Collismycin A biosynthesis in *Streptomyces* sp. CS40 is regulated by iron levels through two pathway-specific regulators

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Two putative pathway-specific regulators have been identified in the collismycin A gene cluster: ClmR1, belonging to the TetR-family, and the LuxR-family transcriptional regulator ClmR2. Inactivation of *clmR1* led to a moderate increase of collismycin A yields along with an early onset of its production, suggesting an inhibitory role for the product of this gene. Inactivation of *clmR2* abolished collismycin A biosynthesis, whereas overexpression of ClmR2 led to a fourfold increase in production yields, indicating that ClmR2 is an activator of collismycin A biosynthesis. Expression analyses of the collismycin gene cluster in the wild-type strain and in ΔclmR1 and ΔclmR2 mutants confirmed the role proposed for both regulatory genes, revealing that ClmR2 positively controls the expression of most of the genes in the cluster and ClmR1 negatively regulates both its own expression and that of *clmR2*. Additionally, production assays and further transcription analyses confirmed the existence of a higher regulatory level modulating collismycin A biosynthesis in response to iron concentrations in the culture medium. Thus, high iron levels inhibit collismycin A biosynthesis through the repression of *clmR2* transcription. These results have allowed us to propose a regulatory model that integrates the effect of iron as the main environmental stimulus controlling collismycin A biosynthesis.

INTRODUCTION

Members of the genus *Streptomyces* are Gram-positive bacteria with a complex life cycle of morphological and physiological differentiation. These micro-organisms produce an extraordinary diversity of antibiotics and other biologically active secondary metabolites of medical and industrial importance (Bérdy, 2012). Biosynthesis of these metabolites is controlled by a complex regulation process (Bibb, 2005; Liu et al., 2013). Complex regulatory cascades link environmental and physiological signals with pleiotropic and pathway-specific regulatory proteins which control the expression of individual antibiotic gene clusters. Pathway-specific regulatory genes are usually clustered with genes for the biosynthesis of the antibiotic and genes involved in its secretion and resistance. These regulators can have either activator or repressor effect on the expression of cluster genes. The number of regulators in a particular cluster is variable, ranging from none, or at least not yet identified (the case of borrelidin or elloramycin gene clusters), to frequently several (as occurs in mithramycin, daunorubicin or salinomycamide A gene clusters) (Olano et al., 2011). In some cases, clusters may contain both activators and repressors, as happens in the tylosin pathway, which contains two activator and two repressor-coding genes (Cundliffe, 2008), or in the daunorubicin pathway, which is controlled by three activators and one repressor (Vasanthakumar et al., 2013). Understanding how expression of a gene cluster is regulated is a powerful tool to increase secondary metabolite yields in producer organisms. In addition, since many novel derivatives generated by genetic engineering are produced in low amounts, manipulation of pathway-specific positive regulators or inactivation of pathway-specific repressors can be used to increase the yields to levels allowing the production of the novel compounds.

Collismycin A (Fig. 1a) is a member of the 2,2′-bipyridyl family of natural products, which also include SF2738B-F (Gomi et al., 1994), pyrisulfoxins (Tsuge et al., 1999) and caerulomycins (Funk & Divekar, 1959; McInnes et al., 1977, 1978). This family of compounds is characterized by containing a 2,2′-bipyridyl ring system which is further modified by some tailoring modifications. These compounds have been shown to exhibit antibacterial, anti-fungal and cytotoxic activities (Gomi et al., 1994; Stadler et al., 2001), and to have potential as anti-inflammatory...
agents through binding to the glucocorticoid receptor (Shindo et al., 1994) or as neuroprotectants by reducing oxidative stress in neurons (Martinez Gil et al., 2008). Both the collismycin A (Fig. 1b) and the caerulomycin gene clusters have been isolated and characterized (García et al., 2012; Qu et al., 2012; Zhu et al., 2012). In addition, several derivatives of collismycin A have been generated by insertional inactivation, biocatalysis and mutasynthesis (García, 2013; Sialer et al., 2013). The collismycin A cluster of Streptomyces sp. CS40 contains two regulatory genes, clmR1 and clmR2, and a set of membrane-transporter-coding genes that could be involved in metabolite import and/or export (García et al., 2012). Here we report the characterization of clmR1 and clmR2 through insertional inactivation, gene expression and transcriptional analysis and we propose a model for regulation of the biosynthesis of collismycin A that includes iron levels as a core element in the regulatory process.

**METHODS**

**Strains and culture conditions.** Bacterial strains used in this work were Streptomyces sp. CS40 (collismycin producer), Escherichia coli DH10B (Invitrogen) and E. coli ET12567 (pUB307) (Kieser et al., 2000). Tryptone soy broth (TSB) was used as growth medium for Streptomyces strains and MA medium was used for spore generation (Fernández et al., 1998). Two different culture media were used for production assays: R5A as standard production medium (Fernández et al., 1998) and MD medium for cultures with iron-controlled levels (2.1 % MOPS, 1 % glucose, 0.35 % K2HPO4, 0.2 % asparagine, 0.06 % MgSO4.7H2O, 0.5 % MnCl2, 0.1 % ZnSO4. H2O, 0.1 % CaCl2; modified from Bascaran et al., 1989). When needed, MD medium was supplemented with different quantities of FeSO4.7H2O from a 5 mg ml⁻¹ stock solution. Intergeneric conjugation between E. coli and Streptomyces was performed following standard procedures (Kieser et al., 2000). E. coli culture media used in this work were those described in Sambrook et al. (1989). When clones containing antibiotic resistance gene markers were grown, the medium was supplemented with the appropriate antibiotics: 100 µg ampicillin ml⁻¹, 20 µg tobramycin ml⁻¹, 25 µg chloramphenicol ml⁻¹, 10 µg tetracycline ml⁻¹, 50 µg nalidixic acid ml⁻¹, 50 µg thiostrpton ml⁻¹ and 25 µg apramycin ml⁻¹.

**DNA manipulation and plasmids.** Plasmids used in this work were pCR-Blunt (Invitrogen) for subcloning and sequencing of PCR products, pBluescript II SK+ (Fermentas) for subcloning, pHZ1358 (Sun et al., 2009) for gene replacement experiments in Streptomyces and pUO9090 (Pelaer et al., 2001) as the source of the apramycin resistance gene aac(3’IV). For gene expression experiments plasmid
Plasmid construction. Inactivation of clmR1 was performed using a pHZ1358-derived plasmid denominated pHZ3KA. In order to generate this plasmid, a 3.6 kb fragment containing clmR1 coding sequence was obtained by pBSK88 through BamHI digestion. This fragment was subcloned into the BamHI site of pBluescript II SK+., generating pBSK3K. Then, a HindIII–EcoRI fragment containing the gene aac3(IV) was excised from pU90900, made blunt and then cloned into the blunt-ended Ncol site from pBSK3K (a unique restriction site within the sequence of clmR1). From the construct generated, called pBSK3KA, a 5.1 kb NotI–EcoRI fragment carrying the replacement cassette was rescued. This fragment was made blunt and then subcloned into the blunt-ended BamHI site from pHZ1358, thus generating pHZ3KA. Inactivation of clmR2 was carried out using plasmid pHZ25AB. For its construction, two PCR fragments of approximately 1.5 kb flanking clmR2 were amplified using oligonucleotides orf5del1/orf5del2 and orf5del3/orf5del4, respectively (Table S1, available in the online Supplementary Material). These two fragments were sequenced and then cloned into sites EcoRI– HindIII and XbaI respectively, flanking the apramycin resistance gene aac3(IV) of plasmid pU90900, generating the construction pUO5AB. The cassette constituted by the apramycin resistance gene and the two flanking regions was excised as a 4.5 kb SpeI fragment, which was then subcloned as a blunt fragment in the blunt-ended BamHI site of pHZ1358, resulting in the construct pHZ25AB.

Gene expression analysis by reverse transcriptase PCR (RT-PCR). RT-PCR analyses were performed with 100 ng total RNA as a template using the SuperScript One-Step RT-PCR with Platinum Taq DNA polymerase (Invitrogen), DMSO, 5% (v/v), and RNAGuard RNase inhibitor (32.2 U per reaction; Amersham Biosciences) were added to all reactions. Negative controls for each pair of primers were carried out with Platinum Taq DNA polymerase (Invitrogen) in the absence of reverse transcriptase to confirm that amplified products did not originate from contaminating chromosomal DNA in RNA preparations. Amplification conditions were the following: 30 min at 50 °C for first-strand cDNA synthesis followed by 2 min at 94 °C and then 33 cycles of 98 °C for 15 s, 62 °C for 45 s and 72 °C for 1 min, with a final extension step at 72 °C for 7 min. Primers used in this work (16–23mers, Tm ~65 °C, for amplification fragments of approximately 400 bp) (Table S1) were designed using Vector NTI Advance 11.5 software (Invitrogen) and validated using the on-line tool NetPrimer (Premier Biosoft). Oligonucleotide primers HRDB-GB1-F and HRDB-GB2-R for lrdII, encoding the constitutively expressed housekeeping sigma factor, were used as an internal control to assess the quality of RNA (Rodriguez et al., 2008). RT-PCR products were separated in agarose gels and visualized by ethidium bromide staining. The identity of the PCR products was verified by direct sequencing with one of the amplification primers.

Analysis of collistymycin A production by ultraperformance liquid chromatography (UPLC) and LC-MS. Collistymycin production by both the wild-type strain and the generated mutants was measured using ethyl acetate extracts of whole cultures. These extracts were analysed by UPLC (Acquity UPLC equipment with a BEH C18 Waters column of 2.1 × 100 mm) and HPLC-MS (Alliance chromatographic system coupled to a Waters ZQ4000 mass spectrometer and a SunFire C18 Waters column of 2.1 × 150 mm) using procedures previously described (Garcia et al., 2013). Seed cultures of the different Streptomyces strains were obtained in 50 ml Erlenmeyer flasks containing 10 ml TSB medium. After 24 h of incubation at 30 °C and 250 r.p.m. these cultures were used to inoculate square deepwell plates consisting of 24 wells of 3 ml culture volume each (Siebenberg et al., 2010) at a 3.5% (v/v) ratio. Production cultures were incubated at 30 °C and 250 r.p.m. for 6 days and then 1 ml samples from each of the wells were extracted with an equal volume of ethyl acetate in order to quantify collistymycin A levels in the cultures.
amplification fragments (approx. 100 bp). Their efficiencies were then measured using a dilution series of chromosomal DNA. Oligonucleotide primers HRDBqRT1 and HRDBqRT2 were used to normalize gene expression levels to the housekeeping gene hrdB (Gómez et al., 2011). qRT-PCR results were analysed using MxPro software (Stratagene).

RESULTS

Genes clmR1 and clmR2 are regulators of collismycin A biosynthesis

Two genes of the clm cluster have been proposed to regulate the expression of this cluster (García et al., 2012). Gene clmR1 encodes a transcriptional regulator of the TetR-family, members of which participate in the regulation of a number of cellular activities such as morphogenesis, osmotic stress response and export of secondary metabolites (Deng et al., 2013; Ramos et al., 2005). ClmR1 contains the characteristic N-terminal helix–turn–helix (HTH) DNA-binding domain of this family (Fig. 1c). To analyse the role of clmR1 in collismycin A biosynthesis, the gene was inactivated by gene replacement through the insertion of an apramycin resistance cassette within the coding region. The achievement of the correct replacement in the resultant clones was verified by Southern hybridization (Fig. 2a). Inactivation of clmR1 did not abolish collismycin A biosynthesis but it caused an increase in production yields (Fig. 2b) and an early initiation of biosynthesis (Fig. 2c). Ectopic expression of clmR1 in the producer organism caused a discrete, but consistent, reduction of collismycin A yields in the wild-type strain (Fig. 2d).

The second regulatory gene product, ClmR2, shows similarity with LuxR-family transcriptional regulators and contains the characteristic C-terminal HTH DNA-binding domain of LuxR regulators (Fig. 1d). Unlike other members of this family, ClmR2 shows no similarity or identifiable domain in its N-terminal region, meaning that it is not a typical response regulator, and it also lacks the characteristic structure of SARP regulators, one of the main families of pathway-specific regulators in Streptomyces (Liu et al., 2013). Gene clmR2 was also inactivated by gene replacement through the insertion of the apramycin resistance cassette (Fig. 3a). Its inactivation completely abolished collismycin A biosynthesis (Fig. 3b, c), whereas complementation experiments showed that the ability to synthesize collismycin A was restored when clmR2 was expressed in the mutant strain (data not shown). Furthermore, ectopic expression of clmR2 in the wild-type strain using an integrative vector caused a fourfold increase in collismycin A production compared with the wild-type strain (Fig. 3d).

![Diagram](image)

**Fig. 2.** Functional analysis of clmR1. (a) Schematic representation of the gene replacement event in the generation of the ΔclmR1 mutant and Southern analysis. (b) Production of collismycin A by the wild-type strain and the ΔclmR1 mutant after 6 days of growth in R5A medium. (c) Time-course of collismycin A production by the wild-type strain (wt) and ΔclmR1 mutant. (d) Effect of clmR1 expression on collismycin A biosynthesis in the wild-type strain. Production experiments were performed in triplicate and the error bars represent the standard deviation from the mean production value in the triplicate.
These results suggest that ClmR1 acts as a repressor and ClmR2 as an activator for the expression of the collismycin A biosynthesis gene cluster.

**Effect of clmR1 and clmR2 on gene expression**

To determine which genes of the collismycin A cluster were expressed in the presence of ClmR1 and ClmR2, transcriptional studies were carried out. Total RNA was obtained from the wild-type strain and from mutants ΔclmR1 and ΔclmR2 after 72 h of incubation (when collismycin A is actively produced) and used as a template for gene expression analysis (Fig. 4). Specific primers for RT-PCR were designed from nucleotide sequences belonging to many of the genes of the cluster, including at least two genes from each of its presumed transcriptional units (Fig. 1b). As an internal control, expression of hdrB, which encodes the major vegetative sigma factor, was determined from the wild-type strain and from mutants ΔclmR1 and ΔclmR2 after 72 h of incubation (when collismycin A is actively produced) and used as a template for gene expression analysis (Fig. 4).

**Fig. 3.** Functional analysis of clmR2. (a) Schematic representation of the gene replacement event in the generation of the ΔclmR2 mutant and Southern analysis. (b) UPLC analyses of collismycin A production in the wild-type strain (wt) and in the ΔclmR2 mutant after 6 days of growth in R5A medium. (c) Time-course of collismycin A production by the wild-type strain (wt) and ΔclmR2 mutant. (d) Effect of clmR2 expression on collismycin A biosynthesis in the wild-type strain. Production experiments were performed in triplicate and the error bars in (c) and (d) represent the standard deviation from the mean production value in the triplicate.

**Fig. 4.** RT-PCR transcriptional analysis of the collismycin A gene cluster in the wild-type strain (wt) and ΔclmR2 and ΔclmR1 mutants. Reactions containing the RNA templates but lacking reverse transcriptase were carried out as negative controls to discard the possibility of DNA contamination in positive results. Expression of hdrB was used as an internal control. The specificity of the amplified fragments was determined by sequencing of the RT-PCR products.
(Gómez et al., 2012) (Fig. 4). In the ΔclmR2 mutant, expression of several genes of the cluster (clmM2, clmG1, clmM and clmL) was not detected, while transcription of some others (clmT1–clmT5, clmG2, clmP, clmN1 and clmT) was greatly reduced. Only a few genes from the cluster (clmR1, clmD2, clmAT, clmM1 and clmAH) seemed to be expressed without major alterations in the absence of ClmR2. On the other hand, no apparent effect was observed on expression for any of the genes in the ΔclmR1 mutant (Fig. 4). For a quantitative evaluation, qRT-PCR experiments were carried out at 24, 48 and 72 h of growth. For these experiments, we analysed the expression of the two regulatory genes of the cluster (clmR1 and clmR2) and two structural genes (clmL, coding for a lysine 2-aminotransferase, and clmM, coding for a monooxygenase) that were previously found by RT-PCR to be under the control of clmR2 (Fig. 4). In addition, the expression of two transporter genes (clmT2 and clmT3), both being the first genes in the corresponding transcriptional units, was also assessed. Expression values were normalized to the expression of hrdB in the respective samples and referred to the expression of each gene at 24 h of growth, to which the arbitrary value of 1 was given. The structural genes clmL and clmM showed a similar expression pattern in the wild-type strain, with equivalent transcription levels until 72 h, when the expression increased approximately fivefold (Fig. 5a).
the ΔclmR1 mutant this pattern was clearly altered: at 24 h the expression of both genes was approximately 15-fold higher than in the wild-type strain, then it decreased to similar values to those in the wild-type strain. On the other hand, no expression of clmL and clmM was detected at any time during growth of the ΔclmR2 mutant (Fig. 5a). In the case of the two transporter genes clmT2 and clmT3, both showed a rising transcription pattern with time, reaching a 20-fold and 40-fold increase in their respective expression levels at 72 h (Fig. 5b). In the ΔclmR1 mutant these two genes also showed an altered expression pattern with respect to the wild-type strain, the main difference being that at 24 h of growth the expression of clmT2 and clmT3 in the mutant was 40-fold and 150-fold higher, respectively (Fig. 5b), suggesting that ClmR1 significantly represses expression of these two genes. Additionally, expression of clmT2 and clmT3 in the ΔclmR2 mutant remained at low basal levels at all times during growth (Fig. 5b), in agreement with the observations made in RT-PCR experiments, thus confirming that their expression is ClmR2-dependent.

Regarding the regulatory genes, the expression of clmR1 decreased with time to a fifth of its initial value. This descending pattern was similar in the wild-type strain and in the ΔclmR2 mutant (Fig. 5c), suggesting that ClmR2 does not exert any regulatory effect on clmR1. However, expression of clmR1 seems to be regulated by its own product since in the ΔclmR1 mutant its transcription was less repressed than in the wild-type strain. Thus, at 72 h of growth clmR1 expression was threefold higher in the ΔclmR1 mutant (this determination was possible since the apramycin resistance cassette was inserted within the clmR1 sequence instead of deleting the gene). Expression of clmR2 in the wild-type strain slightly increased with growth time; however, in the ΔclmR1 mutant, its expression greatly increased (approx. 3.5-fold) at early times of growth (Fig. 5c), suggesting that ClmR1 represses clmR2.

**Effect of iron on collismycin A biosynthesis**

A series of facts led us to consider the possibility that iron could play a role in regulation of collismycin A biosynthesis. On one hand, clmT3, clmT4 and clmT5 are co-transcribed (data not shown) and they could code for an ATP-binding cassette (ABC) import system involved in iron transport. The clmT3, clmT4 and clmT5 gene products are highly similar to FepB, FepD and FepC-like proteins, respectively, which are involved in the uptake of chelated ferric iron–siderophore complexes (Stephens et al., 1995). The deduced product of clmT3 shows high homology with ABC transporter solute-binding proteins (SBPs). Structurally it belongs to type A SBPs, which are involved in the binding and uptake of metal ions (especially iron) alone or in complex with siderophores (Berntsson et al., 2010). Gene clmT4 codes for an ABC system permease component with ten predicted transmembrane helices, a characteristic topology in type 2 import systems, usually in charge of internalizing metallic complexes (Locher, 2009; Oldham et al., 2008). Finally, the clmT5 product is homologous to ATPase subunits from ABC transporters in general, but also those involved in ferric iron uptake (Locher, 2009; Stephens et al., 1995). On the other hand, the 2,2′-bipyridyl structure of collismycin A suggests that it could behave as a chelating agent. 2,2′-Bipyridyls are frequently used as iron or copper chelators with multiple purposes, including the screening for siderophore-producing bacteria or the biochemical characterization of iron-requiring enzymes (Chen-Roetting et al., 2012; Li et al., 2012; Nakouti et al., 2012; Nocentini & Barzi, 1996).

Based on these observations, we decided to assess in vitro the ability of collismycin A to interact with iron. When 20 μg collismycin (final concentration 0.13 mM) was added to solutions with decreasing concentrations of FeSO₄, these turned from colourless to pink, especially when the ratios of collismycin to iron were close to equimolarity (Fig. 6a) suggesting the formation of complexes between collismycin A and iron. UPLC analyses of these solutions showed that, in addition to a peak corresponding to collismycin A, a new peak appeared with a collismycin A-like absorption spectrum but including an additional maximum at approximately 500 nm that might be associated with the formation of collismycin–iron complexes (Fig. 6b). This was confirmed by HPLC-MS analysis of the peak, revealing that it had an m/z value of 605, which is compatible with the estimated mass for a complex formed by two molecules of collismycin A and an iron atom (Fig. 6b).

Once the interaction of collismycin with iron was verified, we determined the effect of adding different iron concentrations on collismycin A yields. With this purpose, the wild-type and ΔclmR1 strains were grown in a defined culture medium lacking iron in its composition (MD) and in the same medium supplemented with increasing concentrations of FeSO₄. In both strains collismycin A production was higher in the absence of added iron or in the presence of low iron concentrations, reaching maximum yields at 0.36 μM (Fig. 6c). With iron concentrations above 0.36 μM, collismycin A yields progressively decreased, suggesting an inhibitory effect of iron on collismycin A production. This effect was quite severe in the wild-type strain, in which no collismycin was detected at 8.99 μM or higher iron concentrations. A similar inhibitory effect could be observed in the ΔclmR1 mutant, even though this strain produced significantly more collismycin A than the wild-type strain and still kept producing it at high iron levels at which the wild-type strain did not. Such differences cannot be attributed to alterations in growth rates since these were equivalent in both cases. This result not only supports the hypothesis that high iron levels repress collismycin A biosynthesis, but also suggests that ClmR1 might be involved in this inhibitory process. With respect to the ΔclmR2 mutant, it did not produce collismycin A even under conditions of iron depletion. However, the lack of collismycin A in these conditions
did not lead to any major changes in the growth or development of the mutant strain (data not shown).

**Effect of iron on gene expression**

We verified whether iron repression was occurring at the gene expression level by qRT-PCR analyses. Total RNA of both the wild-type strain and the ΔclmR1 mutant was isolated after 24, 48 and 72 h of growth in MD medium (lacking added iron) and in MDF medium (containing 17.98 μM FeSO₄). Additionally, collismycin A levels in the mycelium samples used for RNA extraction were also quantified (Fig. 7a). As targeted genes for the qRT-PCR analyses, we used the same genes selected for previous analysis: clmL, clmM, clmT2, clmT3, clmR1 and clmR2. Expression of clmL, clmM, clmT2 and clmT3 mirrored the pattern of collismycin A production in the different samples (Fig. 7a) and was highly repressed by the presence of iron, this repression being more severe in the wild-type strain than in the ΔclmR1 mutant (Fig. 7b, c). With respect to the regulatory genes, expression of clmR1 in the wild-type strain slightly decreased in the presence of iron (Fig. 7d). In contrast, in the ΔclmR1 mutant (in which the negative autoregulatory effect of ClmR1 is absent) its expression increased when iron was added to the culture medium. This apparently opposite effect of iron depending on the presence of ClmR1 suggests that iron repression is somehow mediated by this regulator. On the other hand,
expression of clmR2 was strongly repressed by the addition of iron both in the wild-type strain and in the ΔclmR1 mutant, although in the latter repression was slightly less pronounced (Fig. 7d). This result suggests that iron repression of clmR2 is only partially mediated by the product of clmR1 and an additional iron-dependent repressor mechanism might be involved.

**DISCUSSION**

Previous investigations of the biosynthesis of collismycin A by *Streptomyces* sp. CS40 have revealed that it consists of three main stages. First, a lysine residue is modified by enzymes ClmL, ClmS and ClmAL to generate picolinic acid (Sialer et al., 2013). Second, picolinic acid is used as starter unit for the biosynthesis of the 2,2'-bipyridyl scaffold by a hybrid polyketide synthase–non-ribosomal peptide synthetase (PKS-NRPS) system formed by ClmP, ClmN1, ClmN2, ClmT, and possibly ClmD1 (García et al., 2012). Finally, tailoring enzymes ClmM1, ClmAH, ClmG1, ClmG2, ClmAT, ClmD2, ClmM and ClmM2 catalyse the formation of the oxime group and the methylations that are specific to collismycin A (García et al., 2012). Study of two collismycin A regulatory genes, whose involvement in the collismycin A pathway is addressed in this work, reveals that both of them play an important role in the biosynthesis of this compound. The product of clmR2, a LuxR-family transcriptional regulator, acts as the main and essential activator of collismycin A biosynthesis. This role is consistent with the lack of collismycin A production in a ΔclmR2 mutant, as well as the increase of collismycin A levels observed when this gene was overexpressed in the wild-type strain. ClmR2 controls the transcription of most of the genes in the clm cluster. In the absence of this regulator some of them are not expressed at all (among them clmL and clmM2, which code for the first and last enzymes in the collismycin biosynthetic pathway, respectively) whereas the transcription of others (such as the transporter genes) is greatly decreased. Only the transcription of five genes in the clm cluster (clmR1, clmD2, clmAT, clmM1 and clmAH) is not apparently regulated by ClmR2. On the other hand, clmR1 encodes a TetR-family transcriptional regulator that inhibits collismycin A biosynthesis. Inactivation of this gene leads to a moderate increase of collismycin A yields, as well as a precocious onset of its production. In this case ClmR1 seems to exert its effect at early times of growth, inhibiting the expression
of the biosynthetic genes through the repression of clmR2 transcription. Accordingly, clm genes show an altered expression pattern in the ΔclmR1 mutant, with increased transcription levels at 24 h of incubation that gradually decrease until 72 h, when they reach wild-type strain values. Additional qRT-PCR results revealed that transcription of clmR1 also increases in the clmR1 mutant, suggesting that clmR1 expression is downregulated by its own product. Several examples of negative autoregulation have been described to date, such as the case of heat-shock protein regulator HspR, which negatively regulates its own expression along with that of some other genes coding for different chaperone systems (Salerno et al., 2007), or the IclR-family regulator HpdR, which is involved in calcium-dependent antibiotic production by Streptomyces coelicolor (Yang et al., 2010).

Given previous reports that bipyridyls can act as chelating agents and the existence of a possible uptake system for iron–siderophore complexes in the collismycin A cluster (encoded by clmT3, clmT4 and clmT5), it is plausible that collismycin A could act in a similar way to siderophores, chelating iron atoms to ease their incorporation by the cell. Traditionally, siderophores can be classified in two main groups according to their structure: catecholate-like, including molecules such as enterobactin, and hydroxamate-like, such as desferrioxamines (Miethke & Marahiel, 2007), both binding iron through interaction with oxygen atoms. However, based on the structure of collismycin A, this compound would not belong to either of these categories, but could instead act as a tridentate ligand, interacting with iron through its three nitrogen atoms. A similar type of interaction has already been described for the synthetic molecule 2,2′-bipyridyl-6-carbothiamide, which binds iron through interaction with two nitrogen atoms and one sulphur atom (Antonini et al., 1981; Nocentini & Barzi, 1997). Preliminary assays allowed us to confirm that iron not only interacts with collismycin A but also negatively regulates its biosynthesis. High iron concentrations in the culture medium inhibit the transcription of collismycin biosynthesis and transport genes through the repression of the activator clmR2. This inhibition is partially mediated by the TetR repressor clmR1, which seems to be positively regulated by iron. However, the fact that even in the absence of ClmR1 the transcription of clmR2 is still partially inhibited indicates that iron also represses clmR2 in a ClmR1-independent way. The lack of apparent growth alterations in the ΔclmR2 mutant (unable to produce collismycin A) when cultured under iron-deficient conditions does not dismiss the potential role of this compound as an iron scavenger, since it is known that actinomycetes usually contain gene clusters for the biosynthesis of several different siderophores (Nett et al., 2009) and the absence of collismycin A might be compensated by the biosynthesis of some other iron chelator.

Iron metabolism is strictly regulated in Streptomyces species by a family of pleiotropic transcriptional regulators called DmdR. These regulators sense intracellular iron levels and control the expression of genes encoding several iron-containing enzymes, oxidative stress response systems and siderophore biosynthesis clusters like desferroxamine (Flores et al., 2005). DmdR-like proteins bind distinct DNA sequences called ‘iron boxes’ located upstream of the genes they regulate. It is still unknown if the genome of strain CS40 contains any DmdR orthologue, but the search for possible iron boxes in the collismycin A cluster revealed the presence of a putative DmdR-binding sequence (5′-TTAGGGTACCCTAACATGG-3′) upstream of clmT3.

**Fig. 8.** Model for the regulation of collismycin A biosynthesis in Streptomyces sp. CS40.
that shows a 68.4% identity identity with the DmdR consensus sequence: 5’-taggtAGGCTcCTaA-3’ (Flores & Martin, 2004). Additionally, at least one other level of pleiotropic regulation might be taking place in collismycin A biosynthesis, since the structural gene clmL and the activator clmR2 each contain one TTA codon within their sequence. The presence of this rare codon implies some level of post-transcriptional control over two key genes in the biosynthetic process by the Leu-tRNA encoded by the bldA gene (Chater & Chandra, 2008).

According to these results, at early growth times (and still with high iron levels) collismycin A biosynthesis would be initially inhibited by the TetR repressor ClmR1, whose expression seems to be activated by iron (Fig. 8). This regulator represses not only transcription of ClmR2 but also transcription of most of the genes in the cluster, but also transcription of its own gene. At the same time, clmR2 expression would be further repressed by iron through a still unknown mechanism, ensuring the inactivation of the pathway. Gradually, the negative autoregulatory effect of ClmR1 coupled with the drop of iron concentration in the medium would relieve clmR2 repression, eventually leading to the activation of the whole cluster and the onset of collismycin A production as a response to iron starvation signals. A similar case of siderophore biosynthesis regulation might be that of pyochelin in Streptomyces scabies 87-22. The gene cluster for this siderophore also contains a TetR-family repressor as well as an AfsR-family activator that coordinate the expression of the biosynthetic genes (Seipke et al., 2011). In the case of both pyochelin and collismycin A this regulatory process is likely modulated by several pleiotropic regulators, which would be in charge of coordinating collismycin A biosynthesis with growth and differentiation processes of the strain, as well as with the rest of the secondary metabolism.

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