 µg ml⁻¹), indicating that high levels of resistance to chromomycin are most probably dependent on the simultaneous presence of *cmmRII* and/or *cmrX*, together with *cmrA* and *cmrB*. Therefore, we independently subcloned *cmrA* and *cmrB* genes in combination with either *cmmRII* (pNM30) or *cmrX* (pNM46) (Fig. 2b). In addition, constructs with *cmrX* alone (pNM42), or together with *cmmRII* (pNM39), were assayed (Fig. 2b). Only the construct pNM46, which harbours *cmrA*, *cmrB* and *cmrX*, conferred a high level of resistance to chromomycin A₃ (MIC >100 µg ml⁻¹). Expression of the *cmrX* gene alone conferred a very low level of resistance to chromomycin A₃ (MIC 5 µg ml⁻¹). These results clearly indicate that the coexpression of *cmrA*, *cmrB* and *cmrX* is required to confer a high level of resistance to chromomycin A₃. This is in contrast to what happens in the mithramycin producer, in which expression of the two genes coding for the ABC transporter is sufficient to confer a high level of resistance to mithramycin (Fernández *et al.*, 1996). These results also suggest that the chromomycin ABC transporter and CmrX could act in a cooperative way, since the level of resistance conferred by co-expression of the three genes is higher than the sum of resistance conferred by the CmrAB transporter or CmrX, separately.

Insertional inactivation of *cmrX* and *cmmRII*

To get further information about the roles of *cmmRII* and *cmrX* genes in the biosynthesis and resistance to chromomycin A₃, we decided to inactivate each gene in *S. griseus*. Using gene replacement, we generated two independent mutants, in which we replaced the WT alleles in the chromosome with the alleles that had been mutated *in vitro*. To do this, we generated several constructs in the unstable plasmid pHZ1358, in which each gene was interrupted by an apramycin-resistance cassette, which was inserted in the direction of transcription of the targeted gene, within the corresponding coding regions. The corresponding constructs (pC60RII for *cmmRII*, and pC60X for *cmrX*) were introduced in *S. griseus* by intergeneric conjugation, and transconjugants, in which a double crossover had taken place, were selected for their resistance to apramycin, and susceptibility to thiostrepton.

One colony from each mutant (C60RII and C60X) was selected for further analysis. For both C60RII and C60X, it was verified by Southern hybridization that the gene replacement event had taken place (Figs 3 and 4). Both mutants were then analysed for resistance to, and production of, chromomycin A₃. The C60RII mutant (*cmmR*II mutant), showed levels of resistance to chromomycin A₃ that were similar to those of the WT strain (Fig. 6a), but, surprisingly, the mutant was also very

resistant to mithramycin, without the addition of chromomycin A₃ to the cultures as an inducer (MIC >100 µg ml⁻¹). Notably, production of chromomycin A₃ in this mutant started earlier, and the yields were higher (about 70 % higher), than in the case of the WT strain (Fig. 7). These results suggest that CmmRII could probably act as a transcriptional repressor in chromomycin biosynthesis/resistance. In contrast, the C60X mutant (*cmrX* mutant) was much more sensitive to chromomycin A₃ than the WT strain (Fig. 6a), and it showed a reduction in chromomycin production of about 50 % (Fig. 7). These results confirm that CmrX is implicated in chromomycin resistance, and that it is important in the self-protecting mechanism to chromomycin in *S. griseus*.

Insertional inactivation of the ABC transporter genes was also attempted. However, after three independent attempts no mutant was obtained, suggesting that inactivation of these genes is lethal for the producer strain.

A chromomycin biosynthesis intermediate as a possible substrate for the ABC transporter CmrAB

As mentioned above, it was surprising that the chromomycin ABC transporter conferred a high level of resistance to mithramycin, but only low levels of resistance to chromomycin A₃. This was not what was expected for a transporter system that it is supposed to export a highly potent antibiotic, such as chromomycin A₃. We wondered if the chromomycin ABC transporter could recognize and secrete a biosynthesis intermediate instead of the final product chromomycin A₃. It has been shown (Menéndez *et al.*, 2004b) that the last step in chromomycin A₃ biosynthesis is a sugar-tailoring modification event, consisting of the acetylation of two hydroxyl groups present in two different sugars. A single acetyltransferase CmmaA is responsible for both of the events. By inactivating *cmmaA*, the biosynthesis intermediate DDACA3 (Fig. 1) was isolated. This compound lacks two acetyl groups in sugars

Fig. 6. (a) Susceptibility to chromomycin A₃ of *S. griseus* WT (diamond), *S. griseus* C60RII (square) and *S. griseus* C60X (circle). (b, c) Susceptibility to chromomycin A₃ (b), and DDACA3 (c), of *S. albus* EM4 control (diamond), and *S. albus* NM28 expressing *cmrA* and *cmrB* (square). Mean data from three independent experiments are shown (the replicate values varied by not more than 10 % about the mean).

Fig. 7. Chromomycin A₃ production by *S. griseus* WT (diamond), and mutant strains C60RII (square) and C60X (triangle). Mean data from two independent experiments are shown.

A and E, and shows antibiotic activity, although its activity is lower than that in the final compound. Since DDACA3 is the immediate precursor of chromomycin A₃, and based on the fact that CmmA acetyltransferase is a membrane protein (B. García, N. Menéndez, J. A. Salas & C. Méndez, unpublished), we hypothesized that perhaps DDACA3 could be the actual substrate for the CmrAB ABC transporter. If this was the case, the CmrAB transporter should confer resistance to this compound. Consequently, we tested the susceptibility of *S. albus* recombinant strain NM28 (expressing *cmrA* and *cmrB*) to the dideacetylated intermediate, and compared the results with those for chromomycin A₃ (Fig. 6). As shown in Fig. 6, *S. albus* NM28 was highly sensitive to chromomycin A₃ (Fig. 6b), but highly resistant to DDACA3 (Fig. 6c). However, the control strain (*S. albus* EM4) was sensitive to both chromomycin A₃ and DDACA3. In addition, we also tested the DDACA3 susceptibility of other *S. albus* recombinant strains expressing different combinations of *cmr* genes (Fig. 2b). Only those strains expressing *cmrA* and *cmrB* were highly resistant to DDACA3. These results indicate that the CmrAB ABC transporter efficiently secretes DDACA3, but not chromomycin A₃. On the other hand, strains expressing *cmrX*, but not *cmrAB*, showed a low level of resistance to DDACA3, suggesting that CmrX could play a role in *S. griseus* in self-protection against bioactive intermediates, such as DDACA3, synthesized during the biosynthesis of chromomycin A₃. In agreement with this, analysis of the susceptibility of *S. griseus* C60X (*cmrX* mutant) to DDACA3 showed that, although no inhibition of growth was observed at short incubation times, there was reduced growth over longer time periods. This effect was not observed with either the WT strain or the mutant C60RII, as both showed normal growth (data not shown).

DISCUSSION

Antibiotic-producing micro-organisms protect themselves from the inhibitory/lethal action of antibiotics they produce by developing self-protection mechanisms (Cundliffe, 1989; Martin *et al.*, 2005; Méndez & Salas, 2001). The chromomycin producer *S. griseus* subsp. *griseus* is highly resistant to chromomycin A₃, and the chromomycin gene cluster contains three genes (*cmrA*, *cmrB* and *cmrX*) that are involved in self-resistance to chromomycin A₃. Genes *cmrA* and *cmrB* encode for an ABC transporter system, which is an efflux mechanism for the drug, and *cmrX* encodes a UvrA-like protein of ABC excision nuclease systems, which are responsible for DNA repair. Confirmation of the role of these genes in chromomycin resistance was achieved by independently or jointly expressing these genes in the chromomycin-sensitive strain *S. albus*. The results indicated that expression of the three genes (*cmrA*, *cmrB* and *cmrX*) was required to confer a high level of resistance to chromomycin A₃. Expression of either *cmrA* and *cmrB*, or *cmrX*, resulted in a low level of

resistance to chromomycin A₃. Consequently, *cmrAB* and *cmrX* are two chromomycin-resistance determinants. Additional confirmation of the essential role of *cmrX* in self-resistance to chromomycin A₃ was achieved by insertional inactivation of this gene; this resulted in the generation of a strain that was more sensitive to, and produced less of, chromomycin A₃. In a similar way, we tried to inactivate *cmrAB*, but this was not achieved, and it suggests that mutations in these genes are lethal for the producer strain. A similar situation occurs in the mithramycin producer, in which the inactivation of the mithramycin ABC transporter was also pursued, but not achieved, in the producer strain (Fernández *et al.*, 1998). On the basis of these results, we speculate that CmrX could be a DNA-binding protein that plays a role in self-resistance to chromomycin by inhibiting or destabilizing the interaction of chromomycin A₃ (and precursors) and genomic DNA, thus preventing chromomycin from interfering with cell transcription and/or replication. A similar mechanism has also been proposed for the analogous protein DrrC, which is another UvrA-like protein from the daunorubicin gene cluster that has been shown to behave *in vitro* like an ATP-dependent DNA-binding protein (Furuya & Hutchinson, 1998; Lomovskaya *et al.*, 1996). In addition, CmrX could play a regulatory role in chromomycin biosynthesis, since its absence leads to lower production yields of chromomycin. In relation to this, it has been shown that the CmrX-like protein DrrC can bind the promoter region of the regulatory gene *dnrI*, which is involved in daunorubicin biosynthesis (Furuya & Hutchinson, 1998). In the case of chromomycin biosynthesis, one can speculate that, in the absence of CmrX, the expression of regulatory genes could be affected.

The chromomycin-resistance genes are grouped in the chromosome, together with a gene encoding a transcriptional repressor (*cmmRII*), which most probably plays a role as a transcriptional repressor in chromomycin resistance/biosynthesis in the producer organism. Thus, its inactivation makes *S. griseus* a high producer of chromomycin A₃, and a strain constitutively resistant to mithramycin. However, its presence is not essential to confer resistance to chromomycin A₃ in a heterologous host if the chromomycin-resistance genes are expressed under the control of a constitutive promoter, i.e. the *ermEp* promoter.

The chromomycin ABC transporter appears to play a major role in the self-protection system of *S. griseus*, since its disruption seems to be lethal to the micro-organism, and its expression in *S. albus* resulted in a higher level of resistance compared with the expression of *cmrX*. ABC transporters are frequently found in antibiotic-biosynthesis gene clusters, where they play a role in secreting the antibiotic out of the cell, thus preventing the antibiotic from acting on internal cell structures or enzymes (Fernández *et al.*, 1996; Guilfoile & Hutchinson, 1991; Olano *et al.*, 1995). Surprisingly, the CmrAB transporter confers a low level of resistance to chromomycin A₃, but it

confers a high level of resistance to the biosynthesis intermediate DDACA3; this indicates that this biosynthesis intermediate is the substrate for the CmrAB transporter. Conversion of this compound into chromomycin A₃ is carried out by the action of the acetyltransferase CmmA (Menéndez *et al.*, 2004b), which has been shown recently to be a transmembrane protein (B. García, N. Menéndez, J. A. Salas & C. Méndez, unpublished). Taking all these findings into account, we propose a model for the biosynthesis of, and self-resistance to, chromomycin A₃ in *S. griseus* subsp. *griseus*, by which chromomycin biosynthesis proceeds through intermediates with weak biological activity. CmrX participates in protecting the producer against these compounds that have low bioactivity. The last intracellular product is the dideacetylated, but methylated, intermediate DDACA3. This compound, which also shows weaker antibiotic activity, is recognized by the ABC transporter CmrAB, and then acetylated by the membrane-bound CmmA, and thus converted into the fully biologically active chromomycin A₃. This model of biosynthesis represents an evolutionary advantage for the producer strain to survive during antibiotic biosynthesis, since formation of the antibiotic is not completed inside the cell, but requires a final step. In this way, the organism avoids the interaction of the harmful compound chromomycin A₃ with its intracellular target. In some macrolide producers, such as *Streptomyces antibioticus* (oleandomycin producer) and *Streptomyces venezuelae* (methymycin producer), the active compounds are not found inside the cell; however, in these micro-organisms, the situation is rather different to the chromomycin producer, since the biosynthesis of the antibiotics is actually completed inside the cell, but the antibiotics are modified by glycosylation to render them inactive, and they are then activated once they are outside the cell (Quirós *et al.*, 1998; Zhao *et al.*, 2003). In contrast, in the chromomycin producer, formation of the fully active compound requires the incorporation of additional functional groups (i.e. acetyl groups) to DDACA3, and this probably occurs during the secretion process. Studies now in progress are focused on determining the affinity of the ABC transporter by the deacetylated intermediate, and on elucidating the possible interaction between the ABC transporter and the acetyltransferase CmmA.

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