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Identification and characterization of the *Streptomyces globisporus* 1912 regulatory gene *lndYR* that affects sporulation and antibiotic production

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Here, we report the identification and functional characterization of the *Streptomyces globisporus* 1912 gene *lndYR*, which encodes a GntR-like regulator of the YtrA subfamily. Disruption of *lndYR* arrested sporulation and antibiotic production in *S. globisporus*. The results of *in vivo* and *in vitro* studies revealed that the ABC transporter genes *lndW–lndW2* are targets of LndYR repressive action. In *Streptomyces coelicolor* M145, *lndYR* overexpression caused a significant increase in the amount of extracellular actinorhodin. We suggest that *lndYR* controls the transcription of transport system genes in response to an as-yet-unidentified signal. Features that distinguish *lndYR*-based regulation from other known regulators are discussed.

INTRODUCTION

Streptomycetes are notable for their ability to produce secondary metabolites that are immensely diverse in terms of structure, biogenesis and the biological responses they elicit. The chromosomes of streptomycetes contain many gene clusters for secondary metabolite biosynthesis, although not all of them are expressed in wild-type strains under routine laboratory conditions (Nett *et al.*, 2009). Those that are expressed usually produce only small quantities of the product, creating the need for extensive strain improvement programs for industrial purposes. These observations indicate that streptomycetes have evolved a complicated regulatory web that orchestrates their secondary metabolism. Indeed, available data underscore a great abundance of regulatory genes in actinomycete genomes.

GntR superfamily regulators, which constitute one of the biggest groups of bacterial regulators, contain a typical helix–turn–helix DNA-binding domain at their N terminus (Hoskisson & Rigali, 2009). This superfamily is named after the prototypical member, GntR, a repressor of the *Bacillus subtilis* gluconate operon. The *Streptomyces coelicolor* genome encodes 57 GntR-like regulators (Bentley *et al.*, 2002; Hillerich & Westpheling, 2006), but only a few of these genes have been studied experimentally, namely *whiH*, *devA*, *dasR* and *aglR3R*. Genes *whiH* and *devA* control the early steps of sporulation (Flärdh & Buttner, 2009; Hoskisson *et al.*, 2006), while the other two influence carbohydrate transport and antibiotic biosynthesis (Rigali *et al.*, 2006; Hillerich & Westpheling, 2006). Also, the GntR-like genes *korSA* (*korA*) are known to control conjugal transfer of actinomycete plasmids, such as pIJ101 and pSAM2 (Sezonov *et al.*, 2000). Except for these data and bioinformatic predictions (Rigali *et al.*, 2002, 2004), little is known about the role(s) of many other members of the

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Abbreviations: Act, actinorhodin; EMSA, electrophoretic mobility shift assay; LaE, landomycin E; qPCR, quantitative PCR; Red, prodiginines; RT, reverse transcriptase [PCR].

The GenBank/EMBL/DDBJ accession number for the fragments containing genes *lndY*, *lndYR*, *lndW2* and *lndW* is HM204451.

Ten supplementary figures and a supplementary table are available with the online version of this paper.

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GntR family in streptomyces biology. Our interest in this family arose when we uncovered a new GntR-like gene, *lndYR*, in the vicinity of the landomycin E (LaE) biosynthesis gene (*lnd*) cluster. This cluster is an object of intense investigation in our laboratory, with an emphasis on the genetic regulation of LaE production. To date, two regulatory genes have been shown to control LaE production: one encoding streptomyces antibiotic regulatory protein-like regulator LndI and another that specifies putative proteinase Prx (Ostash *et al.*, 2009). The *lndYR* gene is located between genes for a putative serine-threonine kinase (*lndY*) and a two-component ABC transporter system (*lndW–lndW2*; see Fig. 1). While the function of *lndY* remains unknown, *lndW* has been shown to confer increased LaE resistance under heterologous overexpression conditions (Ostash *et al.*, 2008). Taking into account the physical linkage of *lndYR* to other *lnd* genes and the fact that GntR-like genes often control transport processes (Hoskisson & Rigali, 2009), we set out to investigate the possible functional relationships between *lndYR*, *lndW–lndW2* and LaE production. Here, we report that LndYR influences sporulation and landomycin production in *Streptomyces globisporus*; significant changes in actinorhodin distribution are also seen in *S. coelicolor* overexpressing *lndYR*. As LndYR has numerous homologues in sequenced actinomycete genomes, future studies of this family of regulators may lead to valuable insights into morphogenesis and antibiotic production by members of the order *Actinomycetales*.

METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in LB, 2 × TY or TB for routine applications. Streptomyces strains were grown at 30 °C. Solid oatmeal medium (OM) (Luzhetskiĭ *et al.*, 2001) or mannitol-soy medium (MS) (Kieser *et al.*, 2000) was used for sporulation of streptomycetes and plating of *E. coli–Streptomyces* matings. For isolation of genomic and plasmid DNA, streptomycetes was grown in TSB for 48–60 h. LaE production by *S. globisporus* strains was assayed in liquid media SG (Rebets *et al.*, 2003), TSB, R5A and on solid OM (Rebets *et al.*, 2003), Bennett (Shirling & Gottlieb, 1966), MM, MS, R2YE, and MMGT (Kieser *et al.*, 2000). Total RNA was isolated from *S. globisporus* cultures grown in TSB or R5A (Kieser *et al.*, 2000). Actinorhodin (Act) and prodiginines (Red) production by *S. coelicolor* was determined in liquid medium YMPG (Ishizuka *et al.*, 1992) as previously described (Kieser *et al.*, 2000). Complex agar YMA (Ostash *et al.*, 2007), MS, Bennett, R2YE, MMGT and chemically defined minimal medium (Kieser *et al.*, 2000) and Evans medium (Evans *et al.*, 1970), modified as described by Sun *et al.* (2001), were also used to analyse Act and Red production. Moenomycin production by *Streptomyces ghanaensis* (ATCC14672) was studied in TSB as described by Makitrynskyy *et al.* (2010). Siomycin production by *Streptomyces sioyaensis* was determined in medium SG1 as described by Myronovskyy *et al.* (2009). In all cases, the amount of antibiotic was normalized to equal amounts of biomass (dry weight) and the reported values are the means of three independent experiments. Antibiotic resistance was analysed by using the disc diffusion method.

DNA manipulations. Genomic and plasmid DNA from *Streptomyces* and plasmid DNA from *E. coli* was isolated using standard protocols (Kieser *et al.*, 2000; Sambrook & Russell, 2001). *E. coli* transformation and intergenetic *E. coli–Streptomyces* matings were performed as described by Kieser *et al.* (2000) and Luzhetskiĭ *et al.* (2001). Molecular biology enzymes were purchased from standard commercial sources (MBI Fermentas, NEB, Amersham Biosciences, Invitrogen) and used according to the manufacturer's instructions. quantitative PCR (qPCR) experiments were done on a MyiQ single-colour real-time PCR detection system (Bio-Rad). PCRs were performed using thermal cyclers Mastercycler (Eppendorf) and PTC-100 (MJ Research). All amplicons used in this work were verified by sequencing. DNA sequencing was performed by using the Sanger method using an ABI3700xl instrument. Sequence assembly was carried out using Lasergene software (DNASTAR). The GenBank accession number for fragments containing genes *lndY*, *lndYR*, *lndW2* and *lndW* is HM204451.

Bioinformatic analysis of sequences. FramePlot 2.3.2 (Ishikawa & Hotta, 1999) was used to find open reading frames (ORFs) within a sequenced fragment of the *lnd* cluster. BLAST search tools (on the NCBI server) were used to identify homologues of *lndY*, *lndYR* and *lndW2* genes. The CDD search engine (BLAST) and HHPred, Pfam and TMHMM on the ExPaSy proteomics server were utilized for *in silico* analysis. Phylogenetic trees were constructed by using an online phylogenetic server (http://www.phylogeny.fr; Dereeper *et al.*, 2008). The LndYR tree was rooted using *Bacillus pumilus* ATCC 7061 YtrA protein as an outgroup. The topology of the resultant neighbour-joining tree was evaluated by an approximate-likelihood ratio test (Anisimova & Gascuel, 2006).

Plasmid construction

Sequences of the primers used throughout this work are given in Table 2.

For *lndYR* expression. A 798 bp fragment containing *lndYR* and its 400 bp upstream region was amplified from pSXH4 (Fedorenko *et al.*,

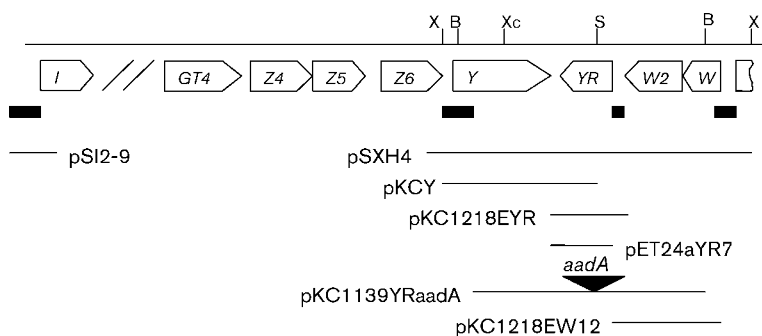

Fig. 1. Genetic organization of the *lnd* cluster in the vicinity of the *lndY–lndW* fragment (not drawn to scale). Subclones used throughout the work are shown below. Restriction sites: X, *Xho*I; B, *Bam*HI; Xc, *Xcm*I; S, *Stu*I. Black rectangles below gene symbols indicate promoter regions used in this work. The black triangle points to the site of insertion of the spectinomycin resistance cassette (*aadA*) into the *lndYR* coding sequence.

Table 1. Strains and plasmids used in this work

Bacterial strain or plasmid	Description	Source or reference
E. coli DH5α	*supE44 λlacU169(Δ80lacZλM15) hsdR17 recA1 endA1 gyrA96 thi*-1 *relA1*	MBI Fermentas
E. coli ET12567 (pUB307)	*dam*-13 : : Tn*9*(Cm^r) *dcm-6 hsdM*; harbours conjugative plasmid pUB307; Cm^r, Km^r	C. P. Smith, UMIST, UK
E. coli BL21(DE3)	F$^-$ *ompT hsdS gal* [*dcm*][*lon*]*hsdS$_B$* (r_B^- m_B^-; an *E. coli* B strain) with DE3, a λ prophage carrying T7 RNAP	Novagen
S. globisporus 1912	Wild-type LaE producer	Rebets *et al.* (2005)
S. globisporus YR	A *lndYR*-disrupted mutant (*lndY* : : *aadA*)	This work
S. coelicolor M145	Model strain, ACT and RED producer	M. Bibb, JIC
S. ghanaensis ATCC14672	Wild-type moenomycin producer	ATCC
S. sioyaensis NRRL-B5408	Wild-type siomycin producer	NRRL
pBluescriptIISK$^-$	General purpose cloning vector; Ap^r	MBI Fermentas
pSTBlue-1	*E. coli* T-cloning vector for PCR products cloning; Ap^r	Novagen
pET24a	Expression vector to purify His-tagged proteins; Km^r	Novagen
pKC1218E	*E. coli*/*streptomyces* shuttle vector with *ermEp* and SCP2* replicon, derivative of pKC1218; Am^r	Rebets *et al.* (2005)
pKC1139	*E. coli*/*streptomyces* shuttle vector, pSG5 replicon; Am^r	Kieser *et al.* (2000)
pHP45Ω	Plasmid carrying Ω interposon with spectinomycin resistance gene cassette *aadA*; Ap^r, Sm^r/Sp^r	Prentki & Krisch (1984)
pSXH4	pBluescriptIISK$^-$ carrying 6 kb *Xho*I fragment of *lnd* cluster (*lndY–lndW* segment)	Fedorenko *et al.* (2000)
pKCYRaadA	pKC1139 carrying the disrupted allele of *lndYR* gene (*lndYR* : : *aadA*)	This work
pKC1218EYR	pKC1218E carrying *lndYR* behind *ermEp*	This work
pKCY	pKC1139 harbouring *lndY* gene	This work
pSI2-9	pSET152 containing *lndI* gene with promoter	Rebets *et al.* (2005)
pET24aYR7	pET24a carrying *lndYR*	This work
pKC1218EW12	pKC1218E carrying *lndW lndW2* behind *ermEp*	This work

2000) with primers Y2upXbaI and Y2rpEcoRI. The resulting amplicon was digested with *Xba*I and *Eco*RI and cloned into the same sites of pKC1218E to give pKC1218EYR.

For *lndY* expression. The *lndY* gene was amplified as a 4.5 kb *Xho*I–*Stu*I fragment from pSXH4, treated with Klenow fragment and cloned into *Eco*RV-digested pKC1139 to give pKCY.

For *lndW–lndW2* expression. The genes *lndW–lndW2* were amplified by using primers W12XbaF and W12EcIR, digested with *Xba*I and *Eco*RI and cloned into the respective sites of pKC1218E resulting in pKCEW12. In this construct, the cloned *lndW–lndW2* segment contains its own ribosome-binding site (15 bp upstream of *lndW*) and its expression is driven only by *ermEp*.

For *lndYR* knockout. A 3.5 kb *Bam*HI fragment of pSXH4 (containing *lndY*, *lndYR*, *lndW2* and *lndW* lacking its first 119 bp; Fig. 1) was cloned into *Bam*HI-digested pBluescriptIISK$^-$ to give pBL35. The latter contains a unique *Stu*I site within the *lndYR* coding region, which was utilized to clone *Sma*I-digested spectinomycin resistance cassette *aadA* to yield pBL35StuIaadA. The mutant *lndYR* : : *aadA* allele was moved as a 5.5 kb *Bam*HI fragment from pBL35StuIaadA to pKC1139 to give the final construct pKCYRaadA.

For *lndYR* expression in *E. coli*. The *lndYR* gene was amplified with primers LdY2EF and LdY2ER, digested with *Nde*I and *Xho*I and cloned into the respective sites of pET24a to give pET24aYR7.

RNA isolation and reverse transcriptase (RT)-PCR analysis. Total RNA was isolated after 3 days of *S. globisporus* growth in TSB or R5A according to the Trizol manufacterer's instructions (Invitrogen). To avoid DNA contamination, samples were treated with RQ1 DNase (Promega). RNA concentration and purity were determined by measuring the ratio of $OD_{260}:OD_{280}$, and an equal amount of RNA from each studied strain was used for the RT reactions. cDNA was obtained by using a cloned AMV first-strand synthesis kit (Invitrogen) and random hexanucleotide primers. PCR was performed using *Taq* DNA polymerase (NEB) and primer pairs specific to each individual *lnd* gene (Table 2). As a positive control, the *rrnA* primer pair specific to 16S rRNA of *S. globisporus* was used. Negative controls were done with *rrnA* primers to confirm the absence of contaminating DNA in the RNA preparations. The PCR parameters for quantitative analysis were as follows: initial denaturation for 5 min at 95 °C, then 35 extension cycles (30 s, 95 °C; 30 s, 53 °C; 30 s, 72 °C) and final denaturation with a gradient of 0.5 °C step every 10 s. C_T was calculated to be 26.0 using the maximum curvation method, and the relative ratios of gene expression were determined. PCR products were analysed by electrophoresis on a 1.5 % agarose gel, and band intensities were measured by using the ImageJ1.36b software (NIH). For qPCR, the QuantiTect SYBR green PCR kit (Qiagen) was used. Data analysis was performed by using the comparative C_T ($2^{-\Delta\Delta CT}$) method using the 16S rRNA signal as an internal reference (Livak & Schmittgen, 2001).

LndYR purification. To obtain C-terminally 6 × His-tagged LndYR, an *E. coli* BL21(DE3) strain carrying plasmid pET24aYR7 was grown overnight at 37 °C. LB (400 ml) containing 50 μg kanamycin ml^{-1} was inoculated with 1 ml of overnight culture and incubated at 37 °C until OD_{600} 0.6. LndYR expression was then induced with 0.4 mM IPTG. After incubation for an additional 6 h at 25 °C, the culture was harvested by centrifugation at 4200 r.p.m. for 10 min at 4 °C and lysed by treatment with BugBuster protein extraction reagent supplemented with Benzonase and rLysozyme (Novagen). Native purification of the LndYR protein on His-Tag binding resin was performed according to Novagen's instructions. LndYR was eluted with 200 mM imidazole and dialysed overnight at 4 °C against the storage buffer (50 mM Tris/HCl, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 10 % glycerol). Protein was stored at –80 °C.

Table 2. Primers used in this study

Purpose/name	Sequence (5′–3′)
To clone *lndYR* for pKC1218E	
Y2upXbaI	AAATCTAGACCACCGTGTTCGTCTGGGACG
Y2rpEcoRI	AAAGAATTCTCAGTCCGGCGCCTCGC
To clone *lndW1W2*	
W12XbaF	ATTCTAGATGGAATCACGATCACGGATCA
W12EcIR	ATGAATTCCGCTCGATTGCCGACACTT
To clone *lndYR* for pET24a	
LdY2EF	TACATATGCGTAAAGTGTCGGCAATCG
LdY2ER	TACTCGAGGTCCGGCGCCTCGCCCG
RT-PCR analysis of *lnd* genes expression	
LdIF	ATACTTCGCACACCGTGTAG
LdIR	AGGTCGAGGAGAACTAGGT
LdEF	ACGCTGCGGTGATCGTTGT
LdER	TGGCCTTGCGTACTGGTCT
LdG4F	TCGAGTTCAACTCGAACCAG
LdG4R	AGCATGCCGTCCATCAGG
LdJF	CGTGGTCGCGATGTTACTC
LdJR	CGGAAGCCGATGAGCTGG
LdYF	CTTCCGTCGGGAGATCGA
LdYR	CGATGCCGAAGTCGATGAC
LdY2F	AAGGTGAAGTCCGTGGTCG
LdY2R	GCGTTCCAGCTCGCGGTA
LdWF	CTGTTCAAGCGGTTCACCG
LdWR	AGGACATCACCACCGTGGT
rrnAF	CACATGCAAGTCGAACGATG
rrnAR	GCTGCTGGCACGTAGTTAG
Promoter fragments for EMSA	
W12PR	TCGGTTCCGTGATCCGTGAT
W12PF	AGCGTGCCGTCGAAGTCGA
LdWF1	GTGATTCCATGAATCAATTAATG
LdWF2	AATGGAATCATGGAGTCGTGA
LdWF3	GTGAGGGAATGACTGTCAAG
LdWF4	CAGTGCCCAGTTGCCGGA
LdWR4	CTTGACAGTCATTCCCTCAC
LdWF5	CTGTCAAGCCGGGGTGGT
LdWF6	TGAATCAATTAATGGAATCATGG
ldYRF	TTGCCGACACTTTACGTAGG
ldYRR	GTCAACGACGTGATCGAGTA
ldYPF	ATCCGCAGGAAGCGAGAGC
ldYPR	TCGTGCACCACCTTCACGG
ldIPF	CGAACCACCGGAAATTGATC
ldIPR	CTGCCGTCTCATTTACTACC
Intact fragment	
intact-up	TGACAGTCATTCCCTCACGACTCCATGATTCCATTAATTGATTCATGGAATCACGATCA
Mutant versions of intact fragment (only one strand is shown for each duplex)	
Δs1s2s3-up	TGACAGAAAAAAACCTCACGACTCCAAAAAAACATTAATAAAAAAATGGAATCACGATCA
Δs1s2s3s4-up	TGACAGAAAAAAACCTCACGACTCCAAAAAAACATTAATAAAAAAATGAAAAAACGATCA
Δs4-up	TGACAGTCATTCCCTCACGACTCCATGATTCCATTAATTGATTCATGAAAAAACGATCA
Δs1-up	TGACAGAAAAAAACCTCACGACTCCATGATTCCATTAATTGATTCATGGAATCACGATCA
Δs2s3-up	TGACAGTCATTCCCTCACGACTCCAAAAAAACATTAATAAAAAAATGGAATCACGATCA

Electrophoretic mobility shift assays (EMSAs). Four gene fragments upstream of *lndI* (*lndIp*; 242 bp), *lndY* (*lndYp*; 293 bp), *lndW* (*lndWp*; 294 bp) and the *lndYR*–*lndW2* intergenic region (*lndYRp*; 169 bp) were used in DNA binding assays. The DNA fragments were amplified from plasmid pSXH4 or pSI2-9 (Fig. 1) using primers described in Table 2. One DNA binding assay contained 40 pM DNA fragment and 0–250 pM LndYR protein in a total volume of 30 μl binding buffer [50 mM Tris/HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 10 % glycerol, 0.5 μg poly(dA)-poly(dT)]. After incubation for 20 min at room temperature,

protein-bound and free DNA were resolved by electrophoresis at 4 °C on a 5–8 % non-denaturing polyacrylamide gel in 0.5 × TBE buffer. The gel was stained with ethidium bromide and analysed with a UV-imaging system (Fluorochem 5330). Short fragments of *lndWp* were also tested to map LndYR binding sites (see Results and Supplementary Fig. S1, available with the online version of this paper), and competitor was not included in these experiments.

RESULTS

Sequencing and *in silico* analysis of novel regulatory genes in the *lnd* cluster of *S. globisporus* 1912

Sequencing of a 4 kb segment between *lndW* and *lndZ6* (Fig. 1) revealed three ORFs, referred to as *lndY*, *lndYR* and *lndW2*. Typically for actinomycetes, these genes are GC-rich (68–73 %) and extremely biased in GC usage in the third codon position (over 90 %). LndY (690 aa) showed local similarity to several known serine/threonine protein kinases from *S. coelicolor* M145 [SCO3621, SCO4487 (PkaG) and SCO4507; more than 60 % similarity over a 300 aa stretch] and *Streptomyces granaticolor* (68 % similarity over a 272 aa segment) (Vomastek *et al.*, 1998). The 5′ end of *lndW2* overlaps by 4 nt with the stop codon of *lndW*. LndW2 (331 aa) is homologous to putative transmembrane subunits of ABC transporters (Méndez & Salas, 2001), and is likely to cooperate with the ATPase subunit LndW to produce a functional two-component ABC transporter (Ostash *et al.*, 2008). According to BLAST and CDD search results, *lndYR* encodes a GntR-superfamily transcriptional regulator, which belongs to the YtrA subfamily of GntR-like regulators. Like other members of this subfamily (Rigali *et al.*, 2002), LndYR is small (144 aa) and contains a greatly reduced C-terminal effector binding/oligomerization domain. LndYR homologues are present in all sequenced genomes of members of the order *Actinomycetales*. Moreover, LndYR-like genes are part of a genomic segment showing good local synteny, e.g. they are located near putative ABC transporter genes, which are the usual targets of YtrA-like proteins (Hoskisson & Rigali, 2009). The YtrA subfamily remains the least studied GntR subgroup due to low sequence similarity and the scarcity of structural data for the operator sites. To our knowledge, no experimental work has been carried out to date on LndYR or any of its homologues. The dense location of *lndW*, *lndW2* and *lndYR* suggests their transcriptional coupling.

We analysed phylogenetic relationships within a group of 18 LndYR homologues of streptomycete origin. The LndYR tree (see Supplementary Fig. S2, available with the online version of this paper) consists of two major clades, one of which contains LndYR and its orthologues (*Streptomyces griseus* SGR3238 and proteins found in partially sequenced genomes of *Streptomyces roseosporus*, *Streptomyces albus* J1074, *Streptomyces flavogriseus*, *Streptomyces* sp. C1, *Streptomyces.* sp. SPB8). The second, larger clade of LndYR paralogues includes *S. coelicolor* SCO0823 and 10 other proteins (Supplementary Fig. S2). Several genomes (*S. griseus*, *S. roseosporus* and *S. albus*) contain both paralogues and orthologues of LndYR, suggesting that gene duplications shaped the evolution of this group of regulators.

An *S. globisporus lndYR* mutant is deficient in antibiotic production and sporulation

lndY, *lndYR* and *lndW–lndW2* were overexpressed individually under the control of *ermEp* in *S. globisporus*, but did not exert any significant effect on landomycin production (in liquid and solid media), resistance to antibiotics or sporulation compared with a control strain carrying the empty vector (data not shown). The *lndYR* disruption was done via insertional inactivation (Kieser *et al.*, 2000), using the temperature-sensitive plasmid pKCYRaadA. The spectinomycin-resistant and apramycin-sensitive (Sp^r Am^s, respectively) strain *S. globisporus* YR, in which *lndYR* is replaced with the *lndYR* : : *aadA* allele, was selected, and the gene replacement event was verified by PCR amplification using primers Y2upXbaI and Y2rpEcoRI. A 0.8 kb DNA fragment corresponding to *lndYR* was amplified from strain 1912, whereas a 2.8 kb DNA fragment was amplified from strain YR (Fig. 2c), indicating that the wild-type copy of *lndYR* had been inactivated by insertion of *aadA*.

The *lndYR*-deficient *S. globisporus* YR differed from the 1912 parental strain in both morphology and landomycin production (Fig. 2). YR did not accumulate LaE or its aglyca during submerged fermentation (Fig. 2b) or growth on agar plates. Poor aerial mycelia were formed after 10 days of growth on solid OM, and sporulation, confined to the edges of the lawn, was scant and sporadic (Fig. 2a). There were no differences in the antibiotic resistance profiles of 1912 and YR. Expression of an intact *lndYR* copy fused to *ermEp* (plasmid pKC1218EYR) in the YR strain restored its ability to sporulate and produce LaE (Fig. 2) to the levels of its parent strain, thus ruling out the possibility of polar effects in the YR mutant and unexpected rearrangements in the YR genome. We grew the YR strain on a variety of minimal and complex solid media, and none of them restored sporulation to the strain or stimulated LaE production (see Supplementary Fig. S3, available with the online version of this paper). Since the loss of *lndYR* function has a strong dual phenotype, affecting both morphology and antibiotic production, we conclude that LndYR is not simply a pathway-specific regulator.

Expression of *lndW* is strongly increased in the *lndYR*-deficient strain

We performed semiquantitative RT-PCR analysis of representative landomycin biosynthetic (*lnd*) genes from two main *lnd* operons: the oxygenase *lndE* operon (*lndFABCD*) and the glycosyltransferase *lndGT4* operon (*lndZ1Z3GT4Z4Z5*). We monitored the proton-dependent landomycin antiporter *lndJ*, the ABC transporter *lndW*, and regulators *lndI*, *lndY* and *lndYR* in both the parent and *lndYR* mutant to determine

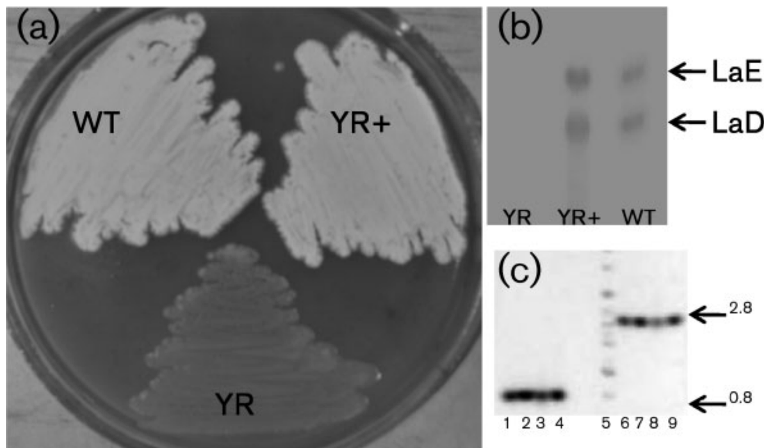

Fig. 2. Phenotype of the *S. globisporus lndYR* mutant. (a) Lawns after 10 days growth of *lndYR* mutant (YR), wild-type (WT) and complemented YR (YR+) on solid OM. (b) Thin layer chromatography of ethyl acetate extracts from YR, YR+ and WT strains grown in liquid SG medium for 4 days. Arrows indicate spots corresponding to LaE and its disaccharide precursor, LaD. (c) Amplicons obtained with *lndYR*-specific primers from pKC1218EYR (lane 1), from WT genome (lanes 3–4, independent clones), pKC1139YRaadA (lane 6) and the YR genome (lanes 7–9, independent clones). Lane 5, DNA ladder (Bionexus); sizes given in kb.

the effect(s) of LndYR deficiency on the expression of those genes. The strains were grown in liquid TSB (supports low landomycin production) and R5A (supports high landomycin production). Despite the lack of landomycin production in the mutant, transcripts for all tested *lnd* genes were observed in this strain. However, there was a dramatic increase in *lndW* transcription in the YR strain (Supplementary Fig. S4, available with the online version of this paper). Hence, we focused on qPCR analysis of *lndW* and *lndI* gene expression to further assess this phenomenon. In TSB, the amount of *lndW* mRNA in the YR strain was 150 ± 9 times higher than in 1912; in R5A, the difference was approximately 430-fold. There was no significant difference in *lndI* expression when YR and 1912 were cultivated in TSB. On R5A, *lndI* showed increased expression but the difference compared with the parent strain was not nearly as dramatic (two- to threefold; data not shown). These results imply that LndYR acts as repressor of *lndW* and all contranscribed genes, most likely *lndW2* and *lndYR*. Although LndYR also affects *lndI* transcription, the latter does not seem to be a primary LndYR target. The cessation of landomycin production by the YR strain is not likely to be a direct result of disrupting repressive LndYR interactions with promoters of structural or regulatory *lnd* genes, since their expression levels are either unchanged or increased. Rather, it could stem from deregulated expression of *lndW–lndW2* and/or other, as-yet-unknown LndYR-dependent genes. For example, LndYR may influence the expression of genes that control landomycin production at a post-transcriptional level (mRNA translation, post-translational Lnd protein modification through phosphorylation or proteolysis). The existence of such regulatory mechanisms is not inconceivable since we have previously shown the essentiality of the putative proteinase gene *prx* for LaE production (Dutko *et al.*, 2006).

LndYR binds to the promoter regions of *lndW* and *lndI*

To confirm DNA binding activity and establish the specificity of LndYR, we purified the protein and performed *in vitro* binding assays using several DNA fragments (Fig. 1) containing the upstream regions of *lndI* (*lndIp*), *lndY* (*lndYp*), *lndW* (*lndWp*) and the *lndYR–lndW2* intergenic region (*lndYRp*). No DNA shift was detected when using *lndYp* or *lndYRp*, whereas significant DNA retardation occurred when *lndWp* was used in the binding assay (Fig. 3); LndYR also interacted with *lndIp* (Fig. 3 and Supplementary Fig. S5, available with the online version of this paper). The presence of several discrete shifted bands in the EMSA assays may stem from the availability of several binding sites (as detailed further in this work) as well as the ability of LndYR (like many other GntR proteins), to bind DNA as both a mono- and an oligomer. In regard to the latter, we noted that even on denaturing gels, a portion of purified LndYR appeared to move as a dimer and, probably, a trimer (Supplementary Fig. S6); this observation was confirmed via FPLC and Western blot (data not shown). Several experiments demonstrated the specificity of LndYR binding to *lndWp* and *lndIp*. For instance, LndYR did not bind all of the four tested promoters, and increased amounts of the protein caused a more complete conversion of free (unbound) *lndWp* into the LndYR-bound form (Fig. 3). Randomly chosen DNA did not interact with LndYR or compete for binding (Supplementary Fig. S1).

We performed multiple sequence alignments of DNA regions that are putative targets for LndYR or its homologues. Such regions are within promoters located upstream of *lndW*, its orthologues from *S. griseus* (*sgr3236*) and *S. roseosporus* (ZP_04710279), and upstream of the *lndYR* paralogue *sco0823*. All sequences aligned well and revealed the presence of a moderately conserved 80 bp region upstream of the *lndW* start codon (Fig. 4a). This region did not seem to contain the consensus sequence that is proposed to be recognized by YtrA-like proteins [$N_{(x)}GTN_{(y)}TAN_{(z)}TAN_{(y)}ACN_{(x)}$]. Nevertheless, visual inspection of the alignment revealed several elements of symmetry. Particularly, an array of imperfect direct and inverted hexanucleotide repeats, embedded into an AT-rich sequence, is present in promoters from orthologous

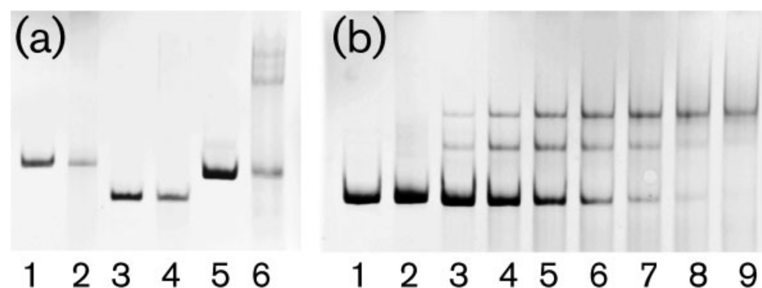

Fig. 3. EMSAs of DNA fragments containing different *lnd* promoters. (a) Binding of LndYR (250 pM) to *lndYp* (4 pM) *lndYRp* (40 pM) or *lndIp* (4 pM). Lanes: 1, *lndYp*; 2, *lndYp*+LndYR; 3, *lndYRp*; 4, *lndYRp*+LndYR; 5, *lndIp*; 6, *lndIp*+LndYR. (b) Retardation of *lndWp* DNA (6 pM) in the presence of increasing concentrations of LndYR. Lanes: 1, *lndWp*; 2, *lndWp*+cell lysate of BL21(DE3); 3–9, *lndWp*+LndYR 16, 40, 80, 110, 160, 200 and 250 pM, respectively.

groups (Fig. 4a). Using small overlapping fragments of *lndWp*, we decided to map the LndYR *cis*-acting element within *lndWp*. Our strategy is outlined in Supplementary Fig. S7 (available with the online version of this paper). We found that a short *lndWp* segment (approx. 60 bp; located 25 bp upstream of the *lndW* start codon), shown in Fig. 4(b) and referred to as the 'intact fragment', contains all of the elements necessary to interact with LndYR and to form two discrete, electrophoretically retarded DNA species (Supplementary Fig. S7). Shorter fragments formed a single shifted DNA band implying that they lack one of the LndYR binding sites (Supplementary Fig. S7), while longer fragments yielded supershifted bands at higher LndYR concentrations (Supplementary Fig. S7c, d). It is possible that LndYR may have additional binding sites far upstream of *lndW*.

Having defined a relatively short LndYR DNA target, we set out to determine the nature and importance of the direct and inverted repeats underpinned by bioinformatic analysis. For this purpose, we carried out EMSAs with the intact fragment and its mutant variants where repeats were replaced with adenines. To this end, we tested several combinations of repeat deletions, as shown on Fig. 4(c). While the removal of a single repeat had little effect on its ability to be recognized by LndYR, deletions of two, three or all four sites demonstrated their importance for LndYR binding. A detailed investigation of the intact fragment can now be performed to obtain more precise information about LndYR *cis*-acting elements.

lndYR affects antibiotic production in heterologous strains

To test whether LndYR influences morphogenesis and secondary metabolism in other streptomycetes, we overexpressed it in the model strain *S. coelicolor* M145 and in two producers of industrially important antibiotics, *S. ghanaensis* ATCC14672, which produces moenomycins, and *S. sioyaensis* NRRL-B5408, which produces siomycins. *S. coelicolor* M145 carrying plasmid pKC1218EY (M145-YR) was grown in parallel with a control strain (carrying an empty vector) and the amount of Act and Red in the cells and spent medium was determined. On average, both strains yielded a similar amount of biomass, but M145-YR accumulated 2.5 times more Act in the supernatant than the control strain (Fig. 5). M145-YR and its control strain differed negligibly in cellular amounts of Act and Red. Overexpression of *lndYR* influenced Act production in *S. coelicolor* during growth on certain solid media. For example, on MMGT plates, strain M145-YR accumulated more Act than did M145 (Supplementary Fig. S8, available with the online version of this paper), probably because M145-YR entered the Act production phase earlier (Supplementary Fig. S8). On replete medium R2YE, M145-YR produced more Act over time (Supplementary Fig. S9). We also grew M145-YR on chemically defined Evans medium and found that on complete, carbon- and phosphate-limited agar plates, M145-YR produced more Act than did M145, while nitrogen-limited medium interfered with Act biosynthesis both in M145-YR and in the control strain (Supplementary Fig. S10). Hence, *lndYR* overexpression appears to affect the export of Act, and its regulatory function is dependent on nutritional status. As a further illustration of the complexity of biological effects caused by *lndYR*, we discovered that its overexpression in *S. sioyaensis* reduced total siomycin production [wild-type, 2.2 ± 0.5 μg (mg dry weight)$^{-1}$; *lndYR*$^{+}$ strain, 1.0 ± 0.2 μg (mg dry weight)$^{-1}$], while it slightly increased moenomycin titres in *S. ghanaensis* [wild-type, 0.9 ± 0.3 μg (mg dry weight)$^{-1}$; *lndYR*$^{+}$ strain, 1.5 ± 0.2 μg (mg dry weight)$^{-1}$].

DISCUSSION

We have identified and characterized a new gene, *lndYR*, encoding a GntR-like regulator (YtrA subfamily) from the LaE producer *S. globisporus* 1912. LndYR homologues are found in most actinomycete genomes, making LndYR an attractive research target. Our studies have revealed the pleiotropic nature of this gene since an *S. globisporus lndYR* mutant was deficient in both morphogenesis and antibiotic production, and these processes were also significantly changed in *S. coelicolor* overexpressing *lndYR*. Genes within the *lndW* operon (*W-W2-YR*) are targets of the LndYR repressor, but most likely not a single major one: the ability of LndYR to bind *lndIp* and to form *lndWp* supershifted bands indirectly supports this suggestion. Since increased transcriptional activity of *lndW*–*lndW2* does not seem to be the immediate cause of the drastic changes in sporulation

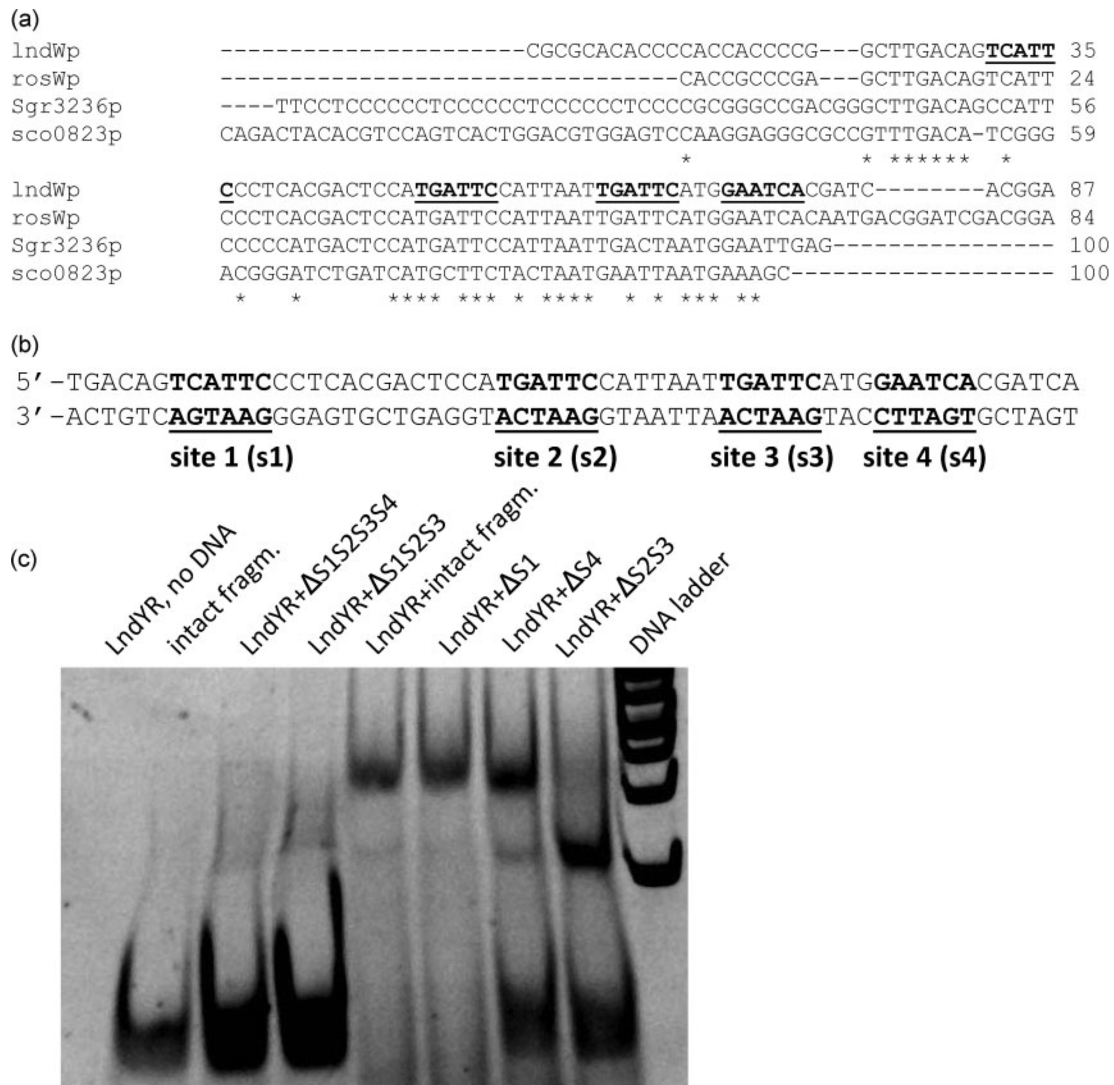

Fig. 4. The *lndW* upstream region contains several LndYR binding sites. (a) Multiple alignment of DNA segments located immediately upstream of genes *lndW*, *sgr3236*, the *lndW* homologue from *S. roseosporus* (rosWp) and *sco0823*. Asterisks show positions conserved in all four sequences. Imperfect direct repeats are shown in bold and underlined. (b) Sequence of the minimal intact fragment of *lndWp* (in double-stranded form) that binds LndYR, as defined by EMSAs of *lndWp* subclones (see Methods and Supplementary Fig. S7). Repeats are marked as s1, s2, s3 and s4 in the intact fragment. (c) Retardation of the intact fragment and its mutant versions carrying the replacement of one or several repeats with hexaadenine sequence. For example, Δs1s2s3s4 is an intact fragment mutant where all four sites (s1, s2, s3, s4) have been replaced with adenines. In all experiments, LndYR was added in molar excess (50 pM, final concentration) with regard to DNA (350 fM).

and landomycin production in the YR strain, other LndYR-regulated genes must exist in the genomes of *S. globisporus* and other species that carry *lndYR* orthologues. Their discovery will be expedited by precise mapping of LndYR operator sequences and further *in silico* screening for genes containing similar motifs. Limited experimental data and comparative genomic analyses point to the fact that YtrA proteins are repressors involved in the regulation of transport processes (Hoskisson & Rigali, 2009), and our experiments with *S. globisporus* and *S. coelicolor* adhere to this hypothesis. We suggest that, besides influencing the expression of adjacent genes *lndW–lndW2* and maybe *lndYR*, LndYR also regulates the transcription of other, currently unidentified, transporter genes. Their inappropriate expression in the *lndYR* mutant may produce its phenotype.

In its influence on morphology and secondary metabolism, *lndYR* resembles gene *agl3R* (SCO7168), a GntR-type regulator that controls ABC transporter genes in *S.*

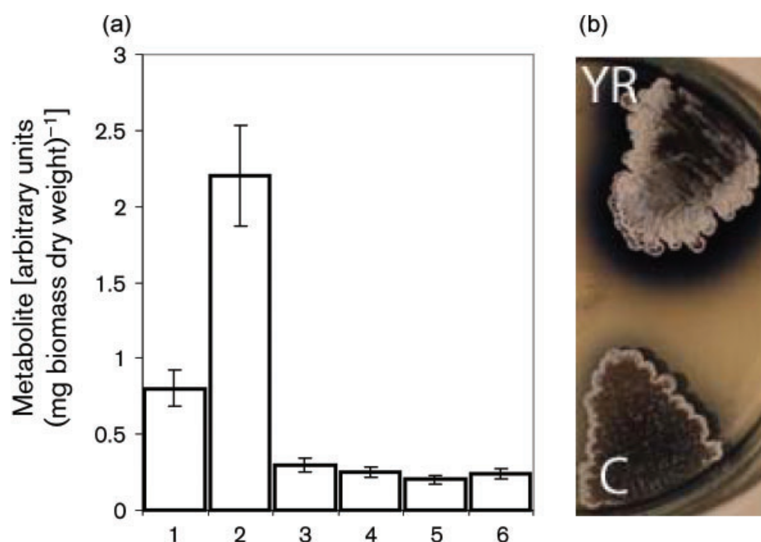

Fig. 5. Overexpression of *lndYR* in *S. coelicolor* M145. (a). Production of Act and Red by *S. coelicolor* strains after 5 days of incubation in YMPG. 1, 2, extracts of Act from spent medium of control and *lndYR*+ strains, respectively; 3, 4, cell extracts of Act from control and *lndYR*+ strains, respectively; 5, 6, extracts of Red from control and *lndYR*+ strains, respectively. (b) Patches of M145-YR (YR) and control (C) *S. coelicolor* strains grown for 5 days on MMGT.

coelicolor and responds to the presence of α-glucosides (e.g. trehalose and mellibiose). It is interesting to note that *lndW* orthologues in *S. roseosporus* and *S. griseus* genomes are situated near genes for trehalose metabolism. We tested several sugars, including trehalose, as potential LndYR ligands but they did not interact with LndYR (data not shown); more detailed screening for an LndYR ligand(s) is underway in our laboratories. Unlike Agl3R and most of the studied GntR-like regulators, LndYR does not bind the upstream region of its own gene, probably because *lndWp* is a single point of control of the entire *lndW–lndW2–lndYR* operon. At the same time, LndYR binds the *lndI* gene upstream region. The biological significance of this binding is unclear, as our RT-PCR analysis showed only slight changes in the transcription of *lndI* in the *lndYR* mutant compared with the parent strain. Nevertheless, the observed binding reinforces the idea that LndYR may regulate several genes scattered throughout the *S. globisporus* genome, one of which is directly connected to the expression of *lnd* genes.

Studies on *lndW–lndW2* have provided valuable initial information about LndYR binding sequences. A consensus recognition sequence for the YtrA subfamily has been proposed [$N_{(x)}\mathbf{GT}N_{(y)}TAN_{(z)}TAN_{(y)}\mathbf{AC}N_{(x)}$; bases essential for binding/recognition are shown in bold] based on a small dataset (Rigali *et al.*, 2002; Hoskisson & Rigali, 2009). Our EMSAs of *lndWp* fragments show that LndYR binding sites are located on a moderately long DNA stretch (approx. 60 bp) that contains several operators. According to sequence analysis, *lndWp* and several other upstream gene regions, also believed to be regulated by LndYR-like proteins, share a moderately conserved AT-rich motif. We suggest that, in *lndWp*, an array of direct ($T_1C/G_2A_3T_4T_5C_6$) and inverted ($T_1G_2A_3T_4T_5C_6$, $G_1A_2A_3T_4C_5A_6$) hexanucleotide repeats (see Fig. 4) serve as an LndYR binding site(s). We note that *lndIp* did not align with *lndWp* or its homologues, and yet it is bound by LndYR. It is possible that LndYR recognizes DNA elements showing no obvious similarity, which would not be unprecedented (McKenzie & Nodwell, 2007; Hoskisson & Rigali, 2009). Uncovering the variations in LndYR binding sites will be key to finding new members of the *lndYR* regulon that influence sporulation and secondary metabolism.

Overexpression of *lndYR* in *S. coelicolor* caused significant increases in Act production and extrusion, both in liquid medium YMPG and on relatively poor MMGT agar. Sporulation was faster on MMGT as well. In contrast, *lndYR* did not exert upregulatory effects when M145-YR was grown on rich medium R2YE. These results, as well as the expression of *lndYR* in moenomycin and siomycin producers, indirectly support the idea that *lndYR* controls gene expression in response to certain nutrients, as many other GntRs (most notably, *agl3R*) do. Hence, the use of different media may lead to different results, since additional regulatory pathways (often linked to both morphogenesis and antibiotic production) are turned on.

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