The clpP multigenic family in Streptomyces lividans: conditional expression of the clpP3 clpP4 operon is controlled by PopR, a novel transcriptional activator

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The *clpP* multigenic family in *Streptomyces lividans*: conditional expression of the *clpP3 clpP4* operon is controlled by PopR, a novel transcriptional activator

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Summary

The *clpP* genes are widespread among living organisms and encode the proteolytic subunit of the Clp ATP-dependent protease. These genes are present in a single copy in most eubacteria. However, five *clpP* genes were identified in *Streptomyces coelicolor*. The *clpP1 clpP2* operon was studied: mutations affected the growth cycle in various *Streptomyces*. Here, we report studies of the expression of the *clpP3 clpP4* operon in *Streptomyces lividans*. The *clpP3* operon was induced in a *clpP1* mutant strain, and the regulation of expression was investigated in detail. The product of the putative regulator gene, downstream from *clpP4*, was purified. Gel migration shift assays and DNase I footprinting showed that this protein binds to the *clpP3* promoter and recognizes a tandem 6 bp palindromic repeat (TCTGCC-3N-GGCAGA). *In vivo*, this DNA-binding protein, named PopR, acts as an activator of the *clpP3* operon. Studies of *popR* expression indicate that the regulator is probably controlled at the post-transcriptional level.

Introduction

Streptomyces are high G+C% Gram-positive soil bacteria that possess an 8 Mb linear chromosome. Sequencing is presently under way and has already revealed many multigene families. Mostly studied for the past 50 years because of its large and diverse production of secondary metabolites, *Streptomyces* is also a model of bacterial differentiation on account of its complex growth cycle. On solid media, spore germination leads to the growth of basal mycelia, which then differentiate into aerial mycelia that finally septate and differentiate into spores. Two classes of differentiation mutants have been characterized: some present a 'bald' phenotype and are arrested at the basal mycelial state, whereas others are 'white' mutants that are unable to sporulate. The mechanisms regulating cell differentiation in *Streptomyces* have not been totally elucidated. As energy-dependent proteases are often responsible for the degradation of regulatory proteins (Gottesman and Maurizi, 1992), the role of one of them, the Clp protease, has been investigated in *Streptomyces* (de Crecy-Lagard *et al*., 1999). The Clp complex is an ATP-dependent serine protease (Maurizi *et al*., 1990). In *Escherichia coli*, it is composed of a tetradecamer of ClpP catalytic subunits, forming a central chamber and associated at one or both ends with a hexamer of ATPase subunits, ClpX or ClpA (ClpC in Gram-positive bacteria) (Kessel *et al*., 1995; Wang *et al*., 1997; Grimaud *et al*., 1998), which confer substrate specificity. Isolated ClpP subunits have been shown to cleave peptides no longer than six amino acids (Woo *et al*., 1989), and the ATPase subunits serve as chaperones (Wawrzynow *et al*., 1995). *clpP* genes are generally present as single copies in eubacteria, but some organisms possess a multigenic *clpP* family. For example, two *clpP* genes are present in *Mycobacterium tuberculosis*, four in the cyanobacterium *Synechococystis* and at least five in *Streptomyces coelicolor*. In *S. coelicolor*, the five genes are organized as two bicistronic operons and one monocistronic gene, all located at different sites on the chromosome. Although *clpP* genes are ubiquitous, they have been analysed in only a few species. Studies in *E. coli*, *Bacillus subtilis*, *Caulobacter crescentus* and *Streptomyces lividans* implicate ClpP in cell cycle development. Indeed, ClpP protease degrades the stationary-phase sigma factor in *E. coli* (Schweder *et al*., 1996), and the *clpP* mutant in *B. subtilis* is affected in competence and sporulation (Msadek *et al*., 1998). In *C. crescentus*, ClpP is required for normal cell division, growth and viability (Jenal and Fuchs, 1998) and, in *S. lividans*, the *clpP1 clpP2* mutant has a bald phenotype, i.e. it is unable to form aerial mycelia and thus unable to complete the normal growth cycle (de Crecy-Lagard *et al*., 1999). In *E. coli*, the Clp proteolytic complex degrades phage replication proteins (Wojtkowiak *et al*., 1993; Mhammedi-Alaoui *et al*., 1994) and proteins tagged by the SsrA system (Gottesman *et al*., 1998). All other known targets of Clp are regulators: the transcriptional regulator ComK that drives competence development in *B. subtilis* (Turgay

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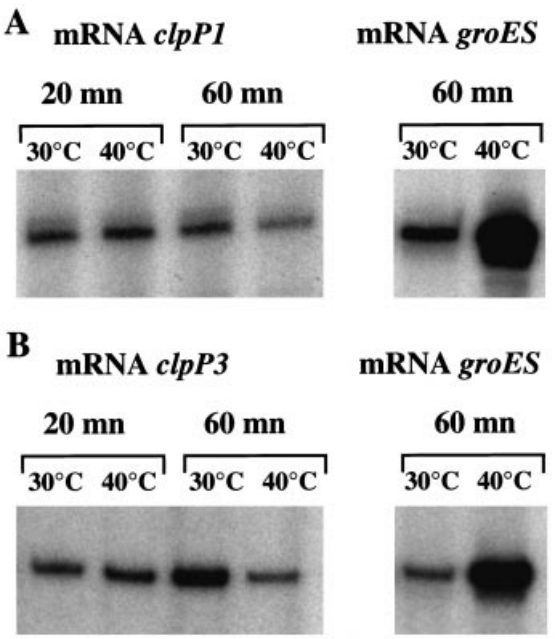

Fig. 1. Studies of the heat shock response of the *clpP1* and *clpP3* operons. Analysis by primer extension was done to determine whether *clpP1* and *clpP3* are heat shock genes.
A. For *clpP1*, mRNAs were extracted from cultures of wild-type *S. lividans* grown at 30°C for 30 h, then kept at that temperature or subjected to heat shock for 20 or 60 min at 40°C.
B. For *clpP3*, mRNAs were extracted from cultures of the *clpP1* mutant subjected to identical heat shock treatments. *groES* was used as a control to verify heat shock conditions.

et al., 1998) and the multicell cycle processes regulator CtrA in *C. crescentus* (Jenal and Fuchs, 1998). The Clp protease probably also plays a more general role. Indeed, although *clpP* genes are constitutively expressed at a low level, most are strongly induced by many and diverse physiological stresses, such as heat shock, ethanol and puromycin treatments (Gerth *et al*., 1998; Frees and Ingmer, 1999; Østeras *et al*., 1999). The stress induction of *clpP* genes indicates that they are subject to regulatory systems. To date, only two systems regulating *clpP* genes are known: σ^{32} the general heat shock sigma factor in *E. coli* (Kroh and Simon, 1990) and the operator–repressor CtsR system identified in *B. subtilis* (Derré *et al*., 1999). In *B. subtilis*, under non-stress conditions, the repressor CtsR binds a heptanucleotide repeat (5′-A/GGTCAAA-NANA/GGTCAAA-3′), which often overlaps the −10 and −35 consensus sequence at the *clpP* promoter. Repression is relieved by an unknown mechanism under heat shock conditions. The control by CtsR is found in many low G+C Gram-positive bacteria. Neither of these two mechanisms has been reported in *Streptomyces,* in which three different modes of heat shock regulation have been described. They are all repressor–operator systems: HrcA/CIRCE controls *groESL* genes (Duchêne *et al*., 1994); HspR/HAIR regulates the *dnaK* operon and *clpB* (Bucca *et al*., 1997; Grandvalet *et al*., 1997; 1999); and RheA is the repressor of *hsp18* in *Streptomyces albus* (Servant *et al*. 2000). None of these operators has been identified upstream from *Streptomyces clpP* genes.

Our experiments gave no evidence that the *clpP1 clpP2* operon of *S. lividans* was induced under heat shock conditions. We examined the transcriptional expression of the *clpP3 clpP4* operon on the premise that it is implicated in the heat shock response. In the conditions we tested, neither *clpP1* nor *clpP3* appeared to be heat shock genes. Our findings indicate that expression of the *clpP3 clpP4* operon is dependent on the genetic background, as its transcription was induced in a *clpP1 clpP2* mutant strain. We also show that this expression is under the control of a specific activator. Thus, we report the first evidence for an interactive regulation between the *clpP* multigenic genes.

Results

Existence of two clpP *bicistronic operons in* S. coelicolor

Sequencing of the *S. coelicolor* A3(2) reference strain is under way at the Sanger Center (http://www.sanger.ac.uk/Projects/S_coelicolor/). The nucleotide sequences of two *clpP* genes, in addition to the known *clpP1* and *clpP2*, have already been determined. These genes, *clpP3* and *clpP4*, are carried by the 5H1 cosmid, mapping at the right extremity of the chromosome. The putative translation initiation codon of *clpP4* is 1 bp downstream from the *clpP3* stop codon, which suggests that they are organized as a single operon. The *clpP3* ATG initiation codon is preceded by a putative Shine–Dalgarno sequence GGAGC 4 bp upstream. The two genes *clpP3* and *clpP4* encode predicted proteins of 217 and 200 amino acids with calculated molecular masses of 23.5 and 21.6 kDa respectively. *S. coelicolor* ClpP1 and ClpP3 are 50.2% similar, and Clp2 and ClpP4 are 53.7% similar. All other sequence pairings are less than 41.5% similar. *clpP3 clpP4* may therefore be a paralogous operon of *clpP1 clpP2*.

In *E. coli*, Ser-97/His-122/Asp-171 are the catalytic triad of the serine protease ClpP, and Ser and His residues are essential for Clp protease activity (Maurizi *et al*., 1990; Wang *et al*., 1997). In *Streptomyces*, the catalytic residues Ser/His/Asp are perfectly conserved in ClpP1, ClpP2 and ClpP3. However, interestingly, in ClpP4, the His residue is substituted by a Gly residue, and the nearest histidine is 6 amino acids upstream from this position. Whether this has a large effect on ClpP4 catalytic activity, as it would in *E. coli*, remains to be determined.

clpP1 *and* clpP3 *operons are not heat induced*

S. lividans 1326 is very closely related to the *S. coelicolor* A3(2) reference strain and it is easier to manipulate. *S. lividans* 1326 was therefore used for the following experiments.

To determine the promoter region of the *clpP3 clpP4* operon, primer extension analysis was performed. No transcripts were revealed using mRNAs extracted from the *S. lividans* wild-type strain. *clpP* genes have been described as heat shock genes in many organisms, so primer extensions were carried out on mRNAs extracted from *S. lividans* cultures after heat shock at 40°C for 20 and 60 min. No heat-induced signal could be detected (not shown). Absence of heat induction was also observed for the *clpP1 clpP2* operon (Fig. 1A). These results suggest that, at least in the standard conditions assayed, which are known to induce many *Streptomyces* heat shock proteins (HSPs) (Puglia *et al*., 1995), *clpP1* or *clpP3* operons are not heat shock induced.

Conditional expression of the clpP3 clpP4 *operon from* S. lividans *and determination of the transcriptional start site*

The *S. lividans clpP1* mutant is unstable, and the bald phenotype could be lost on certain media after subculturing, although the *clpP1* gene is still disrupted (de Crecy-Lagard *et al*., 1999). This indicates that either compensatory mutations appear or usually silent genes are activated. Our results indicated a silencing of the *clpP3* operon in the wild-type strain, and the *clpP3* operon appears to be a paralogue of the *clpP1* operon. Thus, the reversion of the bald phenotype of the *clpP1* mutant may result from the expression of the *clpP3* operon being turned on. mRNAs were extracted from the wild-type strain and from two *clpP1* mutant strains, one bald and one showing a partial revertant phenotype, and used for primer extension analysis. No signal was detected for the wild-type RNA, and strong signals were observed for RNA from both *clpP1* mutants (Fig. 2). mRNA from the glucose kinase gene (*glk*) was used as a quantitative control. Expression of *clpP3 clpP4* was demonstrated consistently only in the *clpP1* mutant; this does not preclude the possibility of a low-level transient expression in a wild-type in conditions that were not established. Indeed, it is well referenced that there are considerable variations in protein synthesis throughout the cell cycle in *Streptomyces*. In view of these results, we reanalysed *clpP3 clpP4* transcripts in heat shock conditions in a *clpP1* mutant. No heat induction was observed (Fig. 1B).

Maintenance of a bald phenotype in the *clpP1* mutant strain expressing *clpP3* and *clpP4* indicates that ClpP3 and ClpP4 are not fully isofunctional to ClpP1 and ClpP2

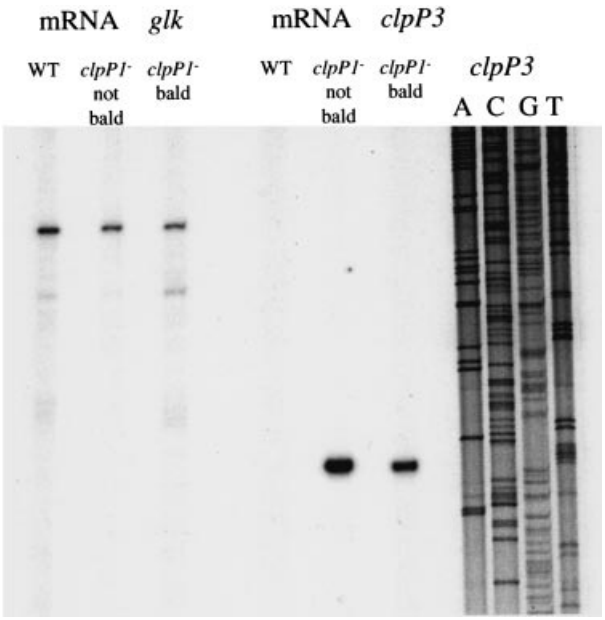

Fig. 2. Expression analysis and mapping of the 5′ end of the c*lpP3* mRNA. In order to determine the transcription start site and to evaluate the level of *clpP3* expression, primer extensions were performed on the *clpP3* mRNA. mRNAs were extracted from cultures of the wild-type strain (WT), a *clpP1* mutant presenting the bald phenotype (*clpP1*$^{-}$ bald) and a *clpP1* mutant strain showing a partial revertant phenotype (*clpP1*$^{-}$ not bald). A control was carried out on the glucose kinase (*glk*) mRNA extracted from same cultures; upper bands correspond to the predicted signal. Lanes A, C, G and T show the product of the dideoxy sequencing realized on pJV37 with the same primer used for the primer extension of the *clpP3* mRNA.

but suggests some specificity of action of the various ClpP paralogues.

A single transcription start site was localized 83 bp upstream from the *clpP3* translation initiation codon. None of the *Streptomyces* promoter consensus sequences described by Bourn and Babb (1995) could be identified in this region.

Overexpression and purification of PopR, a putative DNA-binding protein

Cosmid 5H1 has an open reading frame (ORF) downstream from the *clpP4* gene in the opposite orientation (Fig. 3). This ORF encodes a putative DNA-binding protein of 157 amino acids with a calculated molecular mass of 16.7 kDa. A five-amino-acid segment (RPQAT) is repeated four times in the N-terminal region, and a helix–turn–helix (HTH) motif is present between the 77th and 97th amino acid residues of the protein. The putative protein shows no similarity to proteins in databanks except for three other putative DNA-binding proteins, one in *S. coelicolor* and two in another Actinomycete,

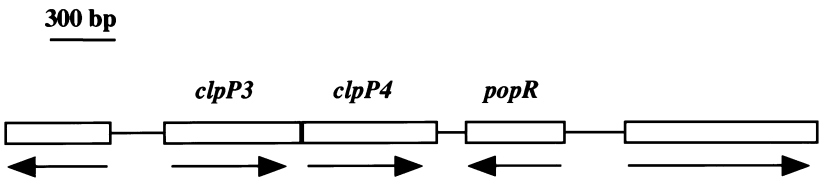

Fig. 3. Genetic organization of the *clpP3* operon region. Arrows indicate the direction of gene transcription.

Mycobacterium tuberculosis. However, most of the sequence identities are in the HTH motif.

We investigated whether this putative DNA-binding protein, called PopR for *clpP3* operon regulator, is a regulator of the *clpP3 clpP4* operon. To assay the DNA-binding activity of PopR, it was overproduced in *E. coli* and purified as follows (Fig. 4). The *popR* gene from *S. lividans* was amplified by PCR, and the product was inserted into the vector pET28. The sequence of the gene was determined: the *S. lividans* PopR is nine amino acid residues shorter than its *S. coelicolor* counterpart because of the absence of one RPQAT motif and a stretch of four amino acid residues in the C-terminal region. The construction, pEt28*popR*, was introduced into *E. coli* for IPTG-inducible T7-driven overproduction of a recombinant PopR protein carrying a His-tag at its N-terminus. The recombinant PopR was purified on column. The His-tag was removed from the PopR recombinant protein by treatment with thrombin, a site-specific protease.

PopR binds directly to the clpP3 *promoter region*

Gel mobility shift DNA-binding assays were used to test the DNA-binding activity of PopR with the *clpP3* promoter region: a fragment of 330 bp corresponding to positions −247 to +82 relative to the transcription start site of *clpP3* was obtained by PCR amplification. Similar experiments were carried out on a 310 bp PCR-amplified fragment corresponding to the upstream region of *popR* to test for possible autoregulation of the gene.

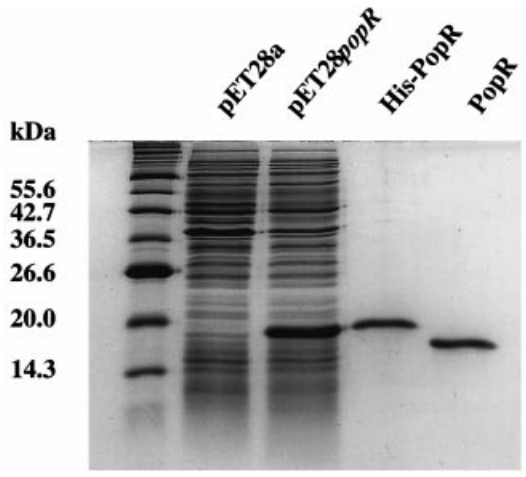

Fig. 4. Overproduction and purification of PopR. Crude extracts were prepared from cultures of *E. coli* BL21λDE3 carrying pET28a (lane pET28a) or pET28*popR* (lane pET28*popR*). These samples, as for His-tag PopR recombinant protein purified on Ni-NTA column (lane His-PopR) and purified PopR after thrombin treatment (lane PopR), were loaded on 15% Tris–glycine SDS–PAGE. Molecular weight standards (Biolabs) are on the left.

The prepared radiolabelled fragments were incubated with increasing amounts of purified PopR. Complete displacement was observed when the *clpP3* promoter region was incubated with the highest quantity of PopR tested (200 ng); no displacement was observed when the *popR* upstream region was incubated with the same quantity of protein (Fig. 5). Thus, PopR bound specifically to the *clpP3* promoter region but was not subject to autoregulation.

PopR recognizes a double-tandem 6 bp palindrome

The operator site in the *clpP3* promoter region recognized by PopR was determined by DNase I footprint assays. A radiolabelled fragment of 360 bp corresponding to positions −160 to +196 relative to the transcription start site was amplified by PCR and treated with DNase I. The PopR protein protects one region extending from positions −75 to −35 relative to the transcription start site (Fig. 6). The following double-inverted repeat, extending from positions −70 to −41 (Fig. 7), was identified in the protected region: TCTGCC-3N-GGCGAA TCTGCC-3N-GGCAGA.

Numerous σ^{70} promoters in *E. coli* subject to activation contain an activator site close to the −40 position (Gralla, 1991). Possibly, the DNA-binding protein PopR, which binds to the *clpP3* promoter, is an activator that allows RNA polymerase recruitment.

PopR activates expression of the clpP3 *operon in* S. lividans

To examine whether PopR is an activator, it was overexpressed in various *S. lividans* strains, and *clpP3 clpP4* operon expression was assessed by primer extension.

A 780 bp fragment corresponding to *popR* and its promoter region was amplified by PCR and inserted into pUWL219. The resulting construction, pJV100, allowed

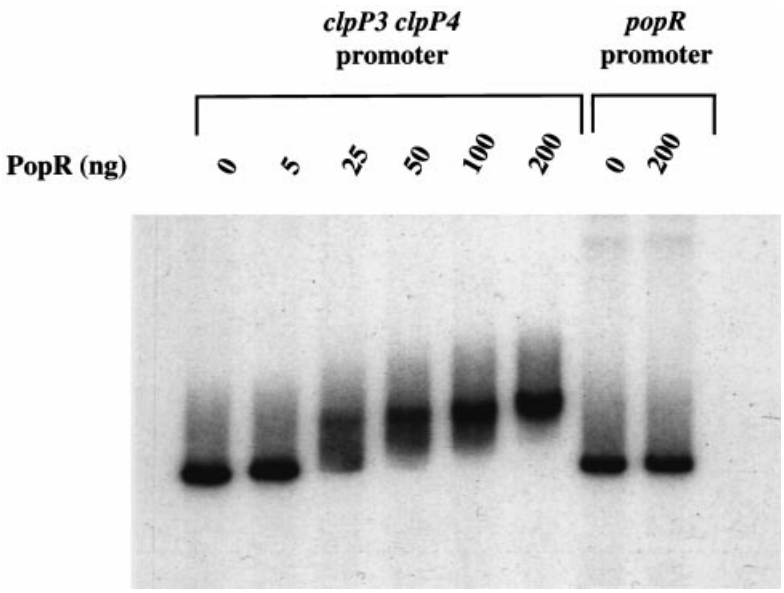

Fig. 5. Specific binding of PopR to the *clpP3* promoter region revealed by the gel shift experiment. The 330 bp DNA fragment probe (10 000 c.p.m.) corresponding to the *clpP3* promoter region was incubated with increasing amounts of purified PopR (0–200 ng). A 310 bp DNA fragment probe (10 000 c.p.m.) corresponding to the *popR* upstream region was incubated with 0 and 200 ng of purified PopR.

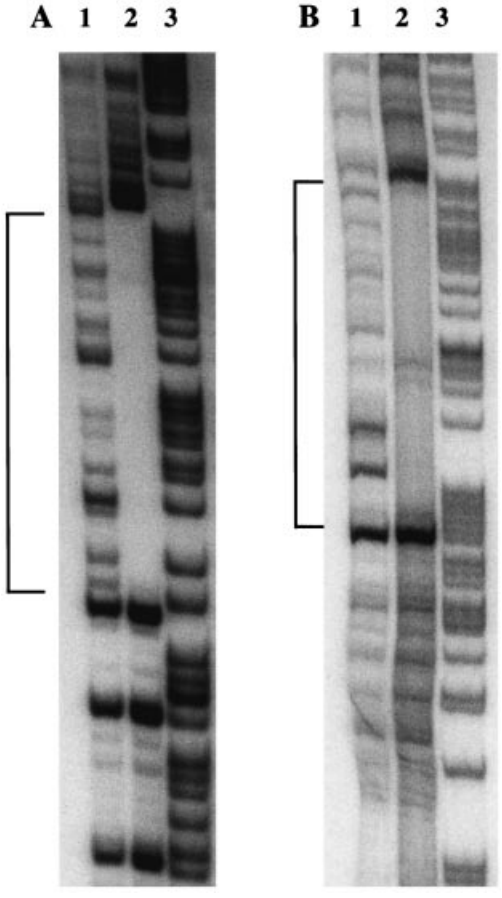

Fig. 6. Determination of the operator site recognized by PopR. Approximately 50 000 c.p.m. of labelled template strand (A) and non-template strand (B) of the *clpP3* promoter region were submitted to DNase I treatment without (lane 1) or with previous incubation with 1 μg of purified PopR (lane 2). G+A Maxam and Gilbert reactions of the appropriate labelled DNA fragments were also loaded (lane 3). Brackets indicate the region protected by PopR.

the expression of *popR* under its own promoter on a multicopy vector. pJV100 was introduced into *S. lividans* 1326 wild-type and *clpP1* mutant strains by conjugation from *E. coli* S17.1. Conjugants were selected on thiostrepton. mRNAs were extracted from cultures of the strains harbouring or not harbouring pJV100. *clpP3* transcription was analysed by primer extension, and *glk* mRNA was used for RNA quantification controls. A strong signal was detected with mRNAs extracted from the *clpP1* mutant strain overexpressing *popR*. The signal was weaker with mRNAs extracted from the *clpP1* mutant. Interestingly, overexpression of *popR* in a wild-type strain led to the expression of *clpP3* (Fig. 8). We concluded that PopR is the activator of the *clpP3 clpP4* operon.

Attempts to obtain a clpP1 popR *double mutant*

We attempted to construct a strain with a *clpP1 popR* double mutation. However, despite considerable effort, no such mutant was obtained. PopR being the activator of *clpP3*, one predicted characteristic of this construction would be the absence of *clpP3 clpP4* transcription. To understand the impossibility of achieving this construction, we tried to obtain a *clpP1 clpP3* double mutant by targeting the structural genes directly. Whereas simple *clpP1* and *clpP3* mutants were readily selected, the double mutant was not obtained. The phenotype of the *clpP3* mutant is different from the phenotype of *clpP1*; whereas *clpP1* is bald, the *clpP3* mutant is not affected in its cell cycle, yet it presents an increased production of a blue pigment (actinorhodin).

Fig. 7. Nucleotide sequence of the *clpP3* promoter region and the deduced amino acid sequence of the beginning of the gene. The putative ribosome binding site is boxed, the +1 position start is indicated by ↱ and nucleotides −10 and −35 are indicated. The Dnase I protected areas are shaded, and the PopR double-inverted repeat recognition target is indicated by inverted arrows in the sequence.

Transcriptional expression of popR

To determine whether *clpP3* activation by PopR in a *clpP1* mutant resulted from *popR* enhanced expression, the rate of transcription of *popR* was evaluated. Primer extension analysis was carried out using two different primers. A unique transcription start point was identified. It is preceded by two hexamers (TAGGCT and TTACGG) at positions −10 and −35. The −10 sequence is in good agreement with the consensus motif recognized by the vegetative HrdB sigma factor (Strohl, 1992), whereas the −35 sequence presents a lower similarity. This transcription start site is located at the initiation codon. Such leaderless mRNAs have been described for other *Streptomyces* genes (Jones *et al.*, 1992). The signals were easily detectable with mRNAs from strains harbouring pJV100, but were very faint with mRNAs from strains possessing only the chromosomal copy of *popR*. The intensity of the signals was similar for the wild-type and *clpP1* mutant strains (Fig. 9). This indicates that ClpP1 has no direct effect on the transcriptional regulation of *popR*. Accordingly, accumulation of *clpP3* transcripts in the *clpP1* mutant suggests a post-transcriptional regulation of the activator.

Discussion

This is the first report describing an interactive regulation among the *clpP* multigenes in *Streptomyces*. In the present study, we have shown that *clpP3 clpP4* operon expression is induced when the *clpP1 clpP2* operon is not functional. This induction involves an activator, PopR, encoded by a gene downstream from *clpP4* and in the opposite orientation. The PopR protein directly binds a

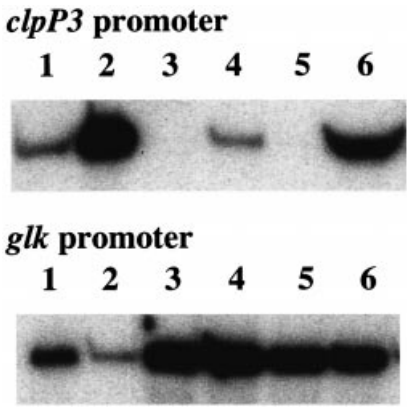

Fig. 8. *clpP3* PopR-dependent expression. Primer extensions were realized on *clpP3* mRNA. mRNAs were extracted from the *clpP1* mutant strain (*S. lividans* 1326 *clpP1::Am*^{R}, lane 1) and from the *clpP1* mutant strain overexpressing *popR* present in multicopy [*S. lividans* 1326 *clpP1::Am*^{R} (pJV100), lane 2]. mRNAs were also extracted from the wild-type strain carrying the empty multicopy vector at 22 h or 32 h of growth culture [*S. lividans* 1326 (pUWL219), lanes 3 and 5 respectively] and from wild-type strain overexpressing *popR* at 22 h or 32 h of growth culture [*S. lividans* 1326 (pJV100), lanes 4 and 6 respectively].

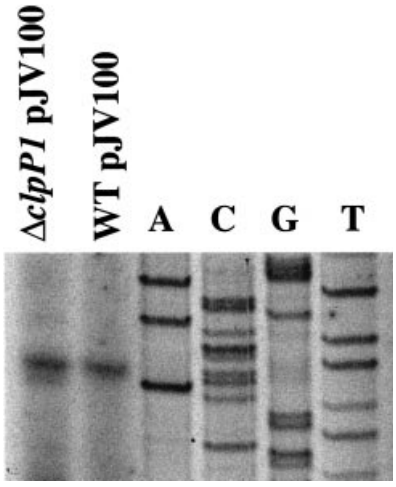

Fig. 9. Expression analysis and mapping of the 5′ end of the *popR* mRNA. In order to determine the transcription start site and to evaluate the eventual effect of the *clpP1* mutant genetic background on *popR* transcription, primer extensions were performed on the *popR* mRNA extracted from cultures of the *clpP1* mutant overexpressing *popR* (*clpP1*$^-$pJV100) or from the wild-type strain overexpressing *popR* (WT pJV100). Lanes A, C, G and T show the product of the dideoxy sequencing realized on pJV100 with the same primer as that used for the primer extension.

double-tandem repeat of a 6 bp palindromic sequence and activates the transcription of the *clpP3 clpP4* genes. PopR is the first activator involved in *clpP* regulation described in bacteria. In the sequence data available for the *S. coelicolor* genome, the complete operator site recognized by PopR is only found upstream from *clpP3*, suggesting that PopR is not a pleiotropic regulator. None of the promoter consensus classes defined by Bourn and Babb (1995) could be identified in the *clpP3* promoter region other than a CNGNNA hexamer. This motif begins at position −13 upstream from the transcription start site, a position rarely used according to the data of Bourn and Babb (1995). This motif is usually associated with another sequence TANNNT or CANNAT, not present in the *clpP3* promoter, probably indicating weak RNA polymerase promoter-binding activity. PopR binding at position −40 presumably recruits the RNA polymerase.

Surprisingly, considering reports on the heat shock induction of *clpP* in many eubacteria, we have not observed heat shock induction either for *clpP1* or for *clpP3* in standard conditions. However, the heat shock modulon presents several peculiarities in *Streptomyces*. Puglia *et al*. (1995) have shown that there is a lot of variation in the patterns of kinetics of heat and developmentally induced synthesis of the different heat shock proteins in *S. coelicolor*, and we have not achieved such an exhaustive study of this phenomenon on *clpP* genes.

The *clpP3* operon and the *popR* gene map at the right extremity of the *S. coelicolor* linear chromosome within an unstable region. Indeed, *Streptomyces* chromosomal ends are subject to substantial genetic instability: large-scale deletions and DNA sequence amplifications may occur with a frequency $>10^{-3}$ (Fischer *et al*., 1998; Volff and Altenbuchner, 1998). Chromosomal ends are proposed to contain non-essential or conditionally essential genes (Redenbach *et al*., 1996). Conditional expression of the *clpP3* operon in a *clpP1* mutant is in agreement with this hypothesis. Furthermore, whereas simple *clpP1* and *clpP3* mutants were readily selected, the double mutant was not obtained. This might suggest that at least one *clpP* bicistronic operon is required for viability. Such a critical role of ClpP in cell viability has been reported in *C. crescentus* (Jenal and Fuchs, 1998). Interestingly, the two *clpP* operons functions overlap only partially. Indeed, *clpP3* and *clpP4* do not fully complement the phenotype of the *clpP1 clpP2* mutant strain: the bald phenotype persists even after induction of the *clpP3* operon. Furthermore, the *clpP3* mutant does not present the bald phenotype. These results point to different specific roles for the various ClpP proteins. Four distinct ATPases belonging to the HSP100 family have been identified in the *S. coelicolor* chromosome: two encoded by *clpC* genes, one by *clpX* and one by *clpB* (no interaction is known with ClpP for the last). There are also five *clpP* genes in this species. It is not known whether there is a specificity of interaction between the different HSP100 Clp proteins and the ClpP protease. If there is, ClpP1 and ClpP2 could have targets other than those of ClpP3 and ClpP4 because substrate specificity is conferred by the HSP100. This could explain why *clpP3* and *clpP4* do not fully complement the phenotype linked to the *clpP1 clpP2* mutations. In view of the substitution of His by Gly in the catalytic triad, the activity of ClpP4 should be examined further. The histidine residue nearest to the position predicted by the alignments is six amino acid residues upstream. It is possible that ClpP4 is inactive. Another organism, the cyanobacterium *Synechocystis*, contains four *clpP* genes, one of which, *clpP4*, lacks all three amino acids residues of the catalytic triad. Porankiewicz *et al*. (1999) proposed that this protein is not a true ClpP but a ClpR, a new uncharacterized class of protease.

PopR is the centre of the regulation system and, thus, the expression of *clpP3 clpP4* is dependent on its cellular level and activity. As the expression of *popR* is similar in the *clpP1* mutant and wild-type strains, the control of the activator is presumably post-transcriptional. ClpP1 and ClpP2 are involved in the stability or activity of PopR, and PopR might be a target for the Clp protease. No specific motif has been characterized in the substrates of the Clp proteolytic complex; however, in *E. coli*, the N-terminal and C-terminal peptide sequences are important for signal recognition. This is illustrated by the N-end rule and by the SsrA-tagging system. The N-end rule is that proteins carrying certain amino acids at the N-terminus are highly unstable and ClpAP is implicated in their degradation (Tobias *et al*., 1991). The SsrA-tagging system, widespread among eubacteria, adds a C-terminal peptide tag to proteins as a degradation signal (Tu *et al*., 1995; Keiler *et al*., 1996; Roche and Sauer, 1999). Among bacteria, there is a quasi-absolute conservation of the Leu–Ala–Ala motif at the C-terminal extremity of the peptide tag. It has been shown that these two Ala are crucial for the degradation by ClpAP or ClpXP proteases (Gottesman *et al*., 1998). The fact that the peptide sequence of PopR ends with Leu–Ala–Ala further sustains the hypothesis that ClpP1 and ClpP2 might degrade this protein. This question will be addressed in future work.

Recently, a fifth *clpP* gene from *S. coelicolor* was sequenced (http://www.sanger.ac.uk/Projects/S_coelicolor/). Nothing is yet known about it. The role of this new gene needs investigation, and its involvement in the general regulatory network of the other *clpP* genes remains to be elucidated.

Experimental procedures

Bacterial strains and media

S. lividans strain 1326 was obtained from the John Innes Culture Collection, and *S. lividans* 1326 *clpP1*::*Am*^{R} was constructed in this laboratory (de Crecy-Lagard *et al*., 1999). YEME medium was used for liquid growth (Hopwood *et al*., 1985). NE medium (Murakami *et al*., 1989) and R5 (Hopwood *et al*., 1985) were used for *Streptomyces* growth on plates. The antibiotics apramycin and thiostrepton were added to final concentrations of 25 μg ml^{-1} to solid medium and to 20 and 10 μg ml^{-1}, respectively, to liquid medium.

E. coli TG1 (Gibson, 1984) was used as the general cloning host, *E. coli* S17-1 (Simon *et al*., 1983) was used as the conjugative host and *E. coli* BL21λDE3 for protein production and purification (Studier and Moffatt, 1986). *E. coli* strains were grown in LB medium. The antibiotics ampicillin and kanamycin were added to final concentrations of 100 and 25 μg ml^{-1} respectively.

DNA manipulation, transformation and conjugation procedures

Plasmid DNA was extracted from *E. coli* using a Qiagen kit. DNA fragments were purified from agarose gels with Ultrafree-DA (Amicon-Millipore). Restriction enzymes were used as recommended by the manufacturers. DNA fragments were amplified by the PCR technique (Mullis and Faloona, 1987; Saiki *et al*., 1988). DNA sequences were determined by the dideoxy chain termination method (Sanger *et al*., 1977) using a modified T7 DNA polymerase (Pharmacia). Standard $CaCl_2$ (Cohen *et al*., 1972) or electroporation procedures were used for *E. coli* transformation.

Streptomyces DNA was prepared as described by Hopwood *et al*. (1985). Intergenic conjugations from *E. coli* S17-1 to *S. lividans* 1326 or *S. lividans* 1326 *clpP1*::*Am*^{R} were performed as described previously (Mazodier *et al*., 1989).

Plasmids and plasmid constructions

The *E. coli* vectors used were pUC19 (Yanisch-Perron *et al*., 1985) for cloning and pET28a (Novagen) for overproduction and purification of proteins. The *Streptomyces*/*E. coli* shuttle vectors used were pUWL219 (Wehmeier, 1995) and pGM160Δ made by J. L. Pernodet and obtained by deleting the Apra^R 1610 bp *Hin*dIII fragment from the thermosensitive vector pGM160 (Muth *et al*., 1989).

The transcription start site of *clpP3* was localized by a sequencing reaction with the oligonucleotide used in primer extension on plasmid pJV37. pJV37 consists of the 1800 bp fragment including the *S. lividans* 1326 promoter region and the *clpP3 clpP4* operon obtained by PCR amplification with primers Ju 40 (see Table 1) and Ju 43, inserted between the *Eco*RI and *Bam*HI sites of pUC19. To overproduce PopR, pET28*popR* was constructed by inserting the 475 bp fragment including the *S. lividans* 1326 *popR* coding sequence obtained by PCR amplification with primers Reg1 and Reg3 between the *Nde*I and *Bam*HI sites of pET28a. This construction led to an N-terminal translational fusion between a peptide of 20 amino acids containing six His residues and PopR under the control of the strong inducible T7 promoter. To overexpress *popR* in *Streptomyces*, pJV100 was constructed. pJV100 is the 780 bp fragment including the promoter region and *popR* gene obtained by PCR amplification with primers Ju46 and Reg3 inserted into the blunted *Bam*HI site of pUWL219. In this construction, *popR* is under the control of its own promoter on a multicopy vector. An *oriT* fragment was inserted into the *Pst*I site to allow conjugation from *E. coli* to *S. lividans*.

Table 1. Primers used in this study.

Ju 13	5′-GGCCGGAAGCCGTGGTCTGCTCGGCGTCGA-3′
Ju 16	5′-CGGCTCCTGGCGCCAGTCGAT-3′
Ju 26	5′-GTCGACCGGCTGGCCGAGG-3′
Ju 40	5′-GAAGAATTCGCGGGTGACGGCGCCGATGCC-3′
Ju 42	5′-CCGCTGGTCGAGCAGCTGCTCGG-3′
Ju 43	5′-GGAGGATCCCTGGCCGCCGCCGCGGGCGCC-3′
Ju 46	5′-GCTGCGGGGGTCCACGACGTC-3′
Ju 49	5′-GGAGGATCCCGGCGCTCCTTGTCGAGTCGTG-3′
Ju 50	5′-GAAGAATTCCTCCTCAGCCTAGGGTTTCGGCC-3′
Ju 51	5′-AGGTGGTGAATCCGTCGGTGCTC-3′
Ju 54	5′-GCAGCGGAATGACGCGGGCTTCG-3′
Ju 64	5′-GGAGGATCCACGCGCCCGGGGGCGCGGCCC-3′
Ju 66	5′-AAGAAGCTTCTAGTGTGCCGGATCAGCTCGCCCTG-3′
Reg1	5′-CATCATATGACCAGCCACGTGCCGAACGAAG-3′
rX2′	5′-GCGGCCTTCGCTCCGGGGGAACC-3′
Reg3	5′-GGAGGATCCTCAGGCGGCCAGGCACATCCCGTC-3′

The *clpP3* gene was disrupted as follows. *clpP3* was PCR amplified using oligonucleotides Ju40 and Ju43 and cloned into pUC19. The resulting plasmid was digested with *Sfi*I, blunt ended by Vent polymerase treatment and ligated with the blunt-ended *Hin*dIII cassette from pHP45Ωkan (Blondelet-Rouault *et al*., 1997) containing the neomycin–kanamycin resistance gene (kan). The *Eco*RI–*Bam*HI *clpP3*::*kan* blunt-ended fragment was cloned into pGM160Δ at the blunt-ended *Bam*HI site to give plasmid pJV42. Treatment and selection of *clpP3* mutants by double recombination was as described by de Crecy-Lagard *et al*. (1999).

The strategy for disrupting *popR* was the following. A 300 bp *popR* internal fragment was PCR amplified using oligonucleotides Ju64 and Ju66. It was *Bam*HI–*Hin*dIII-digested and inserted at the corresponding sites in pGM160Δ to give pJV108. Precultures of the *S. lividans clpP1* mutant transformed with pJV108 were agitated for 3 days at 40°C and plated to give isolated colonies on NE supplemented with thiostrepton.

Heat shock induction in Streptomyces

Cultures of *S. lividans* 1326 wild type or *clpP1* mutant grown for 24 or 30 h in YEME were subjected to 20 or 60 min treatment at 40°C or kept at 30°C. mRNA was extracted from these cultures and analysed.

RNA extraction

A volume of 10–15 ml of *S. lividans* 1326 or *S. lividans* 1326 *clpP1*::*Am*^{R} culture was pelleted. Cells were resuspended in 0.5 ml of cold deionized water and added to 0.5 g of glass beads (212–300 μm; Sigma), 0.4 ml of 4% Bentone (Rheox)

and 0.5 ml of phenol–chloroform–isoamyl alcohol, pH 8.0 (Amresco). The cells were disrupted in a Fastprep disintegrator (Savant; Bio101) for 30 s at 4°C. After centrifugation for 8 min at 4°C and 14 000 r.p.m., supernatants were collected, treated with phenol–chloroform (1:1, v/v) and then with chloroform–isoamyl alcohol (24:1, v/v). RNA was precipitated at −20°C with 850 μl of isopropanol in the presence of 0.2 M NaCl and resuspended in 20 μl of cold deionized water. RNA concentrations were determined by measuring absorbance at 260 nm.

Primer extension experiments

Primer extensions were performed with purified oligodeoxynucleotides Ju 26 and Ju 42 (see Table 1) on the *clpP1* mRNA and *clpP3* mRNA, respectively, and with purified oligodeoxynucleotides Ju 13 and Ju 16 on the *groES* mRNA and *glk* mRNA respectively. Primer extensions were also performed on *popR* mRNA with oligonucleotides Ju 54 and rX2′. The synthetic primer (10 pmol) was 5′ end labelled with 5 μl of [γ-^{32}P]-ATP (3000 Ci mmol^{-1}) using T4 polynucleotide kinase. RNA (40 μg) and 1 pmol of labelled oligonucleotide were annealed in a final volume of 17 μl of 1 × AMV reverse transcriptase buffer (Boehringer Mannheim) for 3 min at 65°C. The mixture was then frozen for 1 min in dry ice and thawed in ice. Twenty-five units of AMV reverse transcriptase (Boehringer Mannheim) and 2 μl of 20 mM dNTP were added. After 30 min of incubation at 42°C, 1 μg of RNase was added, and the mixture was incubated for 10 min at 37°C. The reactions were stopped by adding 5 μl of a loading solution containing 97.5% deionized formamide, 10 mM EDTA, 0.3% xylene cyanol and 3.3% bromophenol blue. Samples were loaded on 6% acrylamide urea sequencing gels.

Overproduction and purification of PopR

pET28*popR* containing the translational fusion 6 His/*popR* under the control of the T7 promoter was introduced into the *E. coli* BL21λDE3 strain in which the T7 RNA polymerase gene is under the control of the IPTG-inducible *lacUV5* promoter. The resulting strain was grown at 37°C in LB medium containing 25 μg ml^{-1} kanamycin until the culture reached an OD_{600} of 0.9; then IPTG was added to a final concentration of 1 mM. Incubation was pursued for 6 h at room temperature. Cells were centrifuged for 10 min at 5000 r.p.m., and the pellet was resuspended in 1/50th of the culture volume of buffer 1 (50 mM $NaPO_4$, pH 8, 300 mM NaCl, 20 mM imidazole). Cells were disrupted by sonication, and cell debris was pelleted by centrifugation for 20 min at 12 000 r.p.m. The *E. coli* crude extract was loaded on a 100 μl Ni-NTA agarose (Qiagen) column previously equilibrated with buffer 1. The column was washed abundantly with buffer 2 (50 mM $NaPO_4$, pH 6, 300 mM NaCl, 30 mM imidazole), and the PopR protein was eluted with an imidazole gradient (30–500 mM). The eluted product was dialysed overnight at 4°C in 50% glycerol, 50 mM $NaPO_4$, pH 6.5, and 300 mM NaCl. The 20 residues brought by the translational fusion were removed by thrombin digestion, which cleaves a specific site just upstream from the *popR* coding sequence, and the thrombin was eliminated as recommended by the manufacturer (Thrombin Cleavage Capture Kit; Novagen).

Total or purified protein extracts were resolved by SDS−15% PAGE according to the method of Laemmli (1970). The protein concentration was determined by the method of Bradford (1976).

Gel mobility shift DNA-binding assays

A 330 bp *Eco*RI–*Bam*HI DNA fragment PCR amplified with primers Ju 40 and Ju 49 (see Table 1), corresponding to the *clpP3* promoter region, and a 310 bp *Eco*RI DNA fragment PCR amplified with primers Ju 46 and Ju 50, corresponding to the *popR* upstream region, were end labelled with [α-^{32}P]-dATP using the Klenow fragment of DNA polymerase I (Gibco-BRL). DNA fragments were then purified using the Qiaquick PCR purification kit (Qiagen). Radiolabelled fragments (10 000 c.p.m.) and various quantities of PopR, from 0 to 200 ng, were incubated for 15 min at 30°C in a 10 μl reaction containing 1 μg of calf thymus (non-specific competitor) DNA and the reaction buffer [25 mM $NaPO_4$, pH 7, 150 mM NaCl, 0.1 mM EDTA, 2 mM $MgSO_4$, 1 mM dithiothreitol (DTT), 10% glycerol]. Samples were then loaded on a 6% polyacrylamide gel (50 mM Tris, 400 mM glycine, 1.73 mM EDTA), and electrophoresis was performed under 100 V tension for 1 h.

DNase I footprint

Purified synthetic primers Ju 42 and Ju 51 were end labelled using [γ-^{32}P]-dATP and the T4 polynucleotide kinase. The *clpP3* promoter region was PCR amplified using end-labelled primers Ju 42 and Ju 51 (20 pmol) and *Pfu* polymerase (Stratagene). Fragments were then purified using the Qiaquick PCR purification Kkit (Qiagen). In a final volume of 50 μl, PopR (1 μg) and end-labelled fragments (50 000 c.p.m.) were incubated for 20 min at room temperature in the presence of 5 μg of calf thymus DNA, 1 μg of BSA and 1 × reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, 4% glycerol). Then, 5 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ were added, followed by 5 ng of DNase I and the mixture was incubated for 1 min. The reaction was stopped with 140 μl of stop buffer (0.4 M sodium acetate, pH 6.7, 2.5 mM EDTA, 50 μg ml^{-1} calf thymus DNA) and 200 μl of phenol. DNA was precipitated with ethanol, resuspended in the loading solution and run on gels as described above for primer extensions. Samples were run alongside a G+A sequencing reaction performed according to the method of Maxam and Gilbert (1980).

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