**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 SARPs (*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), Dmr1 from *Streptomyces peucetius*, 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 pimaricin and nikkomycin (NIK) production, respectively, belong to this group (Anton et al., 2004; Liu et al., 2005).

Poloxins (POLs), a group of nucleoside peptide antifungal antibiotics, were isolated from the culture broths of *Streptomyces cacaoi* subsp. *asoensis*. These antibiotics show...
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-acetylgalactosamine, 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 DH5α 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 µg ml⁻¹ in YEME or MS for S. cacaoi subsp.

![Fig. 1.](image-url)
asoensis, 100 μg ml⁻¹ in LB for E. coli; kanamycin, 10 μg ml⁻¹ in YEME or MS for S. cacaoi 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 φC31 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. cacaoi 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 Framerplot 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. cacaoi 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 BamHI and KpnI, blunted by mung bean nuclease and ligated into the Smal 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. cacaoi 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 φC31 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.** Speros of S. cacaoi 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. 529331), so we set up a separation system for detecting POLL-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 nuclelease mapping.** RNAs for S1 nuclease mapping were isolated from S. cacaoi 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 fmol 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. cacaoi subsp. asoensis genomic DNA using the primers PolRN and PolRC, containing NdeI and EcoRI sites respectively. The PCR product was digested with NdeI and EcoRI and cloned into the corresponding sites of pET28a to generate expression plasmid pET28a::polR. The recombinant plasm-
## Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer and purpose</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>To construct pDR101 for polR disruption</td>
<td></td>
</tr>
<tr>
<td>DR Forward</td>
<td>GAACGCTCCTGGTGCTGCTCATC</td>
</tr>
<tr>
<td>DR Reverse</td>
<td>ACGCCACCTCCAGACCTACA</td>
</tr>
<tr>
<td>To construct pSER for complementation</td>
<td></td>
</tr>
<tr>
<td>polR Forward</td>
<td>TCAGTGCCGGAACGCCT</td>
</tr>
<tr>
<td>polR Reverse</td>
<td>GCCGGTGGACGATGTTCG</td>
</tr>
<tr>
<td>For RT-PCR analysis</td>
<td></td>
</tr>
<tr>
<td>RTS-polR</td>
<td>ACGTGAGATCCAGGAAAG</td>
</tr>
<tr>
<td>RTS-polY</td>
<td>CCTCTACGCAAGTCTCCT</td>
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<td>RTS-polD</td>
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<td>RTS-polG</td>
<td>GAGGAAGCTGATGATG</td>
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<tr>
<td>RTS-polI</td>
<td>GCCCTCCAGACGGCTTAC</td>
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<td>RTS-polH</td>
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<tr>
<td>RTS-polI</td>
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</tr>
<tr>
<td>RTS-polK</td>
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</tr>
<tr>
<td>RTS-polJ</td>
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</tr>
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<td>RTS-polM</td>
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<tr>
<td>RTS-polN</td>
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<td>RTS-polO</td>
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<td>RTS-orf5</td>
<td>AGTCGTCGGCATGTTG</td>
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</table>
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 His$_6$-tagged fusion protein. *E. coli* BL21(DE3) carrying pET28a::polR was grown in 200 ml LB to an OD$_{600}$ 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 NaH$_2$PO$_4$, 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 His$_6$-tagged PolR protein eluted with 250 mM imidazole was analysed by SDS-PAGE. Further desalting and concentration were performed with a Microcon protein eluted with 250 mM imidazole was analysed by SDS-PAGE.

For S1 nuclease mapping

<table>
<thead>
<tr>
<th>Primer and purpose</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-polR-SE</td>
<td>TCGTCGTTGGCTCTTCACC</td>
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<tr>
<td>S-polR-AN</td>
<td>ATGTGTCGATCCATGTCTTCCCAC</td>
</tr>
<tr>
<td>S-polC-SE</td>
<td>GCCGGTAGTGCTTGACAC</td>
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<tr>
<td>S-polC-AN</td>
<td>TCGTGTTGGCCGTCTTCAGC</td>
</tr>
<tr>
<td>S-polB-SE</td>
<td>ATCTCCCACGCGCGTGACCT</td>
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<tr>
<td>S-polB-AN</td>
<td>TCTCATATGATGTATGCAACGGAAGT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer and purpose</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>PolRNN</td>
<td>ATAGAATTCGTGGCCGGAACGCCT</td>
</tr>
<tr>
<td>PolRC</td>
<td>TCTCATATGATGTATGCAACGGAAGT</td>
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</tbody>
</table>

**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. AI250878) (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. anschromogenes* (Liu et al., 2005). PolR also showed full-length similarity to PimR (35 % identity), a positive regulator for pimaricin 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-termi and other similar domains in their C-termi.

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], HCl (pH 7.8), 20 mM boric acid and 1 mM EDTA. Gels were dried and exposed to Biomax radiographic film (Kodak).
Fig. 2. Domain structure and amino acid alignment of parts of the PolR protein. (a) Predicted domain structure of PolR. TRC, \textit{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 \textit{orfR} (AJ250878) from \textit{Streptomyces tendae}; SanG, NIK biosynthesis regulator from \textit{S. ansochromogenes} (AAV31783); PimR, pimaricin biosynthesis regulator from \textit{S. natalensis} (CAE51066); AurD, regulator of the aureothin pathway in \textit{S. thioluteus} (CAE02599); CcaR, activator of both the cephamycin and clavulanic acid pathways in \textit{S. clavuligerus} (CAB03623); RedD, undecylprodigiosin activator in \textit{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 \textit{S. avermitilis} (NP_821585).
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 φC31 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 φC31 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 32P-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).

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 (*z*-amino-δ-hydroxyvaleric acid) to ACV (*z*-amino-δ-carbamoylhydroxyvaleric acid), and PolA was characterized as UMP-enolpyruvyl transferase, which converted UMP and PEP (phosphoenolpyruvate) into 3′-EUMP (3′-enolpyruvylUMP) (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...
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 tsts 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 235 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
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 -225 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(a), 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.
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 tss 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.

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.
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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