

# Coupling of the biosynthesis and export of the DNA gyrase inhibitor simocyclinone in *Streptomyces antibioticus*

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## Summary

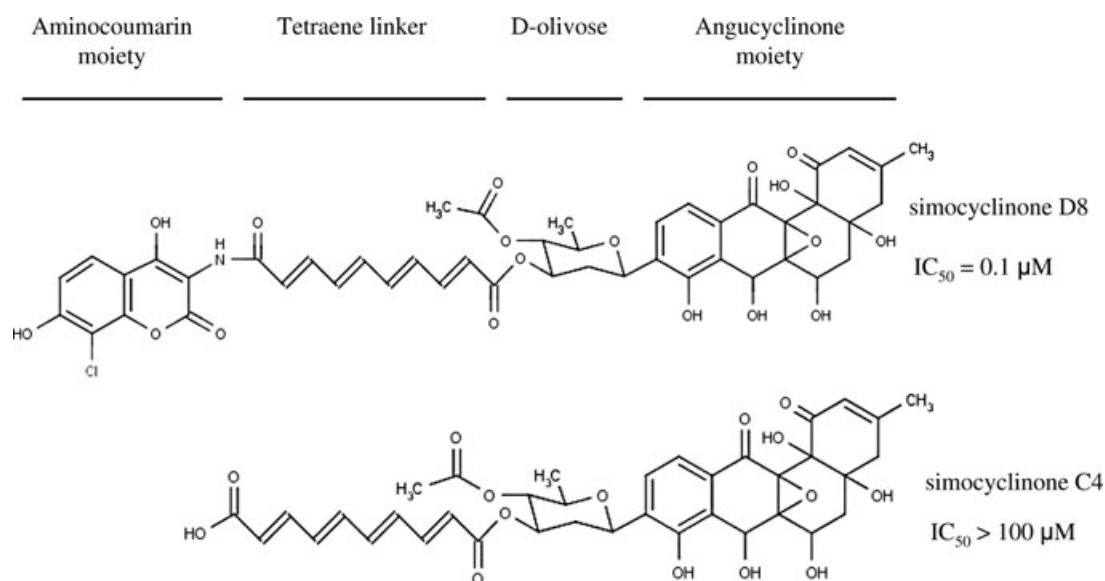
Because most antibiotics are potentially lethal to the producing organism, there must be mechanisms to ensure that the machinery responsible for export of the mature antibiotic is in place at the time of biosynthesis. Simocyclinone D8 is a potent DNA gyrase inhibitor produced by *Streptomyces antibioticus* Tü 6040. Within the simocyclinone biosynthetic cluster are two divergently transcribed genes, *simR* and *simX*, encoding proteins that resemble the TetR/TetA repressor–efflux pump pair that cause widespread resistance to clinically important tetracyclines. Engineered expression of *simX* from a strong, heterologous promoter conferred high level simocyclinone D8 resistance on *Streptomyces lividans*, showing that *simX* encodes a simocyclinone efflux pump. Transcription of *simX* is controlled by SimR, which directly represses the *simX* and *simR* promoters by binding to two operator sites in the *simX*–*simR* intergenic region. Simocyclinone D8 abolishes DNA binding by SimR, providing a mechanism that couples the biosynthesis of simocyclinone to its export. In addition, an intermediate in the biosynthetic pathway, simocyclinone C4, which is essentially inactive as a DNA gyrase inhibitor, also induces *simX* expression *in vivo* and relieves *simX* repression by SimR *in vitro*.

## Introduction

Aminocoumarin antibiotics are active against Gram-positive bacteria and function principally by inhibiting DNA gyrase (Maxwell and Lawson, 2003), an essential DNA topoisomerase found in all bacteria, which catalyses DNA supercoiling (Nollmann *et al.*, 2007). In addition, a likely secondary target of these compounds is topoisomerase IV, which is involved in chromosome decatenation (Hardy and Cozzarelli, 2003; Oppegard *et al.*, 2009). All four known aminocoumarins are produced by *Streptomyces* species. The first three to be discovered, novobiocin, clorobiocin and coumermycin A<sub>1</sub>, each competitively inhibit the ATPase activity of the GyrB subunit of DNA gyrase and exhibit *K<sub>i</sub>* values in the nanomolar range (Gormley *et al.*, 1996). The most recently identified aminocoumarin antibiotic, simocyclinone D8 (Schimana *et al.*, 2000) (Fig. 1), also inhibits DNA gyrase but was unexpectedly found to have a completely novel mode of action, binding instead to the GyrA subunit of the enzyme and preventing its binding to DNA (Flatman *et al.*, 2005; M.J. Edwards *et al.*, unpubl. data). Simocyclinone D8 is a potent inhibitor of DNA gyrase supercoiling with an IC<sub>50</sub> lower than that of novobiocin (Flatman *et al.*, 2005; Oppegard *et al.*, 2009).

The genus *Streptomyces* accounts for the production of approximately two-thirds of the known antibiotics. They expel these compounds into their environment, typically the soil, most probably to give them a competitive advantage over other organisms that share the same ecological niche. Because the antibiotic is often potentially lethal to the producing organism, there must be mechanisms to ensure that the machinery responsible for export of the mature antibiotic is in place at the time of biosynthesis. This export machinery may be sufficient to confer resistance to the antibiotic, or there may be additional resistance mechanisms. For example, in the case of the novobiocin, clorobiocin and coumermycin A<sub>1</sub> producers, the principal mechanism of resistance is production of an aminocoumarin-resistant GyrB subunit (GyrB<sup>R</sup>), encoded within the biosynthetic cluster and activated during antibiotic production (Thiara and Cundliffe, 1988; 1989; 1993; Schmutz *et al.*, 2003). The GyrB<sup>R</sup> subunit replaces the sensitive subunit (GyrB<sup>S</sup>) in the (GyrA)<sub>2</sub>(GyrB)<sub>2</sub> heterotetramer during the production phase. In addition, an aminocoumarin-resistant topoisomerase IV subunit,

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**Fig. 1.** Structures of simocyclinone D8 and its biosynthetic intermediate, simocyclinone C4, and their IC<sub>50</sub>s for *Escherichia coli* DNA gyrase.

ParY<sup>R</sup>, encoded within the clorobiocin and coumermycin A<sub>1</sub> biosynthetic clusters (but not the novobiocin biosynthetic cluster), also confers resistance to these antibiotics when introduced into a naïve host, presumably by an analogous mechanism (Schmutz *et al.*, 2003). However, no DNA gyrase or topoisomerase IV subunits are encoded within the simocyclinone D8 (*sim*) biosynthetic cluster (Galm *et al.*, 2002; Trefzer *et al.*, 2002), leaving unknown the mechanism of resistance in the producing organism, *Streptomyces antibioticus* Tü 6040.

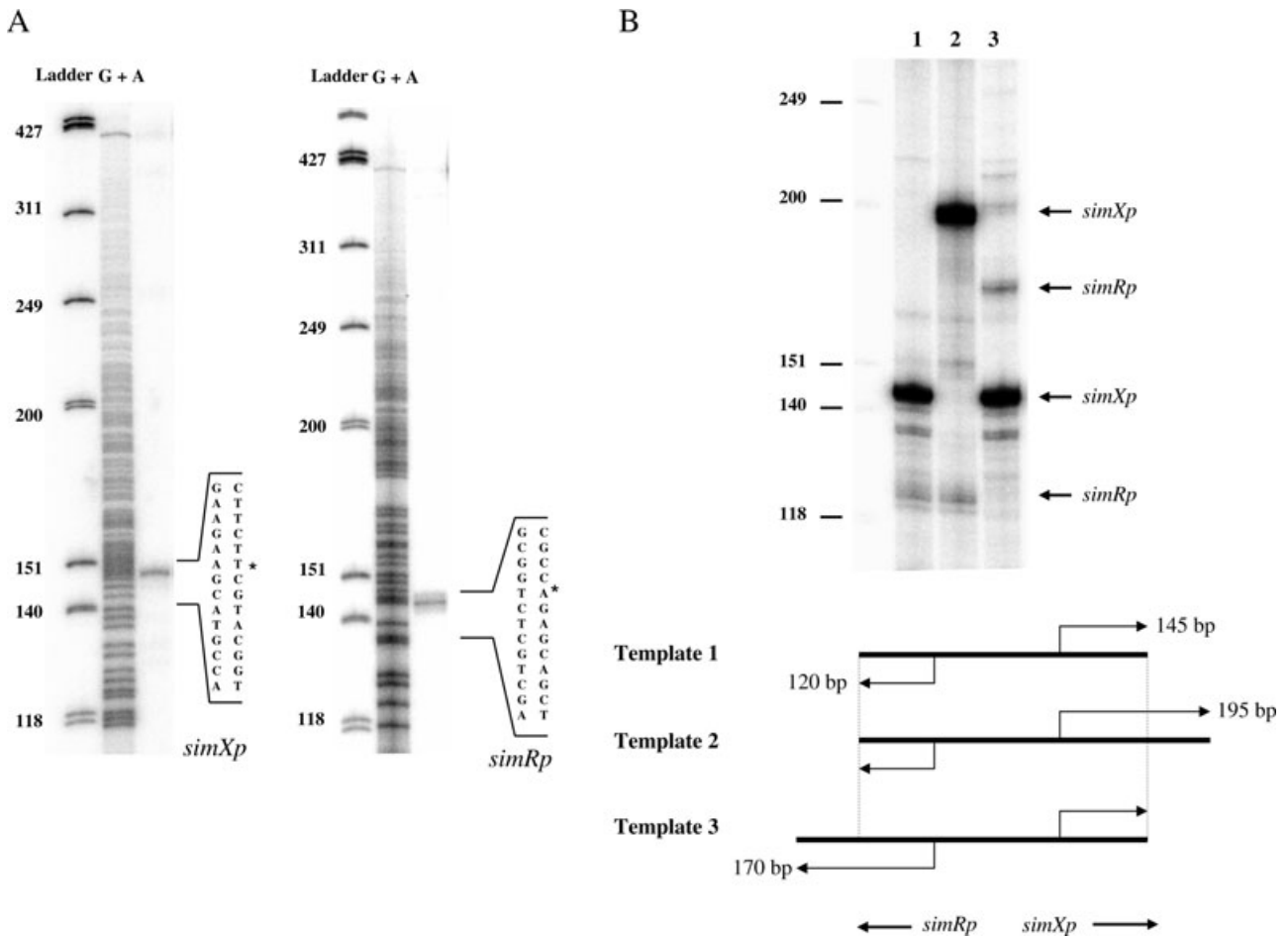
Simocyclinone D8 consists of four different parts, with a halogenated aminocoumarin at one end connected to an angucyclic polyketide at the other end via a tetraene linker and a D-olivose sugar (Galm *et al.*, 2002; Trefzer *et al.*, 2002) (Fig. 1). Within the *sim* cluster, among the genes responsible for the biosynthesis and linking of the four constituents of the antibiotic, are two divergently transcribed genes, *simR2* (hereafter, *simR*) and *simEX1* (hereafter, *simX*) (Galm *et al.*, 2002; Trefzer *et al.*, 2002). The SimR/SimX pair resembles the TetR/TetA repressor–efflux pump pair that causes widespread resistance to clinically important tetracyclines in several human pathogens (Chopra and Roberts, 2001). TetR represses transcription of the divergently transcribed *tetA* gene, encoding a proton-dependent tetracycline efflux pump. Binding of tetracycline to the C-terminal domain of TetR causes it to lose affinity for its operators, derepressing expression of *tetA*, which confers high-level resistance to the drug (Hillen and Berens, 1994; Kisker *et al.*, 1995; Orth *et al.*, 2000; Ramos *et al.*, 2005). The similarity of SimR/SimX to TetR/TetA suggested they might be involved in simocyclinone efflux and, potentially, in simocyclinone resistance (Galm *et al.*, 2002; Trefzer *et al.*, 2002).

Here we show that *simX* encodes a simocyclinone efflux pump, and that transcription of *simX* is controlled by SimR, which directly represses the *simX* and *simR* promoters by binding to two operator sites in the *simR–simX* intergenic region. We show that simocyclinone D8 abolishes DNA binding by SimR, providing an intimate mechanism that couples the biosynthesis of simocyclinone to its export. In addition, we show that an intermediate in the biosynthetic pathway, simocyclinone C4 (Fig. 1), which is essentially inactive as a DNA gyrase inhibitor, also induces *simX* expression *in vivo* and relieves DNA binding by SimR *in vitro*, suggesting a potential ‘feed-forward’ mechanism (Tahlan *et al.*, 2007) that might ensure expression of the SimX efflux pump prior to the build-up of a toxic concentration of the mature, active antibiotic.

## Results

### *SimX* encodes a simocyclinone efflux pump

There are two pump-like transmembrane proteins encoded in the *sim* cluster, SimEX1 (hereafter SimX) and SimEX2 (Trefzer *et al.*, 2002). To determine if either of these two proteins is involved in simocyclinone D8 efflux, we expressed *simX* and *simEX2* from the strong constitutive promoter *ermEp\** using the integrative, single-copy vector pIJ10257 (Hong *et al.*, 2005). We introduced these constructs into the heterologous host *S. lividans* and compared the susceptibility of the resulting strains to simocyclinone D8. The strain carrying *ermEp\*–simEX2* had an MIC of 2 μg ml<sup>-1</sup>, as did *S. lividans* alone, or *S. lividans* containing the parent vector, pIJ10257. In contrast, the strain carrying the *ermEp\*–simX* construct had an MIC of



**Fig. 2.** A. High-resolution S1 nuclease mapping of the 5' ends of the *simR* and *simX* transcripts using PCR-generated probes and RNA from the simocyclinone D8 producing organism, *S. antibioticus* Tü 6040. The most likely transcription start points are indicated by the asterisks. The G+A Maxam–Gilbert chemical sequencing ladder was generated using the same probe as the one used for S1 nuclease mapping assays. The size markers are a radiolabelled *Hinf*I digest of  $\Phi$ X174 DNA.

B. Run-off transcription from the *simR* and *simX* promoters *in vitro* using purified *S. coelicolor* RNA polymerase and the templates illustrated containing the *simR*–*simX* intergenic region. Lane numbers correspond to the different templates shown in the illustration below.

65  $\mu\text{g ml}^{-1}$ . The *ermEp*<sup>+</sup>–*simX* construct did not confer resistance to the structurally related aminocoumarin DNA gyrase inhibitor novobiocin, nor to unrelated antibiotics such as erythromycin, lincomycin, rifampicin, bacitracin and nisin. These results suggested that *simX* encodes a simocyclinone-specific efflux pump. Adjacent to *simX* is the divergent gene *simR*, encoding a potential transcriptional repressor of *simX*. We also cloned *simX* into an integrative vector under the control of its native promoter and in the absence of *simR*. This construct (pJ10461) only mildly enhanced resistance to simocyclinone D8 in *S. lividans*, giving an MIC of 4  $\mu\text{g ml}^{-1}$ .

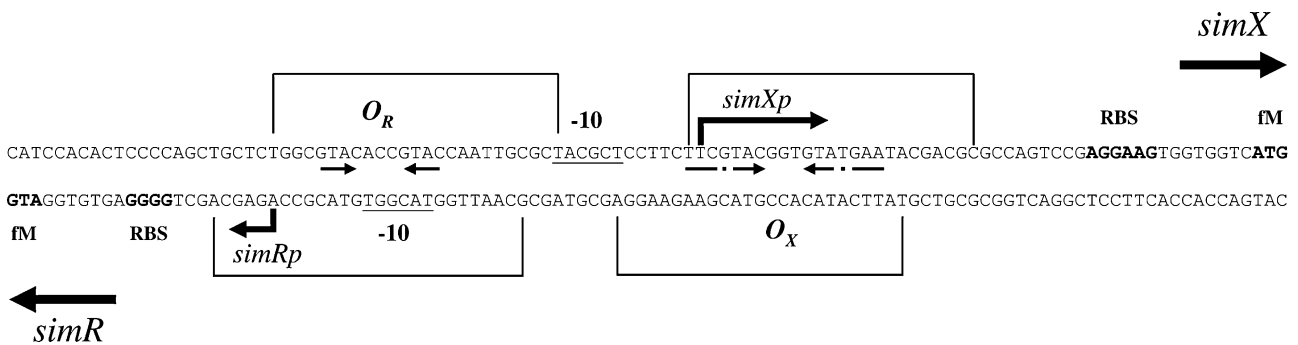
#### Mapping the transcription start points of *simR* and *simX*

High-resolution S1 nuclease mapping of the *simR* and *simX* promoters was performed using RNA isolated from the simocyclinone D8 producing organism, *S. antibioticus*

Tü 6040 (Fig. 2A). A single *simR* promoter (*simRp*) was identified, initiating transcription 20 bp upstream of the *simR* ATG start codon, and a single *simX* promoter (*simXp*) was identified, initiating transcription 47 bp upstream of the *simX* ATG start codon (Fig. 2A and 3). *In vitro* run-off transcription experiments with purified *S. coelicolor* RNA polymerase confirmed the presence and locations of the *simR* and *simX* promoters (Fig. 2B).

#### *SimR* regulates expression of *simR* and *simX*

To investigate the regulation of *simR* and *simX*, we measured *simR* and *simX* promoter activities in the presence and absence of SimR, using the integrative luciferase (*luxAB*) reporter plasmid pJ5972 (Aigle *et al.*, 2000; M. Paget, pers. comm.). Fragments carrying *simRp* and *simXp* were individually cloned into pJ5972, and the resulting reporter constructs were introduced into



**Fig. 3.** Sequence of the *simR*–*simX* intergenic region showing the *simRp* and *simXp* transcription start points and putative –10 sequences, the *simR* and *simX* ribosome binding sites (RBS), the extent of the SimR DNaseI footprints on the  $O_X$  and  $O_R$  operators, and the imperfect inverted repeats within the footprints that may represent SimR binding motifs.

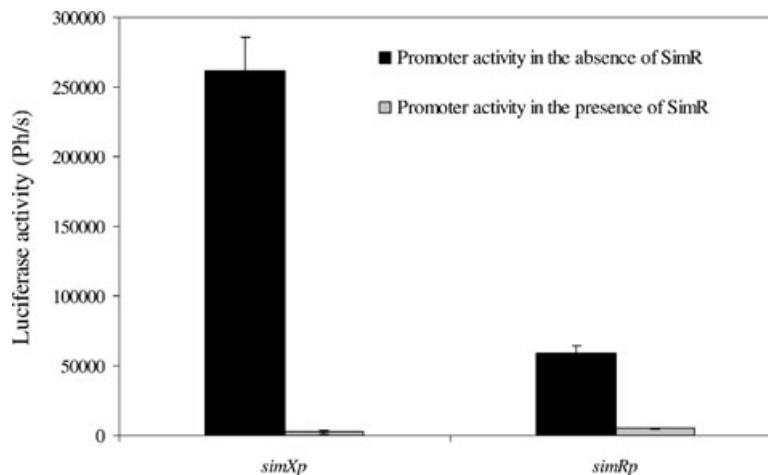
*S. lividans* in order to probe *simR* and *simX* promoter activities in the absence of SimR. To measure *simR* and *simX* promoter activities in the presence SimR, an integrative plasmid (pIJ10469) carrying *simR* under the control of its own promoter was introduced into the strains already harbouring the promoter-probe plasmids. Transformants were grown on Difco Nutrient Agar to promote vegetative growth and delay the formation of aerial hyphae, which may interfere with diffusion of the luciferase substrate (n-decanal) and with light emission. Figure 4 shows that *simX* and *simR* promoter activities were repressed 100-fold and 12-fold, respectively, in the presence of *simR*.

#### Purified SimR binds to the *simR*–*simX* intergenic region at two distinct operator sites

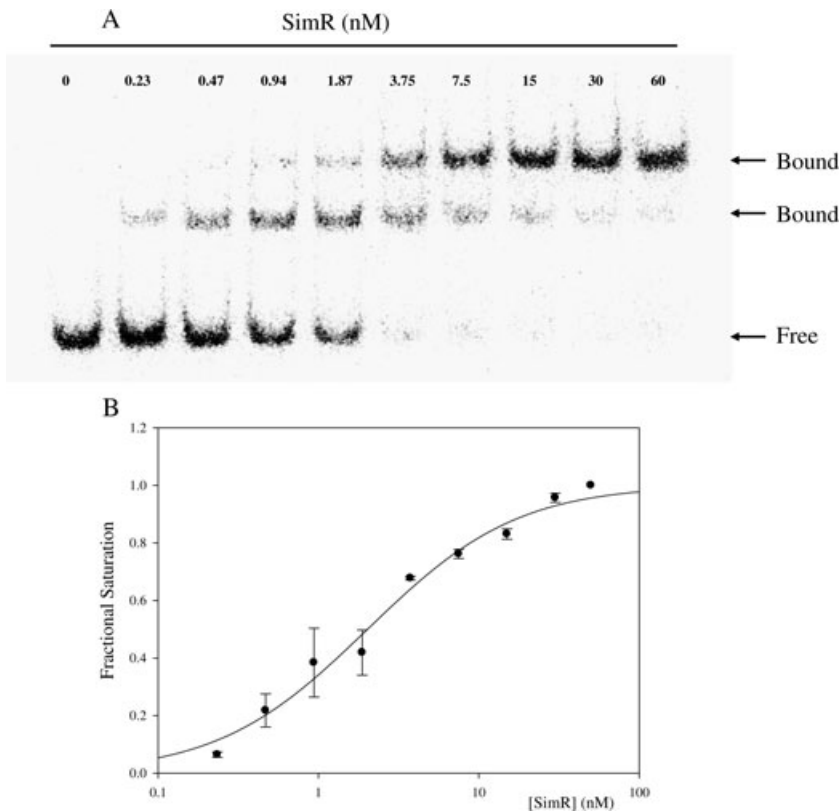
The *lux* reporter data suggested that SimR is a repressor that regulates its own expression as well as that of *simX*.

To test this idea, we monitored SimR binding to the *simR*–*simX* intergenic region by electrophoretic mobility shift assay (EMSA). An N-terminally His<sub>6</sub>-tagged derivative of SimR was overexpressed in *Escherichia coli* and purified to homogeneity. Increasing concentrations of SimR were incubated with a radiolabelled probe spanning the *simR*–*simX* intergenic region and the complexes were resolved on a native gel. Purified SimR bound to the intergenic region at concentrations as low as 0.23 nM and, as the concentration of SimR increased, two sets of shifted protein–DNA complexes became evident, suggesting that there are two SimR binding sites in the *simR*–*simX* intergenic region (Fig. 5A).

DNaseI footprinting on both DNA strands was used to map precisely the SimR operator sites within the *simR*–*simX* intergenic region. Two separate SimR binding sites were observed, consistent with the two shifted species seen in the EMSA experiments: the operator closer to *simX* was designated  $O_X$  and the one closer to *simR* was



**Fig. 4.** Promoter activities of *simR* and *simX* in the presence and absence of *simR*. *simRp*–*luxAB* and *simXp*–*luxAB* transcriptional fusions were created in an integrative luciferase promoter-probe vector (pIJ5972) and assayed in *S. lividans*, in either the presence or absence of *simR*. Plasmid-containing *S. lividans* strains were grown on Difco Nutrient Agar in single wells of a 96-well microtitre plate (Sterilin) for 3 days. Each well was inoculated with approximately  $5 \times 10^4$  spores. Plates were exposed to filter paper impregnated with n-decanal for 5 min and luciferase activities were quantified using a NightOwl camera (Berthold) equipped with WinLight software (Berthold) using a 1 min exposure time. Values given correspond to the average of three biological replicates from three different spore stocks and standard errors are shown.



**Fig. 5.** A. Electrophoretic mobility shift assay showing binding of purified SimR to the *simR-simX* intergenic region. Bands corresponding to protein-DNA complexes (Bound) and free DNA (Free) are indicated. The final concentration of SimR is indicated above each lane.

B. Saturation curve of the data from EMSA experiments. EMSA data were collected and analysed on a PhosphorImager (FujiFilm) using Multi Gauge image analysis software (FujiFilm). Two independent EMSAs were carried out [one of which is shown in (A)] and the mean values calculated. Standard errors are shown. Saturation curves (saturation fraction against concentration of protein) were fitted with SigmaPlot (see *Experimental procedures*) to determine  $K_{d}$ s. In all EMSA experiments, SimR was present in molar excess over the probe.

designated  $O_R$  (Figs 3 and 6). Within the  $O_X$  and  $O_R$  footprints we identified imperfect inverted repeats that may represent the binding sequences for the SimR homodimer. Figure 3 shows these imperfect inverted repeats, the extent of the SimR DNaseI footprints on the sequence of the *simR-simX* intergenic region and the positions of the *simRp* and *simXp* transcription start points and putative  $-10$  promoter sequences in relation to the two SimR operators.

#### *SimR binding to the two operators is non-cooperative*

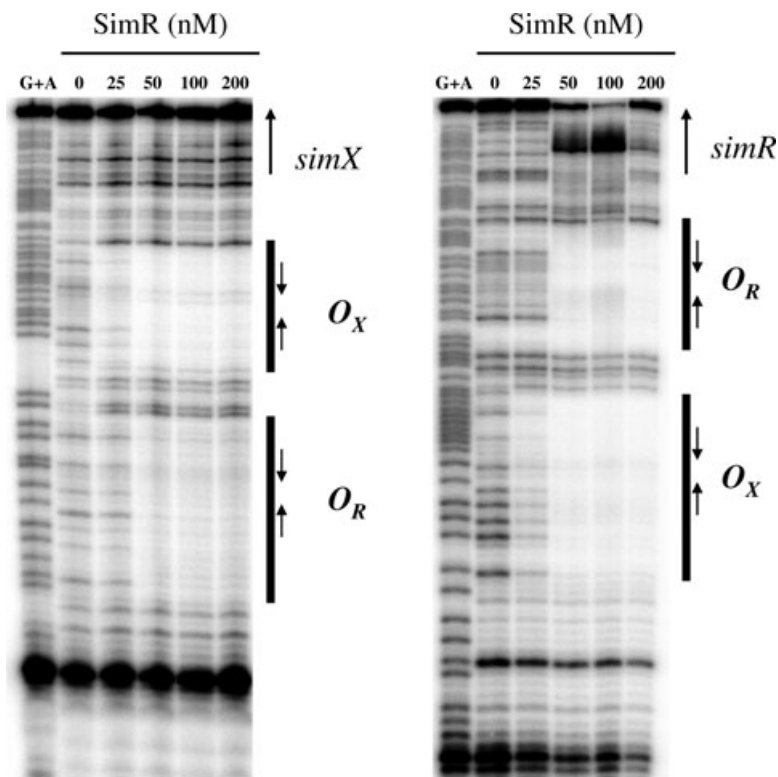
In the DNaseI footprinting analysis,  $O_X$  was occupied at a lower concentration of SimR than was  $O_R$ , suggesting that  $O_X$  has a higher affinity for SimR than  $O_R$ . Competitive EMSA was used to explore this issue further. In these experiments, unlabelled fragments containing either  $O_X$  or  $O_R$  were used to compete with a radioactively labelled *simR-simX* intergenic fragment containing both  $O_X$  and  $O_R$ . The final concentration of SimR used in the competitive EMSA experiments was set at 20 nM such that all the labelled probes were in complex with SimR in the absence of unlabelled competitor DNA. A 1000-fold excess of unlabelled  $O_X$ -containing fragment out-competed the labelled intergenic probe (no complex formation between SimR and the labelled intergenic probe) (Fig. 7). However, the same excess of  $O_R$ -containing frag-

ment could not completely abolish SimR complex formation with the labelled intergenic probe (Fig. 7), confirming that SimR binds  $O_X$  more tightly than  $O_R$ .

Using the EMSA data shown in Fig. 5, we determined the approximate equilibrium dissociation constants ( $K_{d}$ s) for the two complexes to be  $1.2 \pm 0.4$  nM for SimR- $O_X$  and  $3.5 \pm 1.4$  nM for SimR- $O_R$ . In order to determine whether there is cooperativity between SimR binding at  $O_X$  and  $O_R$ , we also determined the  $K_{d}$ s of each SimR-operator complex by EMSA using probes containing only  $O_X$  or only  $O_R$  (data not shown), instead of the full *simR-simX* intergenic region. The  $K_{d}$ s were found to be  $0.9 \pm 0.2$  nM for SimR- $O_X$  and  $3.6 \pm 0.3$  nM for SimR- $O_R$ . The  $K_{d}$ s for the two SimR-operator complexes did not change substantially when the two operators were separated, suggesting that SimR binding to its two operators is non-cooperative.

#### *Exogenous simocyclinone D8 induces expression of the SimX efflux pump in vivo*

In order to determine whether the *simR* and *simX* promoters respond to simocyclinone D8 *in vivo*, we used the *S. lividans luxAB* reporter system to measure *simRp* and *simXp* activities in the presence of SimR and in response to exogenously added antibiotic. Figure 8A shows the response curve of *simR* and *simX* promoter activity to



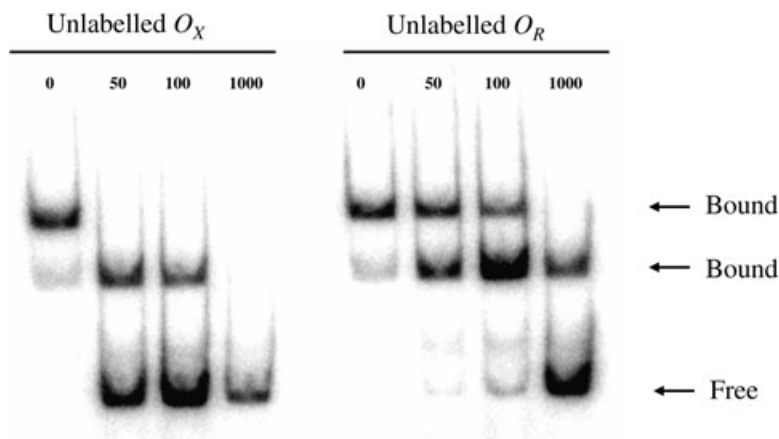
**Fig. 6.** DNaseI footprinting analysis of SimR binding to the *simR*–*simX* intergenic region. A DNA fragment containing the *simR*–*simX* intergenic region, 5′ end-labelled on either the upper strand (left panel) or the lower strand (right panel), was exposed to DNaseI in the presence of increasing concentrations of SimR. The sequencing ladders were generated by subjecting the probes to Maxam–Gilbert G+A chemical sequencing. Regions protected from DNaseI cleavage (operators  $O_X$  and  $O_R$ ) are indicated with vertical bars and inverted repeats within the DNaseI protected regions are indicated by convergent arrows; these features are also highlighted on the DNA sequence in Fig. 3.

increasing concentrations of simocyclinone D8. Both promoters were induced by simocyclinone D8, suggesting that the antibiotic can relieve SimR-mediated repression of both *simRp* and *simXp*. The highest concentration tested was  $0.6 \mu\text{g ml}^{-1}$  because *S. lividans* is sensitive to simocyclinone D8 ( $\text{MIC} = 2 \mu\text{g ml}^{-1}$ ).

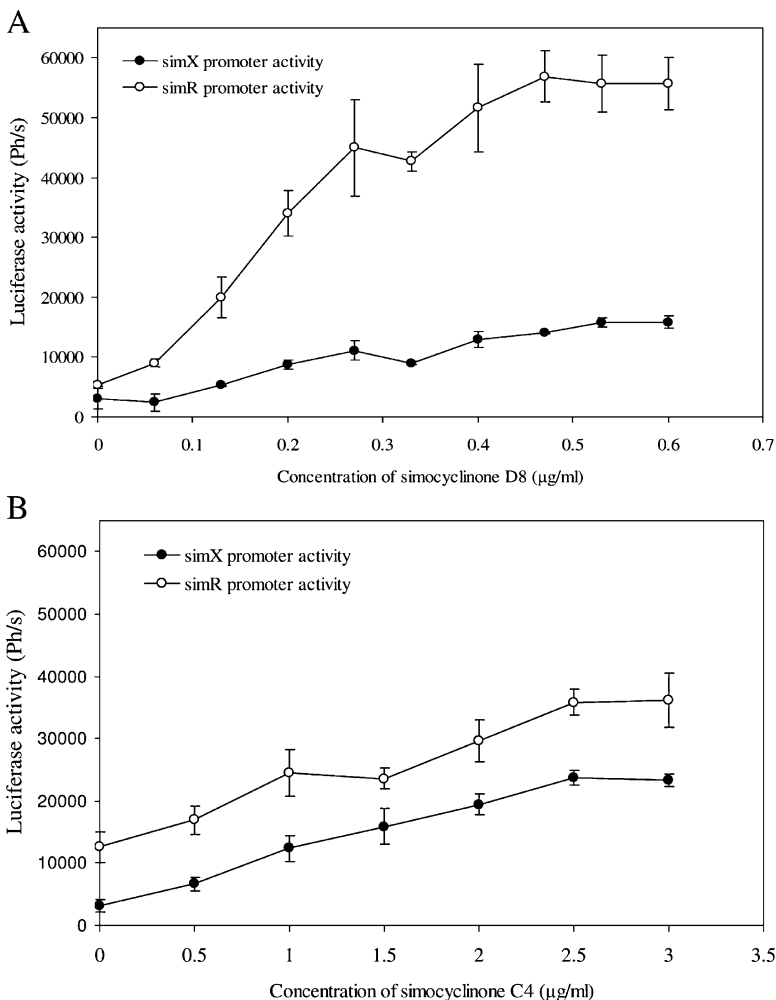
#### *Simocyclinone D8 dissociates SimR from the simR–simX intergenic region*

To determine if SimR responds directly to simocyclinone D8, we examined the effect of the antibiotic on SimR–operator complex formation by EMSA (Fig. 9A). The SimR

concentration was held constant and an increasing concentration of simocyclinone D8 was introduced into the binding reaction. As the simocyclinone D8 concentration increased, there was a progressive decrease in SimR–DNA complex formation and a concomitant liberation of free probe;  $62.5 \mu\text{M}$  simocyclinone D8 was sufficient to dissociate the SimR–DNA complexes almost completely. This effect was not due to DMSO, the simocyclinone D8 solvent, as equivalent amounts of pure DMSO had no effect on SimR–DNA complex formation (data not shown). Furthermore, to test specificity, we examined the effect of simocyclinone D8 on the DNA binding activity of ActR, a TetR homologue that regulates actinorhodin export in



**Fig. 7.** Competitive electrophoretic mobility shift assay comparing the binding affinity of SimR to  $O_X$  and  $O_R$ . All lanes contain SimR at a final concentration of 20 nM and a constant amount of a radiolabelled *simR*–*simX* intergenic probe that carries both  $O_X$  and  $O_R$ . The fold excess (over the radiolabelled probe) of a competing unlabelled fragment containing either  $O_X$  or  $O_R$  is indicated above each lane.



**Fig. 8.** Induction of the *simR* and *simX* promoters *in vivo* by (A) simocyclinone D8 and (B) the biosynthetic intermediate simocyclinone C4. *S. lividans* containing pIJ10469 (carrying *simR* under its native promoter) together with luciferase promoter-probe plasmids pIJ10465 (*simXp-luxAB*) or pIJ10466 (*simRp-luxAB*) were assayed. Values given correspond to the average of three biological replicates from three different spore stocks and standard errors are shown. For further details, see the legend of Fig. 4.

*Streptomyces coelicolor* (see Discussion) and found that 100 µM simocyclinone D8 had no effect on ActR binding to its cognate operator (data not shown). These results show that simocyclinone D8 is able to specifically disrupt SimR–DNA complex formation.

*An intermediate in the simocyclinone biosynthetic pathway induces simX in vivo and dissociates SimR from its operators in vitro*

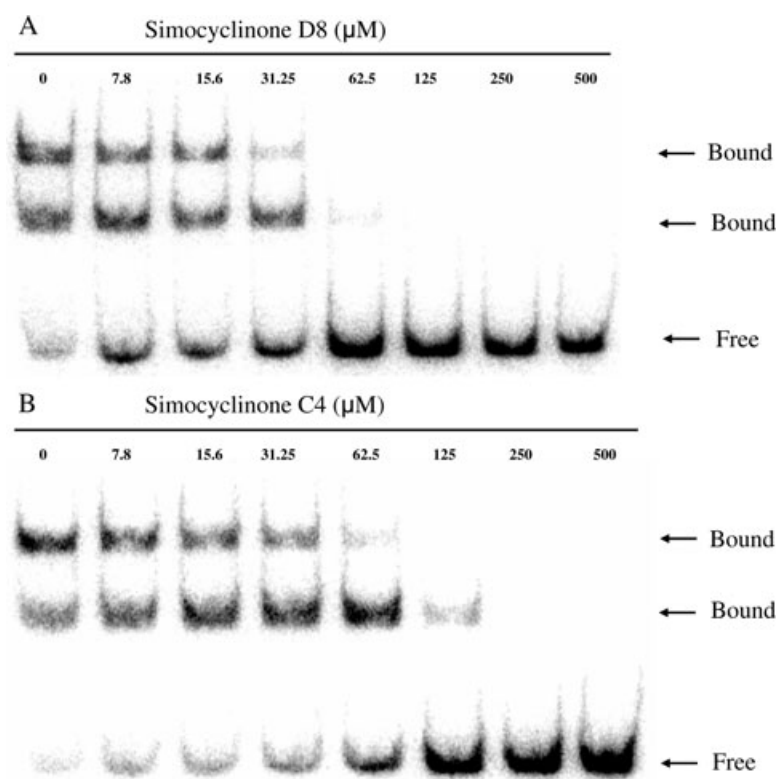
In addition to simocyclinone D8, we also tested the ability of simocyclinone C4, a natural intermediate in the D8 biosynthetic pathway, to induce the *simR* and *simX* promoters *in vivo* and to dissociate SimR from its operators *in vitro*. Simocyclinone C4 lacks the aminocoumarin ring present in the mature antibiotic (Fig. 1) and is essentially inactive as a DNA gyrase inhibitor; the simocyclinone D8  $IC_{50} = 0.1$  µM, whereas the simocyclinone C4  $IC_{50} > 100$  µM (M.J. Edwards *et al.*, unpubl. data). Simocyclinone C4 induced the *simR* and *simX* promoters *in vivo*, although somewhat more weakly than the mature

antibiotic (Fig. 8B). Because simocyclinone C4 is not an active antibiotic, we were able to test higher concentrations than for simocyclinone D8. Consistent with the *in vivo* inductions, simocyclinone C4 also caused SimR to dissociate from its binding sites *in vitro*, although again somewhat more weakly than simocyclinone D8; 250 µM simocyclinone C4 was sufficient to abolish all complex formation (Fig. 9B).

## Discussion

This report shows that *simX* encodes a simocyclinone D8 efflux pump and that the *simX* promoter is directly repressed by SimR, which binds to two operator sites in the *simR–simX* intergenic region. Simocyclinone D8 abolishes DNA binding by SimR, coupling the biosynthesis of simocyclinone to its export.

Simocyclinone D8 is a potent inhibitor of DNA gyrase supercoiling ( $IC_{50} = 0.1$  µM) (Flatman *et al.*, 2005; Oppergard *et al.*, 2009; M.J. Edwards *et al.*, unpubl. data). The recent structure of the antibiotic bound to the DNA gyrase



**Fig. 9.** Electrophoretic mobility shift assays showing that (A) simocyclinone D8 and (B) its biosynthetic intermediate simocyclinone C4 abolish SimR DNA binding activity. All lanes contain a constant amount of SimR and radiolabelled *simR*–*simX* intergenic fragment containing both  $O_x$  and  $O_R$ . Bands corresponding to protein–DNA complexes (Bound) and free DNA (Free) are indicated. The final concentrations of simocyclinone D8 or simocyclinone C4 are indicated above each lane.

A subunit shows that the two moieties at the ends of simocyclinone D8, the aminocoumarin ring and the angucyclic polyketide, bind to two separate, well-defined pockets within the GyrA DNA binding saddle, linked by the intervening tetraene linker and D-olivose moieties (M.J. Edwards *et al.*, unpubl. data). Given the prominence of the aminocoumarin ring in the overall binding of the antibiotic to the GyrA subunit, it is not surprising that the biosynthetic intermediate simocyclinone C4, which lacks the aminocoumarin ring (Fig. 1), is essentially inactive as a DNA gyrase inhibitor ( $IC_{50} > 100 \mu\text{M}$ ). Importantly, however, although simocyclinone C4 is not a DNA gyrase inhibitor, it can efficiently derepress SimR *in vivo* and *in vitro*.

The most striking analogy to the research presented here is the work of Nodwell and colleagues on the regulation of efflux of actinorhodin (Ahn *et al.*, 2007; Hopwood, 2007; Tahlan *et al.*, 2007; 2008; Willems *et al.*, 2008). Actinorhodin is a blue-pigmented, six-ring polyketide antibiotic made by *S. coelicolor* (Bystrykh *et al.*, 1996). Within the actinorhodin biosynthetic cluster are two co-transcribed genes, *actA* and *actII-ORF3*, encoding integral membrane proteins implicated in actinorhodin export (Caballero *et al.*, 1991; Fernandez-Moreno *et al.*, 1991). Expression of these two genes is regulated by a TetR-like protein, ActR, the product of the adjacent, divergently transcribed gene. Nodwell and colleagues have been characterizing ligands that relieve repression by ActR. Importantly, they showed that, in addition to the

mature six-ring antibiotic, three-ring intermediates from the biosynthetic pathway also relieve repression by ActR. From this, they suggested that the ability of actinorhodin intermediates to relieve repression by ActR might provide a 'feed-forward' mechanism that would ensure expression of the ActA efflux pump prior to the build-up of a toxic concentration of the mature antibiotic (Ahn *et al.*, 2007; Hopwood, 2007; Tahlan *et al.*, 2007; 2008; Willems *et al.*, 2008). Similarly, the ability of an inactive simocyclinone intermediate to relieve repression by SimR might also act as a biosynthetic checkpoint to ensure feed-forward regulation of simocyclinone export.

As applied to simocyclinone (or actinorhodin) export, the feed-forward hypothesis is speculative. We have shown that the pathway intermediate simocyclinone C4 induces *simX* expression *in vivo* when applied exogenously. However, for a feed-forward mechanism to operate in the producing organism, simocyclinone C4 or other SimR-binding pathway intermediates would have to accumulate in the cytoplasm to a concentration high enough to trigger *simX* expression, and the cytoplasmic concentrations of pathway intermediates are hard to determine experimentally. However, there are other examples in the literature where the TetR-like protein blocks expression not only of the exporter gene, but also of late biosynthetic genes, and in these cases it seems that an intermediate in the pathway must be responsible for inducing expression of these genes (i.e. feed-forward

activation), as induction of the late biosynthetic enzymes is required to generate the mature antibiotic.

In the landomycin A producer, *Streptomyces cyanogenus*, a TetR-family regulator, LanK, represses expression of *lanJ*, encoding a putative landomycin A efflux pump (Ostash *et al.*, 2008). LanK is derepressed by mature landomycin A, which carries a hexasaccharide chain, but also by intermediates in the pathway that carry only a pentasaccharide or a trisaccharide chain (Ostash *et al.*, 2008). However, *lanJ* is co-transcribed with several downstream biosynthetic genes involved in late glycosylation steps. As a consequence, LanK couples production of intermediates not only to assembly of the export machinery, but also to expression of late biosynthetic enzymes that attach the final sugars to produce mature landomycin A (Ostash *et al.*, 2008). A more complex example concerns the biosynthesis of the clinically important anticancer agents daunorubicin and doxorubicin made by *Streptomyces peucetius*. In this system, binding of daunorubicin–doxorubicin pathway intermediates like rhodomycin D appears to derepress the TetR-like regulator DnrO, activating a cascade involving two further transcription factors, DnrN and DnrI, which leads to expression of the resistance genes and of late biosynthetic genes (Otten *et al.*, 1995; Jiang and Hutchinson, 2006).

In addition to repressing the *simX* promoter, SimR also directly negatively regulates its own expression. In other systems, negative autoregulation has been shown to confer specific functions that are absent in systems that have simple regulation. Many of these studies on the design principles of genetic circuits that incorporate negative autoregulation have exploited TetR, the founding member of the family to which SimR belongs (Becskei and Serrano, 2000; Rosenfeld *et al.*, 2002; D. Madar and U. Alon, pers. comm.). These studies show that negative autoregulatory feedback loops in gene circuits can provide stability, thereby limiting stochastic fluctuations in the system (Becskei and Serrano, 2000). In addition, negative autoregulation can speed the response time of the transcription network to a stimulus (Rosenfeld *et al.*, 2002), and it can broaden the dynamic range of the input signal to which the downstream genes respond (D. Madar and U. Alon, pers. comm.).

An unresolved issue is the basis of resistance to simocyclinone D8 in the producing organism. In the streptomycetes that produce the aminocoumarins novobiocin (*Streptomyces sphaeroides*), clorobiocin (*Streptomyces roseochromogenes*) and coumermycin A<sub>1</sub> (*Streptomyces rishiriensis*), expression of an aminocoumarin-resistant GyrB<sup>R</sup> gyrase subunit encoded within the biosynthetic gene cluster is turned on during antibiotic production, conferring resistance (Thiara and Cundliffe, 1988; 1989; 1993; Schmutz *et al.*, 2003). The absence of an equivalent

*gyrB* resistance gene in the simocyclinone biosynthetic gene cluster was an initial surprise when the *sim* locus was sequenced, until it was subsequently and unexpectedly shown that simocyclinone has a completely different mode of action, binding instead to the GyrA subunit of the enzyme (Flatman *et al.*, 2005; M.J. Edwards *et al.*, unpubl. data). However, no GyrA<sup>R</sup> subunit is encoded within the *sim* cluster either (Galm *et al.*, 2002; Trefzer *et al.*, 2002). Expressing *simX* from the strong, constitutive promoter, *ermEp\**, conferred simocyclinone resistance on the heterologous host *S. lividans* (MIC 65 µg ml<sup>-1</sup> instead of 2 µg ml<sup>-1</sup> for *S. lividans* alone), showing that SimX can act as an effective resistance determinant when expressed at appropriate levels. However, the MIC was only 4 µg ml<sup>-1</sup> when *simX* was expressed from its own promoter in the absence of SimR. This may reflect additional levels of *simX* regulation that have yet to be uncovered. For example, in addition to its repression by SimR, it is possible that *simX* expression in *S. antibioticus* might also be activated by a simocyclinone pathway-specific activator that is absent from *S. lividans*. Alternatively, it is possible that SimX-mediated efflux is not the principal simocyclinone resistance mechanism in *S. antibioticus*.

## Experimental procedures

*Bacterial strains, plasmids, oligonucleotides, culture conditions and conjugal plasmid transfer from E. coli to Streptomyces spp.*

Strains, plasmids and oligonucleotides are described in Table 1. Unless stated otherwise, *S. lividans* 1326 was grown on SFM (Kieser *et al.*, 2000) and *S. antibioticus* Tü 6040 was grown on MYMTap [0.4% (w/v) maltose, 0.4% (w/v) Yeast Extract, 1% (w/v) Malt Extract, supplemented trace elements; made up in tap water]. For conjugal transfer into *Streptomyces* (Paget *et al.*, 1999), plasmids were introduced into the *dam dcm hsdS E. coli* strain ET12567, which carries the non-transmissible, *oriT*-mobilizing plasmid pUZ8002 (Paget *et al.*, 1999). All cloned PCR fragments were verified by sequencing.

### *Fermentation, isolation and purification of simocyclinone D8 and simocyclinone C4*

Simocyclinone D8 was isolated as described by Schimana *et al.* (2000). Briefly, *S. antibioticus* Tü 6040 was fermented in a complex medium consisting of 2% (w/v) mannitol and 2% (w/v) soybean meal in a 20 l fermentor, and simocyclinones were extracted from the mycelium with methanol. Pure simocyclinone D8 was obtained after reversed-phase HPLC using Nucleosil-100 C-18 material and 0.01% trifluoroacetic acid-acetonitrile gradient elution, resulting in a dark yellow powder after drying. Pure simocyclinone C4 was isolated using essentially the same procedure, but fermentation was carried out in a defined medium containing 20% (v/v) glycerol and 0.15% (w/v) L-arginine to maximize simocyclinone C4

**Table 1.** Bacterial strains, plasmids and primers used in this study.

	Relevant genotype/comments	Source/reference
<b>Strains</b>		
<i>Streptomyces</i>		
<i>S. antibioticus</i> Tü 6040	Environmental isolate; original producer of simocyclinone D8	Schimana <i>et al.</i> (2000)
<i>S. lividans</i> 1326	Wild-type SLP2 <sup>+</sup> SLP3 <sup>+</sup>	Kieser <i>et al.</i> (2000)
<i>E. coli</i>		
ET12567 (pUZ8002)	ET12567 containing helper plasmid pUZ8002	Paget <i>et al.</i> (1999)
<b>Plasmids</b>		
pSET152	ΦC311 <i>attP-int</i> derived integration vector for the conjugal transfer of DNA from <i>E. coli</i> to <i>Streptomyces</i> (Apr <sup>R</sup> )	Bierman <i>et al.</i> (1992)
pMS82	ΦBT1 <i>attP-int</i> derived integration vector for the conjugal transfer of DNA from <i>E. coli</i> to <i>Streptomyces</i> (Hyg <sup>R</sup> )	Gregory <i>et al.</i> (2003)
pIJ5972	Integrative <i>Streptomyces</i> promoter-probe plasmid based on TTA codon-free derivatives of the <i>luxAB</i> reporter genes	Aigle <i>et al.</i> (2000); M. Paget, pers. comm.
pIJ10257	Integrative expression vector based on the strong, constitutive <i>ermE</i> <sup>*</sup> promoter ( <i>ermEp</i> <sup>*</sup> )	Hong <i>et al.</i> (2005)
pIJ10461	pSET152 carrying <i>simX</i> under the control of its own promoter, without <i>simR</i>	This study
pIJ10465	pIJ5972 <i>simXp-luxAB</i>	This study
pIJ10466	pIJ5972 <i>simRp-luxAB</i>	This study
pIJ10469	pMS82 carrying <i>simR</i> under the control of its native promoter	This study
pIJ10480	pIJ10257 <i>ermEp</i> <sup>*</sup> - <i>simX</i>	This study
pIJ10481	pIJ10257 <i>ermEp</i> <sup>*</sup> - <i>simEX2</i>	This study
pIJ10490	pET28a derivative expressing His <sub>6</sub> -tagged SimR	This study
<b>Primers</b>		
pX-F-EcoRI	GAATTCGAGCACGAACTCCTGCTGGC	
pX-R-BamHI	GGATCCGACCACCACTTCCCTCGGACTGG	
pR-F-BamHI	GGATCCTCCCCAGCTGCTCTGGCGTACACC	
pR-R-EcoRI	GAATTCGTGAACGTACCGACCATCAGGCCG	
MS82-simR-F-HindIII	GGCAAGCTTTCAAGCCAGTGCTGGACGTTCC	
MS82-simR-R-KpnI	AACGGTACCAACGGCATCCTCATCTGGCATGACC	
intRX-138-F	AAAGATATCCTCGTTCATCCACTCCCC	
intRX-138-R	AAAGGATCCATCTGGCATGACCACCACTTC	
intOX-123-F	CCAATTGCGTACTCGTCCCTTC	
intOX-123-R	CCTGCGCGGAGCCTCCGGAC	
intOR-130-F	CACCCCTCGGTGTCCGCCACC	
intOR-130-R	AACGAGAACGAACCCGTCAG	
simEX1-F-NdeI	GAGCATATGCCAGATGAGGATGCCGTTGC	
simEX1-R-HindIII	TAGAAGCTTCTATCCGGCATTCCGAGCCG	
simEX2-F-NdeI	GGGCATATGACCAGTTTCCAAGTCCAG	
simEX2-R-HindIII	GGGAAGCTTACCTCCCGGCCGACGATAGACC	
simR-int-simX-R	CTATCCGGCATTCCGAGCCG	
S1-probeX-F	GTAGAGGGACATCGTGCCGGC	
S1-probeX-R	GGCCGAGCAGTACGGCCAGC	
S1-probeR-F	GTGTCGGCCACCTTGACGGC	
S1-probeR-R	CCACCGAGCTCTCCGACGATCG	
Invitro1-F	GAACGATCTGGTACGGCTC	
Invitro1-R	AGCAGTACGGCCAGCACCCC	
Invitro2-R	GCAGCGCCGTACCGACGATCACC	
Invitro3-F	CATCGACGCCCTCGACACCC	

production (Theobald *et al.*, 2000). The nature and purity of the simocyclinone D8 and C4 samples were confirmed by nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry (Holzenkämpfer *et al.*, 2002; M.J. Edwards, pers. comm.)

#### Construction of *simX* and *simEX2* expression plasmids and minimal inhibitory concentration (MIC) determinations

*simX* and *simEX2* were amplified by PCR using primers carrying NdeI (upstream) and HindIII (downstream) sites [for

*simX*, *simEX1*-F-NdeI and *simEX1*-R-HindIII; for *simEX2*, *simEX2*-F-NdeI and *simEX2*-R-HindIII (Table 1)]. To express the *simX* and *simEX2* genes from the *ermEp*<sup>\*</sup> promoter, the fragments were cloned into NdeI-HindIII-cut pIJ10257 (Table 1) to generate pIJ10480 and pIJ10481 respectively. To express *simX* from its own promoter, a fragment containing the *simX* coding sequence and 122 bp of DNA upstream of the *simX* translation start codon was amplified by PCR using the primers intRX-138-F and simR-int-simX-R (Table 1), and cloned into the SmaI site of pUC19. The resulting construct was digested with BamHI and EcoRI and the insert was cloned into BamHI-EcoRI-cut pSET152 to give pIJ10461. Constructs were transferred into *S. lividans* by conjugation.

For MIC determinations, approximately  $5 \times 10^4$  spores were added to each well of a 96-well microtitre plate (Sterilin), containing a twofold simocyclinone D8 dilution series in SMMS medium (Kieser *et al.*, 2000). The plates were evaluated everyday for 5 days after inoculation, and the MIC was defined as the lowest concentration at which no growth was observed. Simocyclinone D8 was dissolved in DMSO with the final concentration of DMSO in the agar not to exceed 1% (v/v).

#### Construction of *luxAB* reporter plasmids and luciferase activity measurements

To probe *simXp* and *simRp* activities in the absence of SimR, the promoter regions (500 bp upstream of the translation start codons) were amplified by PCR using upstream primers carrying an EcoRI site and downstream primers carrying a BamHI site [primers pX-F-EcoRI and pX-R-BamHI for *simXp*; pR-F-BamHI and pR-R-EcoRI for *simRp* (Table 1)]. The *simXp* and *simRp* promoter fragments were cloned into EcoRI-BamHI-cut pIJ5972, an integrative, *Streptomyces* promoter-probe plasmid based on TTA codon-free derivatives of the *luxAB* reporter genes (Aigle *et al.*, 2000; M. Paget, pers. comm.), to create plasmids pIJ10465 and pIJ10466, respectively, and these reporter constructs were transferred by conjugation into *S. lividans*. To probe *simXp* and *simRp* activities in the presence of SimR, a plasmid carrying *simR* under the control of its own promoter (pIJ10469) was transferred by conjugation into strains already harbouring the promoter-probe plasmids pIJ0465 and pIJ10466. To construct pIJ10469, *simR* with its promoter was amplified by PCR using an upstream primer carrying a HindIII site and a downstream primer carrying a KpnI site [primers MS82-simR-F-HindIII and MS82-simR-R-KpnI (Table 1)] and the fragment was cloned into HindIII-KpnI-cut pMS82.

Plasmid-containing strains were grown on Difco Nutrient Agar in single wells of a 96-well microtitre plate (Sterilin) for 3 days. Each well was inoculated with approximately  $5 \times 10^4$  spores. Plates were exposed to filter paper impregnated with n-decanal for 5 min and luciferase activities were quantified using a NightOwl camera (Berthold) equipped with WinLight software (Berthold) using a 1 min exposure time. Values given correspond to the average of three biological replicates from three different spore stocks.

#### Overexpression and purification of SimR

The *simR* gene was chemically synthesized with codon optimization (Genescript) for expression in *E. coli* and ligated into pET28a (Novagen) to give pIJ10490, which was introduced into *E. coli* BL21 (DE3) pLys. Recombinant His<sub>6</sub>-SimR was purified from a 250 ml LB culture induced with a 0.5 mM final concentration of IPTG at 30°C for 4 h. The cell pellet was re-suspended in 20 ml Buffer A [10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 50 mM imidazole, 1× Complete Mini, EDTA-free protease inhibitor (Roche)], sonicated (20 s at 10 microns for three cycles), and the lysate was clarified by centrifugation. The filtered cell lysate was loaded onto a 1 ml Ni-loaded Hi-Trap Chelating HD column (GE Healthcare) and His-tagged SimR was eluted with Buffer B [10 mM Tris-HCl,

pH 7.8, 150 mM NaCl, 500 mM imidazole] and dialysed into Storage Buffer [10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10% (v/v) glycerol]. The resulting preparation of SimR was 98% pure as judged by SDS-PAGE and was stored at -80°C.

#### Electrophoretic mobility shift assays and determination of dissociation constants ( $K_{dS}$ )

The EMSA DNA probe spanning the entire *simR-simX* intergenic region was amplified by PCR using primers intrX-138-F and intrX-138-R (Table 1) and then 5' end-labelled using [ $\gamma^{32}$ -P]-ATP and T4 polynucleotide kinase (New England Biolabs). The competitor DNA carrying only  $O_R$  was amplified using primers intOR-130-F and intOR-130-R, and the competitor DNA carrying only  $O_X$  was amplified using primers intOX-123-F and intOX-123-R (Table 1). Binding of SimR to DNA was carried out in 20  $\mu$ l EMSA buffer [20 mM Tris, pH 8.0, 1  $\mu$ g poly(dI-dC), 1 mM EDTA, 100 mM NaCl, 0.5 mM DTT, 8% (v/v) glycerol] containing 0.1 nM radiolabelled DNA (approximately 8000 c.p.m.) and varying amounts of SimR. After incubation at 30°C for 5 min, the binding reaction mixtures were loaded on 5% (w/v) native polyacrylamide gels and run in TBE buffer at 120 V for 45 min. The effect of simocyclinone D8 and simocyclinone C4 on the ability of SimR to bind DNA was tested by adding the compounds to the EMSA buffer. The simocyclinone D8 and simocyclinone C4 stock solutions were made up in 100% DMSO.

EMSA data were collected and analysed on a Phosphor-Imager (FujiFilm) using Multi Gauge image analysis software (FujiFilm). Two independent EMSAs were carried out for each probe and mean values were calculated. In order to calculate  $K_{dS}$  for SimR binding to each operator when the two operator sites were separated, saturation curves (percentage of probe bound against concentration of protein) were fitted using SigmaPlot (one site saturation model). For  $K_{dS}$  of each operator when the two sites were coupled, a random-order binding model was used, where  $Y = [S]/(2(K_{d1} + [S])) + [S]/(2(K_{d2} + [S]))$ , in which  $Y$  is the fractional saturation,  $K_{d1}$  and  $K_{d2}$  are dissociation constant of SimR binding to  $O_X$  and  $O_R$  operators, respectively, and  $[S]$  is the concentration of protein. The fractional saturation was calculated from intensities of EMSA bands in each lane (Fig. 5A) as followed,  $Y = (0.5 \times \text{intensity of middle band} + \text{intensity of top band}) / (\text{intensity of all bands in a lane})$ . The equation was then fitted using SigmaPlot to determine  $K_{dS}$ .

#### DNaseI footprinting

Templates for DNaseI footprinting were amplified by PCR using one unlabelled primer and one primer 5' end-labelled using [ $\gamma^{32}$ -P]-ATP and T4 polynucleotide kinase (New England Biolabs). The primers were intrX-138-F and intrX-138-R (Table 1), the same pair used to generate the *simR-simX* intergenic region probe for the EMSA experiments. DNaseI footprinting assays were performed in 40  $\mu$ l EMSA buffer containing approximately 180 000 c.p.m. radiolabelled DNA and varying amounts of SimR. After incubation at room temperature for 5 min, 10  $\mu$ l of DNaseI (10 units in 10 mM CaCl<sub>2</sub>) was added and the incubation was continued for a

further 60 s. Reactions were stopped by adding 140 µl DNaseI stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, 0.15% SDS and 0.1 mg ml<sup>-1</sup> yeast tRNA), the samples were precipitated with ethanol, and the pellets were dried and dissolved in 5 µl Sequencing Loading Dye [80% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue]. After heating at 80°C for 3 min and cooling on ice, the samples were run on a 6% (w/v) polyacrylamide/8 M urea sequencing gel, which was dried and analysed using a PhosphorImager (FujiFilm). A G+A sequencing ladder was generated from the template DNA by chemical sequencing (Maxam and Gilbert, 1980).

#### RNA preparation, S1 nuclease protection analysis and in vitro run-off transcription

For RNA preparation, approximately 10<sup>9</sup> *S. antibioticus* spores were germinated by heat-shock treatment in 5 ml TES buffer (0.05 M, pH 8) at 50°C for 10 min, then diluted with an equal volume of double-strength germination medium [1% (w/v) Difco yeast extract, 1% (w/v) Difco casaminoacids, 0.01 M CaCl<sub>2</sub>] and incubated with shaking at 37°C for 6 h (modified from Kieser *et al.*, 2000). Germinated spores were inoculated into NMMP (Kieser *et al.*, 2000) and incubated with shaking for a further 15 h at 30°C. RNA was prepared essentially as described by Hesketh *et al.* (2007), but with minor modifications taken from the Qiagen RNA Extraction Kit procedure (Qiagen). Probes for S1 nuclease protection analysis were generated by PCR using a 5' end-labelled oligonucleotide internal to the ORF and an unlabelled upstream primer [for *simXp*, primers S1-probeX-F and S1-probeX-R; for *simRp*, primers S1-probeR-F and S1-probeR-R; (Table 1)]. Hybridizations were carried out in sodium trichloroacetic acid buffer at 45°C overnight after denaturation at 65°C for 10 min (Kieser *et al.*, 2000). A G+A sequencing ladder was generated by chemical sequencing (Maxam and Gilbert, 1980).

RNA polymerase containing a mixture of sigma factors was purified as described previously (Buttner *et al.*, 1988) from *S. coelicolor* M600 grown to exponential phase in YEME (Kieser *et al.*, 2000). *In vitro* transcription was carried out as described previously (Buttner *et al.*, 1987). Three different templates for *in vitro* run-off transcription were generated by PCR that differed in their left or right ends, to allow the *simR* and *simX* transcripts to be identified unambiguously. Templates were generated using the following primers: Template 1, Invitro1-F and Invitro1-R; Template 2, Invitro1-F and Invitro2-R; Template 3, Invitro3-F and Invitro1-R (Table 1).

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