polR, a pathway-specific transcriptional regulatory gene, positively controls polyoxin biosynthesis in *Streptomyces cacaoi* subsp. *asoensis*

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The polyoxin (POL) biosynthetic gene cluster (*pol*) was recently cloned from *Streptomyces cacaoi* subsp. *asoensis*. A 3.3 kb DNA fragment carrying an obvious open reading frame (*polR*), whose deduced product shows sequence similarity to SanG of *Streptomyces ansochromogenes* and PimR of *Streptomyces natalensis*, was revealed within the *pol* gene cluster. Disruption of *polR* abolished POL production, which could be complemented by the integration of a single copy of *polR* into the chromosome of the non-producing mutant. The introduction of an extra copy of *polR* in the wild-type strain resulted in increased production of POLs. The transcription start point (tsp) of *polR* was determined by S1 mapping. Reverse transcriptase PCR experiments showed that PolR is required for the transcription of 18 structural genes in the *pol* gene cluster. Furthermore, we showed that *polC* and *polB*, the respective first genes of two putative operons (*polC*–*polQ2* and *polA*–*polB*) consisting of 16 and 2 of these 18 genes, have similar promoter structures. Gel retardation assays indicated that PolR has specific DNA-binding activity for the promoter regions of *polC* and *polB*. Our data suggest that PolR acts in a positive manner to regulate POL production by activating the transcription of at least two putative operons in the *pol* gene cluster.

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INTRODUCTION

Streptomycetes are Gram-positive filamentous soil bacteria with a complex life cycle, and are also well known for their capacity to produce a vast majority of secondary metabolites, many of which are commercially important antibiotics widely used in medicine and agriculture. Studies on the model organism *Streptomyces coelicolor* and other antibiotic-producing streptomycetes have revealed that regulation of antibiotic biosynthesis is a complex process (Hopwood *et al.*, 1995; Paradkar *et al.*, 1998). Antibiotic production in *Streptomyces* species is generally dependent on the growth phase, and involves the expression of clustered biosynthetic genes. Pathway-specific regulatory genes are usually linked to these gene clusters, and many pathway-specific regulators belong to a protein family called SARP (Streptomyces antibiotic regulation proteins) (Wietzorrek & Bibb, 1997). Members of this family share

an N-terminal OmpR-type DNA-binding domain, usually followed by a bacterial transcriptional activation domain (BTAD) (Tanaka *et al.*, 2007). Examples of this family include the positive regulators ActII-ORF4 and RedD from *S. coelicolor*, which regulate the biosynthesis of actinorhodin and undecylprodigiosin, respectively (Arias *et al.*, 1999; Narva & Feitelson, 1990), DnrI from *Streptomyces peuceitii*, which controls the production of daunorubicin (Stutzman-Engwall *et al.*, 1992), and CcaR from *Streptomyces clavuligerus*, which regulates the production of cephamycin and clavulanic acid (Perez-Llarena *et al.*, 1997). Significantly, recent characterization of several antibiotic pathways has led to the identification of a set of novel transcriptional regulators. This unique group of transcriptional regulators has extra domains in addition to the conserved core of SARP-family proteins, and has a relatively large size compared with those of the typical SARP family. PimR and SanG, positive regulators of pimarin and nikkomycin (NIK) production, respectively, belong to this group (Anton *et al.*, 2004; Liu *et al.*, 2005).

Polyoxins (POLs), a group of nucleoside peptide antifungal antibiotics, were isolated from the culture broths of *Streptomyces cacaoi* subsp. *asoensis*. These antibiotics show

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Abbreviations: NIK, nikkomycin; POL, polyoxin; SARP, Streptomyces antibiotic regulation protein; tsp, transcription start point.

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high activity against phytopathogenic fungi. At least 12 components of POLs (designated alphabetically Pol-A to -L) have been reported (Isono *et al.*, 1969). Since the POLs have a similar structure to UDP-*N*-acetylglucosamine, they are competitive inhibitors of chitin synthase and have proved to be excellent fungicides (Endo *et al.*, 1970). The biosynthesis of POLs has been extensively investigated *in vivo* and a possible biosynthetic pathway was proposed by Isono and coworkers using radiolabelled precursor feeding experiments (Isono, 1988). POLs have a similar nucleoside moiety to that of NIKs (Müller *et al.*, 1981). NIK biosynthesis gene clusters (*nik*) have been cloned from *Streptomyces tendae* and *Streptomyces ansochromogenes* (Bormann *et al.*, 1996; Chen *et al.*, 2000). The biosynthetic pathway and the regulation mechanism of NIK biosynthesis have been widely studied (Bruntner *et al.*, 1999; Lauer *et al.*, 2000, 2001; Liu *et al.*, 2005). Considering the structural similarity between POL and NIK (Fig. 1a), it is likely that some similar gene homologues could be shared for their biosynthesis. Based on this idea, the *pol* gene cluster from *S. cacaoi* subsp. *asoensis* was cloned recently (Chen *et al.*, 2009). The organization of genes with potential functions in the regulation or synthesis of polyoxins is shown in Fig. 1(b). Boundaries of the *pol* gene cluster were determined by gene disruption and polyoxin biosynthesis in a heterologous host. Twenty genes (*polR*, *polY*, *polC*-Q2, *polA* and *polB*) were defined as necessary for polyoxin production. The deduced functions

for most of these genes correlated well with the biosynthetic pathway of polyoxins proposed by Isono and coworkers (Isono, 1988). Remarkably, the *pol* cluster contains two potential regulatory genes, *polR* and *polY*, that are SARP-encoding genes. *polR* is the homologue of *sanG*, which is a regulatory gene for NIK biosynthesis. In this paper, we describe detailed characterization of *polR* and demonstrate its role as a positive regulator of polyoxin biosynthesis in *S. cacaoi* subsp. *asoensis*.

METHODS

Strains, media and chemicals. *S. cacaoi* subsp. *asoensis* AS4.1602, a natural POL producer, was obtained from CGMCC (China General Microbiological Culture Collection Centre). *Alternaria longipes* was used as indicator strain for POL bioassay. *Escherichia coli* DH5 α was used as a host for propagating plasmids. *E. coli* ET12567(pUZ8002) was used for transferring DNA from *E. coli* to *Streptomyces* by conjugation (Liu *et al.*, 2005). *E. coli* BL21(DE3) (Novagen) was used as a host for the overexpression of *PolR*.

S. cacaoi subsp. *asoensis* was grown on mannitol/soya (MS) medium at 28 °C (Kieser *et al.*, 2000). For genomic DNA extraction, *S. cacaoi* subsp. *asoensis* was grown in YEME medium, which was also used as a seed medium (Kieser *et al.*, 2000). SP medium (3 % mannitol, 1 % soluble starch, 0.75 % yeast extract and 0.5 % soy peptone, pH 6.0) was used for POL production (Bruntner *et al.*, 1999).

When necessary, antibiotics were used at the following concentrations: apramycin, 10 $\mu\text{g ml}^{-1}$ in YEME or MS for *S. cacaoi* subsp.

Fig. 1. (a) Chemical structures of polyoxin D and the structurally related nikkomycin Z. These two antibiotics share a similar nucleoside moiety. (b) Organization of the POL biosynthesis gene cluster. Each arrow indicates a separate ORF and orientation of transcription. The black arrow indicates *polR*, a putative pathway-specific regulatory gene described in this paper.

asoensis, 100 µg ml⁻¹ in LB for *E. coli*; kanamycin, 10 µg ml⁻¹ in YEME or MS for *S. cacaui* subsp. *asoensis*, 100 µg ml⁻¹ in LB for *E. coli*.

Plasmids and DNA manipulation. pBluescript KS(+) was used for routine DNA cloning. pUC119::neo was the source of the kanamycin resistance gene. The *E. coli*-*Streptomyces* shuttle vector pKC1139, which contains a *Streptomyces* temperature-sensitive origin of replication from pSG5 (Bierman *et al.*, 1992), was used to construct a recombinant plasmid for gene disruption. pSET152, which can integrate into the *Streptomyces* chromosome by site-specific recombination at the phage ϕ C31 attachment site (*attB*) (Bierman *et al.*, 1992; Kuhstoss *et al.*, 1991), was used to create a recombinant plasmid for introducing a single copy of *polR* into *Streptomyces*. pET28a (Novagen) was used to construct the *polR* expression plasmid.

Plasmids and chromosomal DNA were isolated by standard techniques (Kieser *et al.*, 2000). Conjugal transfer from *E. coli* ET12567(pUZ8002) to *S. cacaui* subsp. *asoensis* was carried out as described by Kieser *et al.* (2000). The digoxigenin-11-dUTP labelling and detection kit (Roche Diagnostics) was used in Southern blot experiments according to the protocols provided by the manufacturer.

DNA sequence analysis. DNA sequences were analysed with the NCBI worldwide web BLAST and Frameplot 2.3 server. Protein domain architectures were analysed by using the SMART, Pfam and CDD databases and software tools.

Construction of *polR* disruption mutants. Disruption of *polR* was performed by gene replacement via homologous recombination. For this purpose, a 1885 bp *polR* fragment was amplified from *S. cacaui* subsp. *asoensis* genomic DNA by PCR using primers DR Forward and DR Reverse (Table 1). PCR amplification was carried out using KOD plus DNA polymerase (TOYOBO Co.). The resulting DNA fragment was ligated into the *EcoRV* site of pKC1139 to generate pKC1139::*polR*. The kanamycin-resistance gene (*neo*) was obtained from pUC119::neo after digestion with both *Bam*HI and *Kpn*l, blunted by mung bean nuclease and ligated into the *Sma*I site in pKC1139::*polR* to give pKC1139::*polR*::*neo*, in which the *neo* gene has the same orientation as *polR*. The recombinant plasmid, pKC1139::*polR*::*neo*, was designated pDR101.

pDR101 was first passed through *E. coli* ET12567(pUZ8002) and then introduced into *S. cacaui* subsp. *asoensis* via conjugation (Kieser *et al.*, 2000). The resulting exconjugants were incubated on MS agar to form spores. Grey spores were harvested and spread on MS agar containing kanamycin. After incubation at 40 °C for 3 days, colonies that conferred kanamycin resistance (Kan^R) and apramycin sensitivity (Apr^S) were selected and further confirmed as *polR* disruption mutants of *polR* by Southern blotting analysis. The *polR* disruption mutant selected for further study was named strain DMR.

Complementation of the *polR* mutant. For complementation analysis, a 3.8 kb DNA fragment containing intact *polR* and its flanking sequence was amplified by PCR using *polR* Forward and *polR* Reverse primers (Table 1). The amplified fragment was inserted into the *EcoRV* site of pSET152 to generate pSET152::*polR*, which was designated pSER.

Subsequently, pSER was integrated into the chromosomal ϕ C31 *attB* site of the *polR* disruption mutant by conjugation (Kieser *et al.*, 2000). pSER was also used for the overexpression of *polR* in the wild-type strain.

Bioassay and HPLC analysis of POLs. Spores of *S. cacaui* subsp. *asoensis* were inoculated in YEME. The cultures were grown at 28 °C on a rotary shaker (220 r.p.m.) for 36 h and used as seed cultures.

Mycelia from the seed culture were inoculated into SP medium for POL production.

POL production was measured by a disk agar diffusion method using *A. longipes* as indicator strain. A 100 µl sample of culture filtrate of each strain was used in the bioassay. POL production was further confirmed by HPLC analysis. Because POLs consist of at least 11 active components with similar structure (Isono, 1988), it is difficult to separate and identify all the components by HPLC at the same time. Of all the POL components reported, only POL-D is commercially available (Calbiochem cat. no. 529313), so we set up a separation system for detecting POL-D. An Agilent 1100 HPLC system equipped with an SB-C18 reverse-phase column was used. A mixture of 95 % solvent A (10 mM tetrabutylammonium hydroxide, adjusted to pH 4.0 using glacial acetic acid) and 5 % solvent B (acetonitrile) was applied with a constant flow rate of 1 ml min⁻¹ for 20 min. The detection wavelength was 290 nm. Under these conditions POL-D had a retention time of 10 min.

S1 nuclease mapping. RNAs for S1 nuclease mapping were isolated from *S. cacaui* subsp. *asoensis* after incubation in SP liquid medium for 48 h. Mycelia were collected, quickly frozen in liquid nitrogen and ground into a fine white powder. RNA samples were then extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. S1 nuclease mapping was performed as described previously (Liu *et al.*, 2005).

All the primers used for S1 nuclease mapping are listed in Table 1. For *polR*, the hybridization probe was prepared by PCR using the unlabelled oligonucleotide primer S-polR-AN and labelled primer S-polR-SE. Primer S-polR-SE was labelled at the 5' end with [γ -³²P]ATP using T4 polynucleotide kinase. For *polC*, the probe was generated by PCR with the unlabelled primer S-polC-AN and labelled primer S-polC-SE. For *polB*, the probe was amplified using the unlabelled primer S-polB-AN and labelled primer S-polB-SE. The DNA sequencing ladders were generated using the *fmo*I DNA cycle sequencing kit (Promega) with the corresponding labelled primers. Protected DNA fragments were analysed by electrophoresis on 6 % polyacrylamide gels containing 7 M urea.

Reverse transcriptase PCR (RT-PCR). RNA samples were isolated from wild-type and DMR strains grown in SP liquid medium at 28 °C for 48 h, using Trizol reagent (Invitrogen). The transcription profiles of *pol* genes were compared between the wild-type and *polR* mutant strain by a semiquantitative RT-PCR analysis.

The Qiagen One-Step RT-PCR kit was used with primers specific for each gene and designed to produce PCR products ranging from 300 to 600 bp. Forward and reverse primers are shown in Table 1. Each primer pair was first tested using chromosomal DNA as a template. Absence of DNA contamination in the RNA samples was assessed by PCR. Reaction mixtures contained 6 pmol of each primer and 150 ng RNA in a total volume of 20 µl. RT-PCR conditions were as follows: first-strand cDNA synthesis, 50 °C for 30 min and 55 °C for 30 min followed by 95 °C for 15 min; amplification, 27 or 32 cycles of 94 °C for 45 s, 50 °C to 60 °C (depending on the set of primers used) for 45 s, and 72 °C for 1 min; final extension, 72 °C for 10 min. Twenty-seven cycles of PCR were routinely employed; whenever no product was detected, 32 cycles of PCR were repeated to detect transcripts present at low levels. Products were detected by 1 % agarose gel electrophoresis and visualized by staining with ethidium bromide.

Expression and purification of recombinant PolR. The coding region of *polR* was amplified by PCR from *S. cacaui* subsp. *asoensis* genomic DNA using the primers PolRN and PolRC, containing *Nde*I and *Eco*RI sites respectively. The PCR product was digested with *Nde*I and *Eco*RI and cloned into the corresponding sites of pET28a to generate expression plasmid pET28a::*polR*. The recombinant plas-

Table 1. Primers used in this study

Primer and purpose	Sequence
To construct pDR101 for <i>polR</i> disruption	
DR Forward	GAACGCTCCTGGTCGCTCATC
DR Reverse	ACGCCACCCTCCAGACCTACA
To construct pSER for complementation	
polR Forward	TCAGTGGCCGGAACGCCT
polR Reverse	GCCGGTGGACGATGTTCCG
For RT-PCR analysis	
RTS-polR	ACGTGAGATCCAGGAAGG
RTA-polR	GCAACGGAAGTACACCAG
RTS-polY	CCTCTACCGCAGTCTCCT
RTA-polY	TGTCCTCGTCCTCCAGTT
RTS-polC	AAGCACTACCGGCTCTGG
RTA-polC	CTCCTTCTTTCGGGCATC
RTS-polD	CGTGGTGAGCGACCTGTAC
RTA-polD	AGAGGATGGCGGTGATGG
RTS-polE	CTGCGAGGACGACGAGAA
RTA-polE	GGCGAAGAAGCGATGTG
RTS-polF	CGCCTTCCAGACCGTCTACC
RTA-polF	GGTGTCTCGTCGCTGACCT
RTS-polG	GCAGGAGCTGATCGTGGTC
RTA-polG	TCGGAGTAGGGCTGGTAGG
RTS-polH	ATCGGCTGCTCGTACTGC
RTA-polH	CCTGCTGCGACTTGTGCT
RTS-polI	TACGAGGGCTGGCAGAAA
RTA-polI	GGCCGAAGCACTTGGAGA
RTS-polJ	ACACCCCGCCGAGCGAACT
RTA-polJ	TGCCGACCGTGCAGCAGAC
RTS-polK	CCGGGGCAAGCACTACCT
RTA-polK	GAGCGAACCAGCACCACC
RTS-polL	TGGCGCAAGTACGAGGAG
RTA-polL	CGAGGCAGTCGGTGAAGA
RTS-polM	CACCAACGTGAGCGGACT
RTA-polM	CGACAGGCACCACAGGAC
RTS-polN	GCGTGAGGACGTCGTCGTA
RTA-polN	CATGTGCTGGGGTTCGGTG
RTS-polO	CCCGTGGAACCTGTGCTT
RTA-polO	TAGGCGGTGAGGATGTCG
RTS-polP	GAGGTCTGTCGTCATGGTC
RTA-polP	AGCAGCAGCAGGGTGTCC
RTS-polQ1	GAACAGGTCTGTCGGCAAGG
RTA-polQ1	TGTCAGGCACAGCAGGAGG
RTS-polQ2	GGGAAGACCTCGCTCGCC
RTA-polQ2	AGACGGACCACGGGAACG
RTS-polA	CGCAGATCCAGGTGACGA
RTA-polA	GCTGACGGGCTGTGAGAT
RTS-polB	GCCGTCTCCATCTGTTCCG
RTA-polB	ATCTCGCCGTCGTCAAGG
RTS-orf1	AGGTCGTGACGCCGTAGGT
RTA-orf1	TGGCTCACCACGCAGAACA
RTS-orf2	AAGCGGAGGGTGAAGTCG
RTA-orf2	CATCCCCAAGGAGATGGC
RTS-orf3	ATGACGAGCAGCACCACG
RTA-orf3	CTCCCCGACATCTCACCC
RTS-orf4	GTCGTCCTCCTGCGTGAA
RTA-orf4	GGCGTGGCTCGACTACTT
RTS-orf5	CCGCTCGCTCCACATCAAC
RTA-orf5	AGCCAGGAGTGGGTGAGGT

Table 1. cont.

Primer and purpose	Sequence
RTS-orf6	GGTGTTCGTCGTCGCA
RTA-orf6	CTTCGGTCCTCGTGGCTC
hrdB Forward	GCCGACCCGGTCAAGGACTAC
hrdB Reverse	CACCGGGATACGGATGGTGC
For S1 nuclease mapping	
S-polR-SE	CGTCGAAGATGAGACATGTAGGTC
S-polR-AN	ATGTGTCGATCCATGTCTTCCCAC
S-polC-SE	GCCGGTAGTGCTTGTCGA
S-polC-AN	TCGACGACATGGGACACC
S-polB-SE	TCGTCTGTTGGCGTCTTCACC
S-polB-AN	ATCTCCCACGCCCGTGACCT
For PolR overexpression in <i>E. coli</i>	
PolRN	TCTCATATGATGTATGCAACGGAAGT
PolRC	ATAGAATTCGTGGCCGGAACGCCTGG

mid was further confirmed by DNA sequencing and then introduced into *E. coli* BL21(DE3) for protein overexpression. PolR protein was expressed as an N-terminally His₆-tagged fusion protein. *E. coli* BL21(DE3) carrying pET28a::polR was grown in 200 ml LB to an OD₆₀₀ of 0.6 at 37 °C and was induced with 0.1 mM IPTG at 20 °C overnight. The cells were harvested by centrifugation and resuspended in binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole pH 8.0) and disrupted by sonication. The cleared lysate was applied to a nickel-NTA column (Qiagen). The His₆-tagged PolR protein eluted with 250 mM imidazole was analysed by SDS-PAGE. Further desalting and concentration were performed with a Microcon YM-30 centrifugal filter unit (Millipore).

Electrophoretic mobility-shift assays. The following DNA fragments were used in gel mobility-shift assays: (1) the 429 bp polC promoter fragment amplified using genomic DNA as template and the primer pair S-polC-SE/S-polC-AN; (2) the 384 bp polB promoter fragment amplified using genomic DNA as template and the primer pair S-polB-SE/S-polB-AN. The unrelated DNA fragment used as a negative control was generated by PCR using genomic DNA as template and the primer pair RTS-polF/RTA-polF (corresponding to the internal coding sequence of polF). These PCR products were labelled at the 5' ends with [γ -³²P]ATP and T4 polynucleotide kinase.

For electrophoretic mobility-shift assays, the binding reaction (20 μ l) contained 1 μ g poly(dI-dC), purified protein at various concentrations and a constant concentration of labelled DNA fragment (300 c.p.m.) in a buffer composed of 10 mM Tris/HCl (pH 7.5), 2 mM DTT, 5 mM MgCl₂, 0.5 mg calf BSA ml⁻¹ and 5% (v/v) glycerol. After incubation at ambient temperature for 30 min, DNA-protein complex and free DNA were separated by electrophoresis on non-denaturing 5% polyacrylamide gels (mono/bis, 80:1) at 6 V cm⁻¹ with a running buffer of 0.5 $\times$ TBE containing 40 mM Tris/HCl (pH 7.8), 20 mM boric acid and 1 mM EDTA. Gels were dried and exposed to Biomax radiographic film (Kodak).

RESULTS

polR is a putative regulatory gene for POL biosynthesis

The polR gene in the pol gene cluster encodes a putative polypeptide 1111 aa in length with a predicted molecular

mass of 119.3 kDa. Sequence similarity search showed that PolR is most similar to several regulatory proteins including the deduced product of orfR (accession no. AJ250878) (55% identity), a putative regulatory gene for NIK biosynthesis in *S. tendae*, and SanG (AY631852) (54% identity), a pathway-specific transcriptional regulator for NIK biosynthesis in *S. ansochromogenes* (Liu *et al.*, 2005). PolR also showed full-length similarity to PimR (35% identity), a positive regulator for pimarin biosynthesis in *Streptomyces natalensis* (Anton *et al.*, 2004). All of these proteins are relatively large (1061–1198 aa) and all contain SARP-like domains in their N-termini and other similar domains in their C-termini.

Domain prediction of PolR with SMART, Pfam and CDD confirmed that PolR has similar domain architecture to SanG and PimR (Fig. 2a). The N-terminus of PolR contains a trans-Reg-C (transcriptional regulatory C-terminal) domain (amino acids 30–110) (Pfam no. PF00486) and an accompanying BTA (bacterial transcriptional activator) domain (amino acids 117–262) (Pfam no. PF03794). This region of PolR showed significant similarity to several proteins belonging to the typical SARP family. The highest scores are AurD from the aureothin pathway in *Streptomyces thioluteus* (38.6% identity) (He & Hertweck, 2003), CcaR from the cephamycin and clavulanic acid pathways in *Streptomyces clavuligerus* (32.6% identity) (Perez-Llarena *et al.*, 1997) and RedD from the undecylprodigiosin pathway in *S. coelicolor* (31.1% identity) (Narva & Feitelson, 1990) (Fig. 2b). The C-terminus of this SARP-like region of PolR displays an AAA domain (amino acids 361–546) (SMART no. SM00382). This domain is characteristic of AAA proteins, which constitute a large family of ATPases associated with diverse cellular activities. They are thought to exert their activity through the energy-dependent unfolding of protein (Patel & Latterich, 1998). At the sequence level, the AAA domain contains conserved Walker A and B motifs. The Walker A motif typically adopts the sequence pattern Gx4GK[T/S],

Fig. 2. Domain structure and amino acid alignment of parts of the PolR protein. (a) Predicted domain structure of PolR. TRC, trans-Reg-C domain (Pfam no. PF00486); BTAD, bacterial transcriptional activator domain (Pfam no. PF03794); AAA, domain characteristic of ATPases associated with a variety of cellular activities (SMART no. SM00382). (b) Alignment of the SARP-like domain of PolR with related proteins. Numbers indicate amino acid residues from the N-terminus of the protein. Identical amino acid residues are highlighted in black, and similar residues in grey. ORFR, deduced product of *orfR* (AJ250878) from *Streptomyces tendae*; SanG, NIK biosynthesis regulator from *S. ansochromogenes* (AAV31783); PimR, pimarin biosynthesis regulator from *S. natalensis* (CAE51066); AurD, regulator of the aureothin pathway in *S. thioluteus* (CAE02599); CcaR, activator of both the cephamycin and clavulanic acid pathways in *S. clavuligerus* (CAB03623); RedD, undecylprodigiosin activator in *S. coelicolor* (P16922). (c) Comparison of the AAA domain of PolR with those of other proteins. The Walker A and B motifs are shown. Numbers indicate amino acid residues from the N-terminus of the protein. Identical amino acid residues are highlighted in black, and similar residues in grey. PteR, a putative regulatory protein from *S. avermitilis* (NP_821585).

Fig. 3. HPLC analysis and bioassay of POL production. (a) Culture filtrates from the wild-type strain; (b) culture filtrates from the *polR* disruption mutant (DMR); (c) culture filtrates from the complemented strain (DMR/pSER); (d) POL-D standard sample. The arrows indicate the peak corresponding to POL-D. mAU, milliabsorbance units.

where x is any residue. The Walker B motif is composed of a conserved aspartate (or, less commonly, glutamate) immediately after a hydrophobic strand (Leipe *et al.*, 2003) (Fig. 2c). The C-terminal part of PolR has several dispersed low-complexity regions without known motifs.

Like many pathway-specific regulator genes in *Streptomyces*, *polR* contains a rare TTA codon (codon 64). This indicates that the expression of *polR* may depend on the *bldA* gene, which encodes a tRNA responsible for translating TTA (UUA in mRNA) into leucine (Leskiw *et al.*, 1991).

The *polR* mutant has lost the ability to produce POLs and *polR* complementation restores POL production

To determine the role of *polR*, a *polR* disruption mutant was constructed via homologous recombination. The disruption of *polR* was confirmed by Southern blot analysis using a truncated fragment of *polR* as a probe (data not shown). The resulting *polR* disruption mutant was designated DMR.

DMR and wild-type strains of *S. cacaoi* subsp. *asoensis* were tested for POL production using *A. longipes* as an indicator strain in bioassay. A large zone of growth inhibition was observed with the culture filtrates of the wild-type strain (Fig. 3a), whereas no growth-inhibiting activity against *A. longipes* was detected for DMR (Fig. 3b). This result indicated that the DMR strain had lost the ability to produce active POLs. The inability of DMR to synthesize POLs was further confirmed by HPLC. No peak corresponding to POL-D standard sample was present in the culture filtrates of DMR, but a distinct peak corresponding to POL-D was clearly exhibited in the culture filtrates of the wild-type strain (Fig. 3). The DMR strain showed growth and morphology identical to those of the wild-type on MS or R2YE agar media, indicating that *polR* has no significant effect on growth and differentiation of *S. cacaoi* subsp. *asoensis*.

POL production by the DMR strain was restored by introducing pSER, although at a reduced level compared to the wild-type strain (Fig. 3c). This result further confirmed that the abolition of POL production in DMR was due to *polR* disruption, suggesting that PolR plays a key role as a positive regulator of POL biosynthesis in *S. cacaoi* subsp. *asoensis*. The incomplete restoration of POL production by introducing pSER into strain DMR could be due to the ϕ C31 *attB* site on the chromosome not being neutral for POL production. In fact, when the empty vector, pSET152, was introduced into the wild-type strain, the resulting transformants showed up to 30% reduction in POL production (data not shown).

An additional copy of *polR* leads to overproduction of POL

It has been reported that overexpression of pathway-specific activators can lead to overproduction of the

corresponding antibiotics (Chater, 1990). In our study, pSER constructed for gene complementation was used to increase gene dosage in the wild-type strain. When pSER was integrated into the ϕ C31 *attB* site in the chromosome of the wild-type strain, POL production was increased about threefold in comparison with that of the wild-type strain carrying pSET152 (Fig. 4a), although the biomass of these strains was similar (Fig. 4b). This result indicated that POL biosynthesis in *S. cacaoi* subsp. *asoensis* is limited by the availability of PolR, and also reinforced the evidence that PolR is a positive regulator for POL production.

The transcription start point of *polR*

To determine the tsp of *polR*, an S1 nuclease protection assay was performed. Total RNAs isolated from *S. cacaoi* subsp. *asoensis* were hybridized with 32 P-labelled probe (see Methods; Table 1). The result showed that *polR* has a single tsp, which is localized at the nucleotide C at position 38 bp upstream from the *polR* translation start codon (Fig. 5a, b).

Fig. 4. Growth and POL production of *S. cacaoi* subsp. *asoensis* containing pSER. POL yields were normalized relative to that of the wild-type strain containing pSET152 alone (120 h fermentation), which was set at 100%. (a) POL production; (b) growth curves. ●, Wild-type strain with pSET152; ■, transformants with pSER (pSET152::*polR*). Data are the means $\pm$ SD of three duplicate flasks.

Furthermore, a region in the promoter of *polR* shows similarity to the consensus ARE sequence (Folcher *et al.*, 2001), a potential target site for a γ -butyrolactone receptor (Fig. 5c), suggesting that transcription of *polR* might be regulated by a γ -butyrolactone receptor involved in the hierarchy of γ -butyrolactone regulation (Horinouchi, 2007).

Gene expression analysis in the wild-type and strain DMR

Because of the similarity of the N-termini of PolR with SARP-family regulators, it was possible that the transcription of some *pol* genes might be regulated by PolR. In order to determine whether these genes are potential targets of PolR, total RNAs were isolated from the *S. cacaoi* subsp. *asoensis* wild-type and DMR strains after fermentation for 48 h (when POL is actively produced) and used as templates for analysis of gene expression by RT-PCR. Primers for RT-PCR were designed to detect specific sequences of *pol* genes (Table 1). A primer pair designed to amplify cDNA of *hrdB*, encoding an essential sigma factor (accession no. EU123870) in *S. cacaoi* subsp. *asoensis*, was used as an internal control.

Among the genes of the *pol* cluster, seven genes (*polA*, *polB*, *polD*, *polH-K*) were expected to encode the enzymes responsible for the biosynthesis and modification of the nucleoside moiety; eight genes (*polL-P* and *polC*, *polE* and *polF*) might encode enzymes responsible for the biosynthesis of peptidyl moieties; *polG* was expected to encode the carboxylase responsible for the condensation between nucleoside and peptidyl moieties to form active POLs; and two genes (*polQ1* and *polQ2*) are likely to be responsible for transport. A recent study has demonstrated that both PolO and PolA are required for polyoxin production. PolO was characterized as O-carbamoyltransferase, which catalysed the conversion of AHV (α -amino- δ -hydroxyvaleric acid) to ACV (α -amino- δ -carbamoylhydroxyvaleric acid), and PolA was characterized as UMP-enolpyruvyl transferase, which converted UMP and PEP (phosphoenolpyruvate) into 3'-EUMP (3'-enolpyruvyl-UMP) (Chen *et al.*, 2009). Since PolR regulates POL production, these genes are potential targets of PolR and were selected for transcription analysis by RT-PCR.

Transcripts of the 18 structural genes (*polC* to *polQ2*, *polA* and *polB*) were distinctly visualized in the wild-type strain of *S. cacaoi* subsp. *asoensis* after 27 cycles of PCR, whereas no transcripts could be detected in the DMR strain under the same conditions, indicating that the transcription of these genes is probably regulated by PolR and that the promoters of these genes are likely to be the targets (directly or indirectly) of PolR.

For transcriptional analysis of *polR*, a pair of primers located before the insert site of the kanamycin resistance gene was used. A similar transcription pattern was observed for *polR* in the wild-type and DMR strains, indicating that *polR* was

Fig. 5. Transcriptional analysis of *polR*. (a) High-resolution S1 nuclease mapping of *polR*. Lanes G, A, T and C, DNA sequencing ladder; lanes 1 and 2, RNAs isolated from *S. cacaoi* subsp. *asoensis* after fermentation for 48 h. The asterisk indicates the tsp of *polR*. (b) The nucleotide sequence covering the promoter region. The ARE (autoregulatory element), a conserved DNA motif recognized by many γ -butyrolactone receptors (Folcher *et al.*, 2001), is underlined; the tsp is indicated by an arrow; the ribosome-binding site (RBS) is boxed. (c) Alignment of the putative γ -butyrolactone receptor binding site with the ARE consensus (Folcher *et al.*, 2001). The distance between the ARE site and the translational start codon of *polR* is indicated. The letters Y, K and W represent C or T, G or T, and A or T, respectively.

not auto-regulated. An interesting and unexpected finding in transcript analysis was that *polY* transcripts could not be detected in either the wild-type or the DMR strain even when the number of PCR cycles was increased to 32. Sequence analysis suggested that *polY* might be another regulatory gene in the *pol* cluster. Further studies need to be done to determine the hierarchy of *polR* and *polY*.

The transcription patterns of six other adjacent genes (*orf-1* to *orf-6* in Fig. 1b) were also analysed. They have been experimentally confirmed to be unrelated to POL biosynthesis (Chen *et al.*, 2009). Their transcripts could not be detected in either the wild-type or DMR strains after 27 cycles of RT-PCR. When the number of PCR cycles was increased to 32, transcripts of the six genes were detected in both strains (Fig. 6), suggesting that these six genes are expressed constitutively at a low level *in vivo* and their expression is independent of PolR.

The presence of promoters upstream of *polC* and *polB*

The data presented above indicated that the expression of 18 *pol* genes is positively regulated by PolR. These 18 genes are probably transcribed from at least two transcriptional units based on their genetic organization (Fig. 1b), although this will require further confirmation. The 16 genes from *polC* to *polQ2* are transcribed in the same direction. Half of these 16 genes (*polG-K* and *polM-O*) are translationally coupled as judged by their overlapping

codons, and no predicted transcriptional terminator could be found in the short intergenic regions between other adjacent genes, indicating that these 16 genes might form a transcriptional unit. *polB* and *polA* are divergently transcribed and they might constitute another operon as deduced by the lack of an obvious terminator in their intergenic region.

If this is the case, *polC* and *polB* must have their own promoters since they are the first genes in the two respective putative operons. Therefore, S1 mapping experiments were carried out to determine the tsp of *polC* and *polB*. As expected, *polC* has a single tsp, which is localized at the nucleotide C (Fig. 7a). *polB* is also transcribed from a single promoter, and its tsp is localized at the nucleotide T (Fig. 7a). Interestingly, similar heptameric direct repeats (5'-CGGCAAG-3') with little variation were identified in the respective -35 regions of the promoters (Fig. 7b). We propose that these heptameric repeats might be the target sequences of PolR.

Interaction of PolR with the promoter regions of *polB* and *polC*

In order to determine whether PolR directly acts on the DNA regions mentioned above, PolR was expressed as an N-terminally His₆-tagged protein in *E. coli*. Upon cultivation at 37 °C and induction with 0.1 mM IPTG at 18 °C overnight, the additional protein was detected in the crude lysate of the *E. coli* strain carrying pET28a::*polR*. The

Fig. 6. Transcriptional analysis of the *pol* cluster by semiquantitative RT-PCR. The top panel shows the RT-PCR products from the RNAs of the wild-type strain (WT); the bottom panel shows the RT-PCR products from the RNAs of the *polR* disruption mutant (DMR). Total RNAs were isolated from mycelia after fermentation for 48 h; *hrdB* transcript was used as an internal control. Twenty-seven cycles of PCR were routinely employed (genes represented by black arrows); whenever no product was detected, 32 cycles of PCR (genes represented by grey arrows) were repeated to detect low-level transcripts.

supernatant fraction was applied to an Ni-affinity resin column, from which N-terminally His₆-tagged PolR was purified. SDS-PAGE analysis showed a band of about 120 kDa, which is similar to the expected size of PolR (Fig. 8a).

Gel mobility-shift assays were carried out using the purified PolR and PCR-amplified DNA promoter regions of *polC* and *polB*, respectively. The 429 bp fragment containing the *polC* promoter region was located at position –257 bp to +172 bp with respect to the *tsp* of *polC*. The 384 bp

fragment containing the *polB* promoter region was located at position –205 bp to +179 bp with respect to the *tsp* of *polB*. As shown in Fig. 8(b), purified PolR showed clear binding to both labelled promoter fragments (*polC* and *polB* probes). To examine the binding specificity, a 424 bp DNA fragment corresponding to an internal coding sequence of *polF* was used as a negative control. Purified PolR did not display binding activity with the unrelated DNA fragment (*polF* probe) (Fig. 8b). These results demonstrated that PolR protein has a specific binding activity for the promoter regions of *polC* and *polB*.

Fig. 7. Determination of the *tsp*s of *polC* and *polB*. (a) Transcriptional analysis of *polC* and *polB* by S1 mapping. Lanes G, A, T and C, DNA sequencing ladder; lanes 1 and 2, S1 nuclease-treated samples; the S1 nuclease reactions were carried out with total RNAs isolated from 48 h culture of *S. cacaoi* subsp. *asoensis*. The *tsp*s of *polC* and *polB* are indicated by asterisks. (b) Alignment of the promoter regions of *polC* and *polB*. The 7 bp direct repeats and –10 regions in the cognate promoter sequences are compared. The *tsp*s and transcription direction of *polC* and *polB* are indicated with bold letters and bent arrows. The distance between a *tsp* and the start codon of the downstream gene is indicated.

Fig. 8. Binding of purified PolR to the promoter regions of *polC* and *polB*. (a) SDS-PAGE analysis of purified PolR. M, molecular mass markers. (b) PolR binds to the promoter regions of *polC* and *polB*. An increasing amount of purified PolR was added to the labelled *polC* and *polB* probes; an internal fragment of *polF* coding sequence was used as a negative control. DNA-protein complexes and free probes are indicated by arrows.

DISCUSSION

The genetic analysis of the biosynthetic pathway for the two 'classical' nucleoside peptide antibiotics, namely NIKs and POLs, revealed that 12 genes in the *pol* cluster are likely to be shared by the *nik* gene cluster. This is not surprising, since the structures of these antibiotics share some similar features. The evidence that *polR* in the *pol* cluster is essential for POL production, like its counterpart in the *nik* cluster, *sanG*, which is essential to activate NIK production, strongly suggested that similar regulatory cascades exist in both POL and NIK biosynthesis. The finding of the rare TTA codon in both *polR* and *sanG* implies that they are both translationally controlled by *bldA*, the structure gene of tRNA^{UUA} (Leskiw *et al.*, 1991; Liu *et al.*, 2005). Furthermore, the conserved binding sites of the γ -butyrolactone receptor are present in the promoter regions of both *polR* and *sanG*, suggesting that they are likely to be transcriptionally regulated by a γ -butyrolactone receptor-like regulator *in vivo* (Folcher *et al.*, 2001; Liu *et al.*, 2005). Despite the similarities mentioned above, PolR and SanG also showed different regulatory patterns. In POL biosynthesis, PolR positively regulates the genes responsible for the synthesis of both the peptidyl moiety and the nucleotide skeleton, while in NIK biosynthesis, SanG controls only the expression of the *sanO* and *sanN* operons, which contain the genes mainly responsible for the biosynthesis of the peptidyl moiety (Liu *et al.*, 2005). In addition, PolR in *S. cacaoi* subsp. *asoensis* only affects POL production, while SanG in *S. ansochromogenes* influences

both NIK production and colony development (Liu *et al.*, 2005).

RT-PCR analysis revealed that the disruption of *polR* impaired the expression of 18 structural genes in the *pol* cluster, implying that the potential targets of PolR might exist in these genes. Based on the organization of the *pol* gene cluster, these 18 genes are probably transcribed from at least two transcriptional units. Therefore, the promoter regions of *polC* and *polB* may contain the binding sites of PolR. Gel mobility-shift assays demonstrated that PolR does bind to the promoter regions of *polB* and *polC*. We have determined the tsps of *polC* and *polB*, and their deduced promoters showed a similar structure. Both of them contain heptameric direct repeats (consensus: 5'-CGGCAAG-3'), which overlap with the -35 region of the corresponding promoter, and the distance between the centres of the direct repeats is 11 bp, corresponding to one complete turn of DNA helix (Fig. 7b). These promoter characteristics are well consistent with the common features of promoters recognized by SARP-family proteins (Tanaka *et al.*, 2007). Considering the similarity of the N-terminal section of PolR with proteins of the SARP family, it is likely that PolR follows a regulatory pattern similar to those of SARPs and activates transcription of the *polC* and *polB* operons by binding to the heptameric direct repeats. Domain prediction indicates that PolR has an ATPase domain in addition to the SARP-like domain. Thus, the functions of the ATPase domain and the C-terminal part of PolR are interesting topics for further investigation.

Domain prediction indicates that PolY consists of an N-terminal SARP-like domain, a central ATPase domain and a C-terminal tetratricopeptide repeat (TPR) domain. The domain composition of PolY is identical to that of AfsR (Horinouchi, 2003). Gene disruption experiments indicated that *polY* is also indispensable for POL production (our unpublished data). Unexpectedly, *polY* transcripts could not be detected in either the wild-type or the DMR strain after incubation for 48 h. Further experimental analysis in progress will hopefully elucidate the exact role of *polY* in POL biosynthesis.

The similarity between the biosynthetic gene clusters of POL and NIK provides an opportunity to carry out combinatorial biosynthesis for production of novel nucleoside peptide antibiotics. Further understanding of PolR will not only be helpful for construction of engineered strains with higher production of known metabolites, but will also facilitate the detection of novel compounds resulting from combinatorial biosynthesis by improving the expression of genes involved in secondary metabolism (Hranueli *et al.*, 2005).

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