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ClpP-dependent degradation of PopR allows tightly regulated expression of the *clpP3 clpP4* operon in *Streptomyces lividans*

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Summary

Five *clpP* genes have been identified in *Streptomyces coelicolor*. The *clpP1* and *clpP2* genes form one operon, the *clpP3* and *clpP4* genes form another, and *clpP5* is monocistronic. Previous studies in *Streptomyces lividans* have shown that the first operon (*clpP1 clpP2*) is required for a normal cell cycle. Expression of the second operon (*clpP3 clpP4*) is activated by PopR if the first operon is non-functional. We show here that PopR degradation is primarily dependent on ClpP1 and ClpP2, but can also be achieved by ClpP3 and ClpP4. The carboxy-terminus of PopR plays an essential part in the degradation process. Indeed, replacement of the last two alanine residues by aspartate residues greatly increased PopR stability. These substitutions did not impair PopR activity and, as expected, accumulation of the mutant form of PopR led to very strong expression of the *clpP3 clpP4* operon. Increased PopR levels led to delayed sporulation. The results obtained in this study support the notion of cross-processing between ClpP1 and ClpP2.

Introduction

Energy-dependent proteases play a key role in cells, degrading non-functional proteins and specific short-lived regulators. Several ATP-dependent proteases have been characterized in *Escherichia coli*, including Lon, FtsH, HslUV and Clp (Gottesman, 1996). The proteolytic Clp complex consists of two types of subunit: the proteolytic ClpP subunit and the ATPase subunit, ClpA or ClpX. ClpP subunits are organized into two superimposed heptameric rings, which form a central chamber. Amino acid residues Ser-97, His-122 and Asp-171 of the 14 catalytic triads are located within the proteolytic chamber (Maurizi *et al.*,

1990a; Wang *et al.*, 1997). A hexamer of ATPase subunits binds to one or both ends of the tetradecamer (Grimaud *et al.*, 1998), resulting in the recognition, unfolding and translocation to the proteolytic chamber of the substrate (Weber-Ban *et al.*, 1999; Kim *et al.*, 2000; Ishikawa *et al.*, 2001). Clp proteases are not only involved in the degradation of misfolded proteins (Frees and Ingmer, 1999; Krüger *et al.*, 2000), but also in the degradation of specific regulators. For example, the starvation sigma factor σ^S in *E. coli* (Schweder *et al.*, 1996), the CtrA response regulator and the McpA chemoreceptor in *Caulobacter crescentus* (Jenal and Fuchs, 1998; Tsai and Alley, 2001) are degraded by ClpXP, whereas the ComK transcriptional regulator (Turgay *et al.*, 1998) and the CtsR class three heat shock gene repressor in *Bacillus subtilis* are degraded by ClpP (Derré *et al.*, 2000) associated with the ClpC HSP100 ATPase subunit (Krüger *et al.*, 2001). Another set of Clp targets has been described in *E. coli* and *B. subtilis*: the SsrA-tagged proteins. SsrA (small stable RNA) functions as both a tRNA and an mRNA. The mRNA encodes a small peptide that is added co-translationally to truncated proteins. These tagged polypeptides are then targeted for degradation (for a review, see Karzai *et al.*, 2000). ClpAP in *E. coli* and ClpXP in *E. coli* and *B. subtilis* are among the proteases that ensure the degradation of these SsrA-tagged proteins in the cytoplasm (Gottesman *et al.*, 1998; Wiegert and Schumann, 2001). Several studies have highlighted the importance of the carboxy-terminal sequence of various substrates for recognition by ClpX. This is the case for the SsrA-tagged proteins, the MuA transposase in *E. coli* (Levchenko *et al.*, 1997) or the McpA chemoreceptor in *C. crescentus* (Tsai and Alley, 2001). However, in the case of the bacteriophage λ O replication protein, it is the N-terminus that carries the recognition signal (Gonciarz-Swiatek *et al.*, 1999). ClpA has been shown to recognize the N-terminal extremity of RepA (Hoskins *et al.*, 2000) and the N-terminus of the molecule subjected to the N-end rule (Tobias *et al.*, 1991).

Streptomyces are Gram-positive soil bacteria with a high G+C content. They present a particularly complex growth cycle, during which numerous secondary metabolites are produced. Indeed, bacteria of the *Streptomyces* genus produce 70% of all commercially available antibiotics. The genome of this bacterium has now been entirely

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sequenced (http://www.sanger.ac.uk/Projects/S_coelicolor/). It is 8.7 Mb long, almost twice the length of the *E. coli* genome. Several multigene families have been identified. For example, five *clpP*-like genes have been identified in *Streptomyces coelicolor*, whereas only one *clpP* gene is present in most bacterial genomes. These *clpP*-like genes are organized into two apparent operons, one corresponding to *clpP1 clpP2* and the other to *clpP3 clpP4*, and a monocistronic transcription unit corresponding to *clpP5*. ClpP4 has a modified catalytic triad, in which the His residue is out of alignment, and the Ser residue of ClpP5 is shifted out of the consensus alignment by one position. This raises questions concerning the possible role of ClpP4 and ClpP5 as bona fide ClpP proteases. We studied this gene family in *Streptomyces lividans*, a species very closely related to *S. coelicolor*. It has been shown that a mutation in *clpP1* leads to growth cycle alteration. Both *clpP1* and *clpP2* are required to restore the wild-type phenotype, leading to the suggestion that the *clpP1* mutation has a polar effect on *clpP2* and that *clpP1* and *clpP2* form an operon (de Crecy-Lagard *et al.*, 1999). As for *clpP3* and *clpP4*, the putative translation initiation codon of *clpP4* is 1 bp downstream from the *clpP3* stop codon, which suggests that these two genes are organized as an operon. The study of this multigene family recently led to the characterization of interactive regulation between the two apparent operons. Indeed, the *clpP3* gene is silent in the wild-type strain, but its expression was strongly induced in the *clpP1* mutant strain. This induction has been shown to be mediated by an activator named PopR (Viala *et al.*, 2000). Increased expression of the *clpP3* gene in *clpP1* mutants is not caused by induction of the activator because the level of *popR* transcription is low, but similar in both wild-type and *clpP1* strains (Viala *et al.*, 2000). We investigated *clpP3* induction in the *clpP1* mutant by analysing PopR stability in wild-type and *clpP1* bacteria. We found that ClpP operon products have a key role in PopR degradation. We also showed that the carboxy-terminal residues of PopR play an essential part in the degradation signal.

Results

PopR is stabilized in a clpP1 mutant

Previous results have shown that the *clpP3* operon is induced if the *clpP1 clpP2* operon is not functional. This induction is mediated by the PopR activator. However, the level of transcription of *popR* is similar in wild-type and *clpP1* mutant strains (Viala *et al.*, 2000), suggesting that PopR may be controlled at the post-transcriptional level. As the ClpP1 proteolytic subunit is involved in the induction of the *clpP3 clpP4* operon, this suggested that PopR may be subject to proteolysis. We therefore investigated

the stability of PopR. We carried out a Western blot with polyclonal antibodies directed against PopR, using crude extracts of various strains, *S. lividans* 1326 wild type (WT) and the *clpP1* mutant, as well as derivatives of these strains in which *popR* was overexpressed. We were unable to detect PopR in the wild-type strain (Fig. 1, lane 1), but detected a small amount of this protein in the *clpP1* mutant strain (Fig. 1, lane 2). Overexpression of *popR*, from a multicopy plasmid, under the control of its own promoter in strain WT (pJV100), allowed us to detect a weak signal corresponding to PopR (Fig. 1, lane 5). The introduction of the same construct into a *clpP1* mutant strain resulted in a strong signal (Fig. 1, lane 6). As expected, crude extracts from strains carrying the control vector, pUWL219, did not display this higher level of PopR (Fig. 1, lanes 3 and 4). Thus, PopR was preferentially detected in a *clpP1* mutant, indicating that PopR was stabilized in the absence of proteins encoded by the *clpP1* operon. These results also show that the machinery that degrades PopR is probably saturated if multiple copies of *popR* are present because, under these conditions, PopR was detected in the wild-type strain.

Complementation of the clpP1 mutant by the complete clpP1 operon restores PopR degradation

We complemented the *clpP1* mutant strain with one or both genes of the *clpP1 clpP2* operon and measured PopR levels. To facilitate PopR detection, we used the *clpP1* (pJV100) strain, which overexpresses *popR*, into which we introduced the integrative control vector, pHM11a, or one of the following constructs: pJV50, pJV51 or pJV52, carrying, respectively, the complete *clpP1*

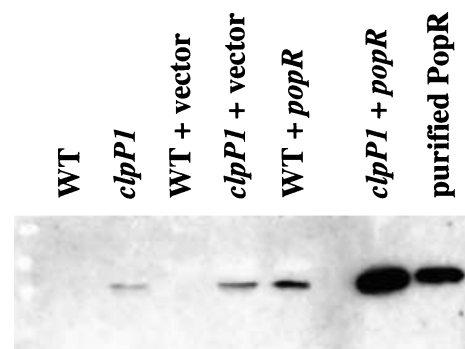


Fig. 1. Detection of PopR by Western blotting using 50 µg of crude extract of WT (lane 1), *clpP1* (lane 2), WT (pUWL219) (lane 3), *clpP1* (pUWL219) (lane 4), WT (pJV100) (lane 5), 20 µg of crude extract of *clpP1* (pJV100) (lane 6) and 5 ng of purified PopR (lane 7) and polyclonal anti-PopR antibodies (1:500). pUWL219 is the control multicopy vector, and pJV100 is the multicopy vector containing *popR*. An exposure time of 15 min was required to detect all the signals.

operon, the *clpP1* gene or the *clpP2* gene, under the control of the strong constitutive promoter, *P_{erm}*. As each of the two genes has several possible initiation codons (Fig. 2A), we generated constructs encoding proteins with N-termini chosen according to the codon usage rule, as described by de Crecy-Lagard *et al.* (1999). We therefore introduced a translation initiation codon downstream from *P_{erm}*, such that the sequences of the ClpP1 molecules encoded by pJV50 and pJV51 began with MTNLMPS, whereas the sequence of the ClpP2 molecule encoded by pJV52 began with MNDFPG. Complementation with the complete *clpP1 clpP2* operon restored PopR degradation (Fig. 2B, lane 2), but no degradation was observed if the mutant was complemented with either *clpP1* (Fig. 2B, lane 3) or *clpP2* (Fig. 2B, lane 4) alone. Thus, both ClpP1 and ClpP2 seem to be necessary for the degradation of PopR. This suggests that the functional proteolytic complex may be a heterologous complex consisting of both ClpP1 and ClpP2 or that ClpP1 and ClpP2 must interact in some way before acting separately.

Control of ClpP production and evidence for cross-processing of ClpP1 and ClpP2

The previously complemented *clpP1* (pJV100) strains harbouring pHM11a, pJV50, pJV51 or pJV53 were analysed by Western blot experiments with polyclonal anti-ClpP1

antibodies and compared with the wild-type strain. In wild type (Fig. 3A, lanes 1 and 2) and in *clpP1* (pJV100) carrying pJV50 (*clpP1* and *clpP2* genes; Fig. 3A, lane 3), two signals were detected at around 23 kDa. These two signals may correspond to ClpP1 and ClpP2 or to two forms of ClpP1, given that ClpP is autoprocessed in *E. coli* to release the first 14 N-terminal residues (Maurizi *et al.*, 1990b). In *clpP1* (pJV100) carrying pJV51 (*clpP1* gene), only the signal corresponding to the larger protein was observed (Fig. 3A, lane 4) and, in *clpP1* (pJV100) carrying pJV52 (*clpP2* gene), no signal was detected (Fig. 3A, lane 5), suggesting that the antibodies used were highly specific for ClpP1 or that ClpP2 was not produced. We hypothesized that the antibodies were highly specific for ClpP1 and that the lower molecular weight signal corresponded to a processed form of ClpP1. We carried out immunoprecipitation with antibodies against ClpP1, using crude extract from *clpP1* (pJV100) (pJV50), and the N-terminus of the smaller of the two proteins was sequenced. The N-terminal sequence was AGEPS. This corresponds to an internal sequence located eight amino acid residues downstream from the experimentally introduced translation initiation codon: MTNLMPSAAGEPS (Fig. 2A). This processed form of ClpP1 was detected only in the presence of the *clpP2* gene, i.e. in the wild type and the *clpP1* mutant carrying pJV50 (*clpP1* and *clpP2* genes). These data suggest that ClpP1 is not autoprocessed, but cross-

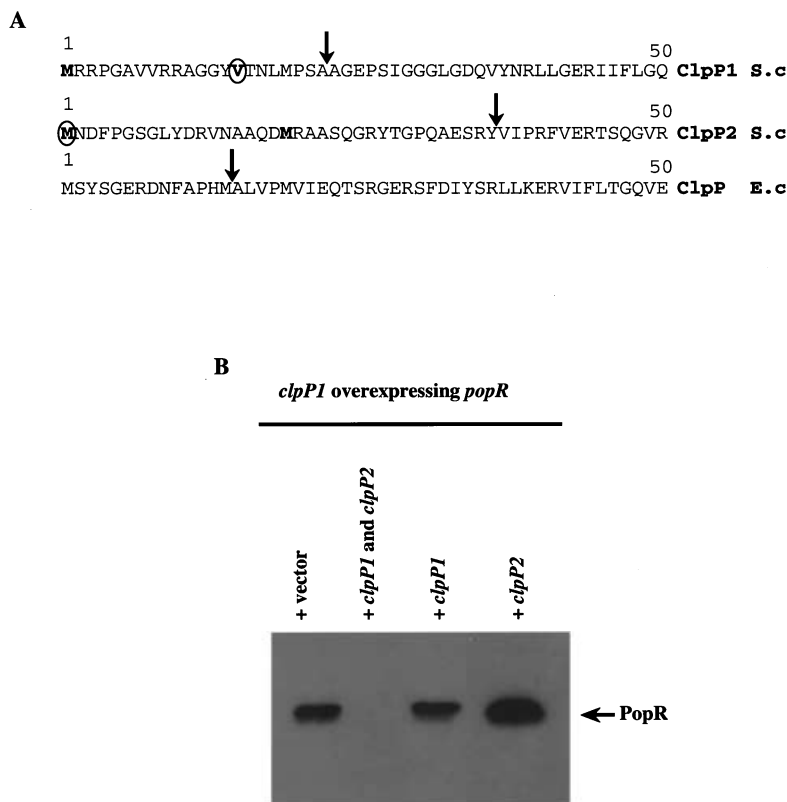


Fig. 2. Restoration of PopR degradation by complementation with ClpP1 and/or ClpP2. A. N-terminal sequences of *S. coelicolor* ClpP1 (ClpP1 S.c.), *S. coelicolor* ClpP2 (ClpP2 S.c.) and *E. coli* ClpP (ClpP E.c.). The putative *Streptomyces* ClpP initiation codons are shown in bold; the most likely according to the pattern of codon usage in *Streptomyces* are circled. Arrows indicate the position of the *Streptomyces* ClpP and *E. coli* ClpP processing sites (Maurizi *et al.*, 1990b; de Crecy-Lagard *et al.*, 1999; this study). B. Crude extracts (20 µg) of strain *clpP1* (pJV100), carrying the control vector pHM11a (lane 1), pJV50 overexpressing the *clpP1 clpP2* operon (lane 2), pJV51 overexpressing *clpP1* (lane 3) and pJV52 overexpressing *clpP2* (lane 4) were analysed by Western blotting with polyclonal anti-PopR antibodies (1:1000). pJV100 is the multicopy vector containing *popR*.

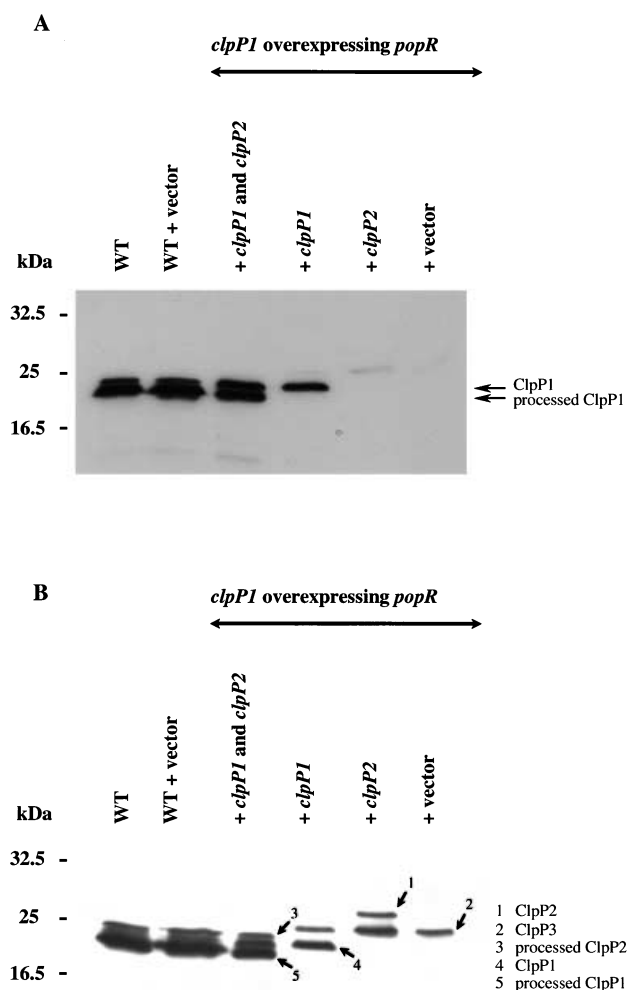


Fig. 3. Cross-processing of ClpP1 and ClpP2. A. Western blot analysis with anti-ClpP1 antibodies. B. Western blot analysis with anti-ClpP1 antibodies purified on ClpP2. Crude extracts (30 μ g) of WT (lane 1) and WT pHM11a (lane 2) and 3 μ g of crude extracts of *clpP1* (pJV100) carrying pJV50 (*clpP1 clpP2*) (lane 3), pJV51 (*clpP1*) (lane 4), pJV52 (*clpP2*) (lane 5) or pHM11a (control; lane 6) were analysed.

processed by its counterpart, ClpP2. The processed form of ClpP1 accumulated in the wild-type strain (Fig. 3A, lanes 1 and 2).

The absence of a ClpP2-specific signal (Fig. 3A, lane 5) suggested that the polyclonal anti-ClpP1 antibodies did not recognize ClpP2. We investigated whether this was indeed the case and assessed ClpP2 production by enriching the anti-ClpP1 antiserum in antibodies directed against ClpP2 epitopes by immunopurification on a ClpP2 column. The previous Western blot was analysed with these enriched antibodies (Fig. 3B). The signals corresponding to the native and processed forms of ClpP1 were again detected (Fig. 3B, lanes 1–4). However, new signals were clearly visible on the Western blot. An additional band appeared in lanes 4–6, in which we expected

to detect ClpP3 according to our results concerning PopR stability in these strains; this band was referred to as ClpP3. ClpP2 production from pJV52 (overexpressing *clpP2*) was clearly detected (higher molecular weight band in Fig. 3B, lane 5). The absence of this band from the wild type (Fig. 3B, lanes 1 and 2) and from the strain overexpressing *clpP1* and *clpP2* (Fig. 3B, lane 3) suggested that ClpP2 was processed. This is consistent with the characterization of a shortened ClpP2, with VIPRFV as its N-terminal sequence (de Crecy-Lagard *et al.*, 1999). We suggest that this processed ClpP2 is the new signal, the higher molecular weight band appearing in Fig. 3B but not in Fig. 3A, lanes 1–3. Finally, the absence of a processed form if only ClpP1 or ClpP2 is produced (Fig. 3B, lanes 4 and 5) suggests that these two proteases are cross-processed.

The PopR degradation signal includes the two C-terminal alanines

The substrate degradation motif recognized by the Clp ATP-dependent protease has not been identified in all cases, but is often located in the N-terminal or C-terminal amino acid residues. The C-terminal SsrA tag has been well characterized. The SsrA system adds a peptide tag to the C-terminus extremity of incomplete polypeptides on stalled ribosomes. These tagged polypeptides are thus targeted for proteolysis (Karzai *et al.*, 2000). This system is widespread among eubacteria, and the sequence tag is well conserved in the three last amino acid residues (<http://www.indiana.edu/~tmrna/>), Leu–Ala–Ala (Fig. 4A). The alanine residues are essential for the degradation signal (Gottesman *et al.*, 1998).

The PopR peptide sequence ends in Leu–Ala–Ala, suggesting that this sequence may act as an SsrA-like tag (Fig. 4A). We investigated the possible involvement of these residues in PopR stability by replacing the two alanine residues with two aspartates. This new gene, 'popR-DD', was inserted into the multicopy vector pUWL219, under the control of its own promoter, to give pJV110. This construct was produced in the same way as pJV100, which contains the 'popR-AA' gene. Crude extracts of the wild-type strain harbouring pUWL219 (control vector), pJV100 (*popR-AA*) or pJV110 (*popR-DD*) were prepared, and PopR levels were determined by Western blotting (Fig. 4). An exposure time of 15 s was sufficient to detect a strong PopR-DD signal (Fig. 4B, lane 3), but too short to detect PopR-AA (Fig. 4B, lane 2). PopR-AA was detected after exposure for a longer period of time (data not shown). If a large amount of PopR was present in the crude extract, a weak band was also detected at 32 kDa (Fig. 4B, lanes 3 and 6), probably corresponding to a dimeric form of PopR. Cross-linking experiments using glutaraldehyde and purified PopR

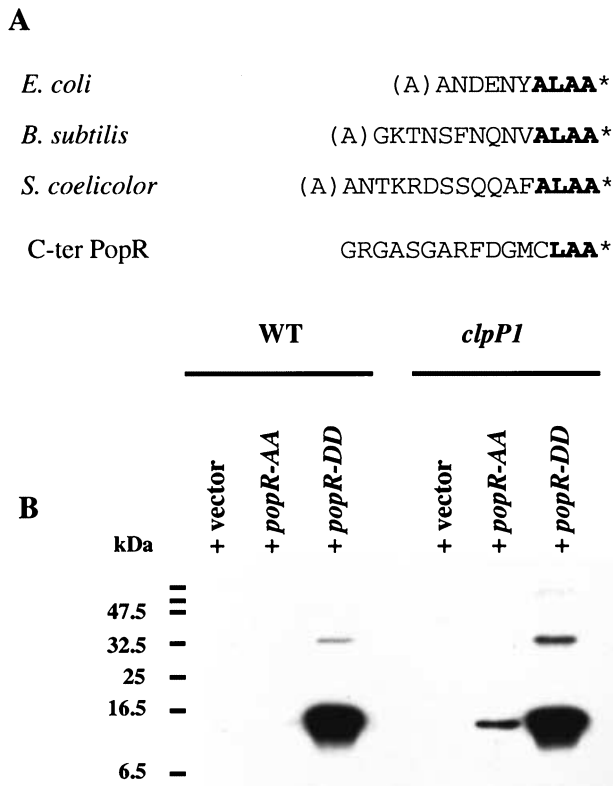


Fig. 4. Stabilization of PopR-DD with respect to PopR-AA in wild-type and *clpP1*.

A. Tag encoded by *ssrA* in *E. coli*, *B. subtilis*, *S. coelicolor* and C-terminal extremity of PopR.

B. Crude extracts from WT carrying pUWL219 (control; 50 µg) (lane 1), pJV100 (*popR*-AA; 50 µg) (lane 2), pJV110 (*popR*-DD; 20 µg) (lane 3) and crude extracts from *clpP1* harbouring pUWL219 (control; 50 µg) (lane 4), pJV100 (*popR*-AA; 20 µg) (lane 5) and pJV110 (*popR*-DD; 20 µg) (lane 6) were analysed by Western blotting with polyclonal anti-PopR antibodies. To avoid saturation of the signal in lanes 3 and 6, we used a very short exposure time (15s).

indicated that PopR was able to dimerize (data not shown).

Finally, Western blot experiments indicated that PopR-DD accumulated to very high levels in the wild type, so the two last alanine residues at the C-terminus are indeed essential to the degradation signal of PopR, as in the *ssrA* tagging system.

Other proteases recognize the PopR degradation signal

We compared the levels of PopR-AA and PopR-DD in a *clpP1* genetic background, assuming that PopR-AA and PopR-DD levels should be similar if ClpP1 and ClpP2 were entirely responsible for PopR degradation. Crude extracts of the *clpP1* mutant strain harbouring pUWL219 (control vector), pJV100 (*popR*-AA) or pJV110 (*popR*-DD) were prepared, and the amount of PopR was determined by Western blotting (Fig. 4). PopR-DD levels were similar

in the wild type and in the *clpP1* mutant (Fig. 4 lanes 3 and 6). However, for equivalent amounts of protein loaded, the PopR-DD signal (Fig. 4 lane 6) was considerably stronger than the PopR-AA signal (lane 5) in the *clpP1* mutant.

This result suggests that other proteases also recognize the Ala-Ala motif on PopR for the degradation of this regulator.

ClpP3 and ClpP4 degrade PopR

As shown above, ClpP1 and ClpP2 are not the only proteases involved in controlling PopR levels within the cell. Likely candidates include ClpP3 and ClpP4. We therefore complemented the *clpP1* (pJV100) strain, which overexpresses *popR*, with pJV41 carrying the *clpP3 clpP4* operon downstream from the strong promoter *Perm*. Crude extracts were analysed by Western blot experiments with anti-PopR antibodies (Fig. 5). Efficient, but not total, PopR degradation was observed (Fig. 5, lane 3). Control experiments with the vector pHM11a (Fig. 5, lane 1) or with pJV50 carrying *clpP1* and *clpP2* (Fig. 5, lane 2) showed accumulation or total PopR degradation respectively.

These results indicate that ClpP3 and ClpP4 can also participate in PopR degradation, controlling their own synthesis through a negative feedback loop.

Phenotype linked to PopR accumulation

The modified protein, PopR-DD, was more stable than the

clpP1 overexpressing *popR*

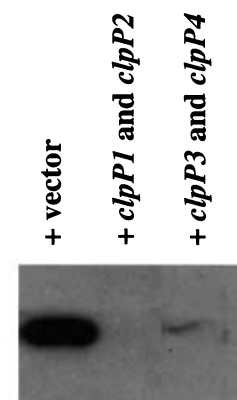


Fig. 5. Degradation of PopR by ClpP3 and ClpP4. Crude extracts (20 µg) of *clpP1* (pJV100), carrying the control vector pHM11a (lane 1), pJV50 overexpressing the *clpP1 clpP2* operon (lane 2) or pJV41 overexpressing the *clpP3 clpP4* operon (lane 3) were run on a 15% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, and PopR levels were analysed by probing with anti-PopR antibodies (1:1000).

wild-type protein. We therefore expected the level of *clpP3* expression to be higher unless the amino acid substitution impaired PopR activity. We assessed *clpP3* expression by primer extension experiments with mRNA extracted from the wild-type strain overexpressing *popR-DD*. Very strong expression was observed (data not shown), indicating that the PopR-DD form was active. The WT pJV110 strain expressing *clpP1 clpP2* and overexpressing *clpP3 clpP4* presented reduced red pigmentation (Fig. 6A) and slight growth retardation on solid R5 medium. Indeed, the aerial mycelium began to appear within 48 h in the wild-type strain harbouring the control vector (pUWL219) or multiple copies of *popR-AA* (pJV100) but not in the strain harbouring multiple copies of *popR-DD* (pJV110) (Fig. 6A). After 1 week, the wild type was sporulating, whereas the strain overexpressing *popR-DD* was forming aerial mycelium (Fig. 6B). The *clpP1* mutant strain in which *popR-DD* was overexpressed also presented reduced red pigmentation with respect to the control strains. Interestingly, some of the bacteria appeared to undergo differentiation after 1 week (Fig. 6C). These differentiated structures, which did not complete sporulation, appeared preferentially in the mass of inoculum rather than at the periphery and did not spread throughout the population.

Which ATPase is involved in PopR recognition?

In *Streptomyces*, three ATPases, ClpX, ClpC1 and ClpC2, could potentially associate with ClpP proteins to form the proteolytic complex. As substrate recognition is controlled by the ATPase subunit, we considered it interesting to determine which ATPase worked with ClpP in PopR degradation. The construction of a *clpX* mutant in *S. lividans* (J. Viala and P. Mazodier, submitted) enabled us to carry out *in vivo* analysis of the role of ClpX in PopR degradation. Thus, PopR was detected by Western blotting, and *clpP3* expression was analysed by primer extension in a *clpX* mutant. PopR did not accumulate in this genetic background, and *clpP3* expression was not induced (data not shown), in contrast to what was observed in a *clpP1* mutant. Thus, ClpX does not seem to be involved in the degradation of PopR, which suggests that one or both the ClpC ATPases may play a role in this process. Unfortunately, we could not test the involvement of ClpC1 in PopR degradation given the failure to obtain a mutation in this gene (de Crecy-Lagard *et al.*, 1999).

Discussion

Previous studies have shown that *clpP3* expression is induced in the *clpP1* genetic background and that the PopR activator mediates this induction. PopR binds specifically to the *clpP3* promoter and does not autoregulate its own synthesis (Viala *et al.*, 2000). Here, we

show that the *clpP3* induction observed in a *clpP1* mutant resulted from the stabilization of PopR in this genetic background. The degradation of PopR is ClpP1 ClpP2 dependent, but can also be carried out by ClpP3 and ClpP4. This observation points to the existence of an interactive network within the *clpP* multigenic family. PopR is the first target of a Clp protease to be described in *Streptomyces*.

The degradation of PopR requires a signal corresponding to the C-terminal amino acid residues Leu-Ala-Ala. Indeed, replacement of these two carboxy-terminal alanine residues by two aspartate residues greatly increases the stability of PopR. These features are reminiscent of those of the SsrA system. The most highly conserved part of the SsrA tag is the C-terminal Leu-Ala-Ala motif, and the two alanine residues are crucial for degradation by the Clp protease (Gottesman *et al.*, 1998). Thus, PopR is a naturally tagged protein. Such dependence on the presence of two alanine residues at the C-terminus has been described for the proteolysis of CtrA, a general regulator in *C. crescentus* (Domian *et al.*, 1997). Are two alanine residues at the C-terminus sufficient for degradation by ClpP1 and ClpP2 in *Streptomyces*? Apparently not, as the Lon protein, which ends in V-A-A at the C-terminus, does not accumulate in a *clpP1* mutant (A. Bellier, unpublished results). Two alanine residues at the carboxy-terminus therefore seem to be necessary but not sufficient for degradation. This raises questions as to what else might be required for degradation: the Leu residue? Flynn *et al.* (2001) have studied recognition determinants within the 11-residue SsrA tag for degradation by ClpXP or ClpAP in *E. coli*. The tripeptide Leu-9-Ala-10-Ala-11 is very important for degradation by ClpXP. Furthermore, the tripeptide is sufficient to mark a protein as a substrate for degradation in some, but not all, cases. This suggests that other sequence features or structural characteristics could have an effect. The Leu-9 residue has also been shown to play an important role for degradation by ClpAP in *E. coli*, in addition to Ala-1, Ala-2, Ala-8 and Ala-10.

The *clpP1* mutant was complemented with the *clpP1* and/or the *clpP2* genes, and the level of PopR was assessed in these strains. Both ClpP1 and ClpP2 were required for PopR degradation. There are several possible explanations for this. First, it is possible that the only functional complex is made up of ClpP1 and ClpP2. Secondly, ClpP1 and ClpP2 may need each other to generate a mature protein and to form a complex together. Thirdly, ClpP1 and ClpP2 may need to interact to generate a mature form before acting independently. We investigated whether this was the case by producing the mature form of ClpP1 directly from pJV54, and the potential mature form of ClpP2 described by de Crecy-Lagard *et al.* (1999) from pJV53. These plasmids were used in

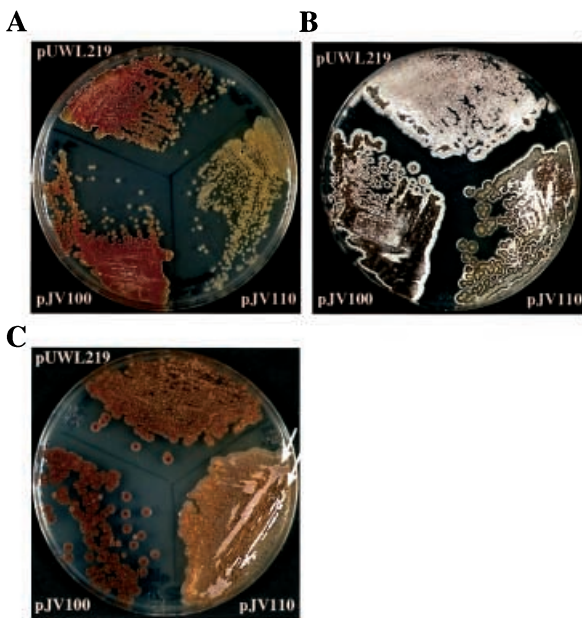


Fig. 6. Phenotype on R5 plates of *S. lividans* WT, after 2 days (A) or after 1 week (B) and *clpP1* after 1 week (C). The strains carried plasmids pUWL219 (control), pJV100 (*popR*-AA) or pJV110 (*popR*-DD). White arrows indicate differentiated patches in *clpP1* (pJV110).

complementation experiments, and PopR stability was assessed. PopR was not degraded, but ClpP1 and ClpP2 were not produced from these constructs in large amounts (data not shown). Furthermore, the ClpP1 and ClpP2 proteins produced may be non-functional, as the proregion of proteases is commonly involved in the proper folding of the mature protease (Baker *et al.*, 1993). To date, the role played by the ClpP propeptide is unclear. In *E. coli*, ClpP autoprocesses the amino-terminal 14-amino-acid propeptide. Cleavage requires an intact active site, and it has been shown that mutations modifying the active site result in the accumulation of an unprocessed form of ClpP. The unprocessed ClpP is able to self-

associate, and *in vitro* experiments have shown that this molecule also interacts with ClpA. However, as the mutation affects the active site, the activity of this unprocessed form could not be tested (Maurizi *et al.*, 1990a). The unprocessed form was analysed by electron microscopy, and the internal cavity was found to be filled or occluded by the propeptide (Kessel *et al.*, 1995).

ClpP proteins are thought to act with an ATPase in the protein degradation process. No induction of *clpP3* expression was observed in the *clpX* mutant, and PopR degradation persisted, indicating that ClpX is not essential for PopR recognition. This suggests that the ClpC ATPases may play a key role.

It was also shown that ClpP3 and ClpP4 could be involved in PopR degradation. Hence, ClpP3 and ClpP4 could exert a negative feedback control on their own synthesis. Interestingly, we observed complete degradation of PopR by ClpP1 and ClpP2, but slightly incomplete degradation of PopR by ClpP3 and ClpP4 (Fig. 5). This feature, if it is physiological, may correspond to tight post-translational control of PopR action by allowing total silencing of the *clpP3 clpP4* genes in the presence of functional *clpP1 clpP2* genes and adapted expression of *clpP3 clpP4* under specific conditions.

These results led us to suggest a model of regulation for *clpP3* operon expression (Fig. 7). In the wild-type strain, ClpP1 and ClpP2 proteins are produced and form a proteolytic complex with a Clp ATPase. This complex degrades the transcriptional activator PopR required for the expression of *clpP3*, preventing the expression of *clpP3* and *clpP4*. In a *clpP1* mutant, ClpP1 and ClpP2 are not produced, and PopR accumulates in the cell. PopR binds to the promoter region of the *clpP3* operon and activates its transcription. The ClpP3 and ClpP4 proteins are produced, form a proteolytic complex and degrade PopR, leading to a steady-state equilibrium of ClpP3 and ClpP4 within the cell.

In *E. coli*, redundant activities have been demonstrated between ATP-dependent proteases. Thus, SsrA-tagged

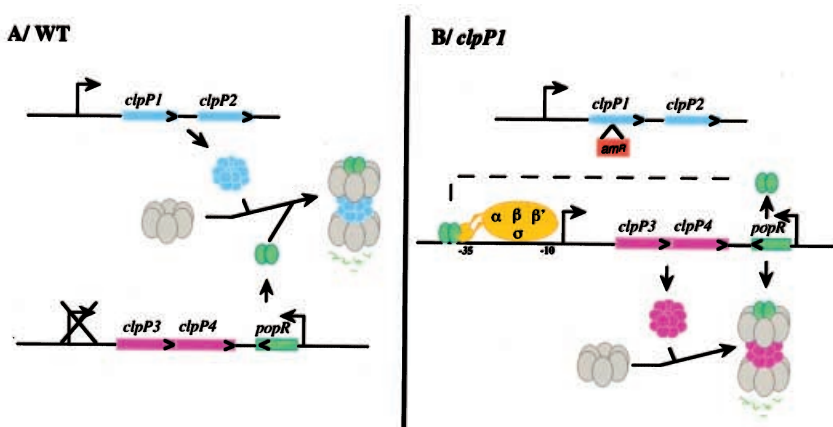


Fig. 7. Model of regulation of *clpP3* operon. A. PopR degradation by ClpP1 and ClpP2 proteins forming a proteolytic complex in a wild-type strain.

B. Stabilization of PopR and activation of *clpP3* and *clpP4* in the *clpP1* mutant strain.

proteins are essentially degraded by ClpP proteases, but they can also be removed by FtsH (Herman *et al.*, 1998); the σ^{32} sigma factor is degraded by FtsH but also by ClpYQ (Kanemori *et al.*, 1997), and Lon and ClpYQ share a common substrate. Indeed, the cell division inhibitor SulA, normally degraded by Lon, is also a substrate of ClpYQ (Kanemori *et al.*, 1999). Although ClpYQ ensures this activity, the *lon* mutant displays UV sensitivity resulting from the stabilization of SulA. This phenotype can be abolished by ClpYQ overproduction (Wu *et al.*, 1999). Here, ClpP3 and ClpP4 also appear to be partially redundant with ClpP1 and ClpP2, as they are able to degrade PopR but do not reverse the bald phenotype resulting from the *clpP1* mutation. Only strong overexpression of the *clpP3* operon in the *clpP1* mutant allows some bacteria to differentiate, as in the case of the *clpP1* mutant accumulating PopR-DD. The overproduction of ClpP3 ClpP4 is likely to lead to the degradation of some ClpP1-specific targets involved in differentiation. As ClpP3 and ClpP4 cannot replace ClpP1 and ClpP2 at the differentiation level, the question remains as to why the organism has established interactive regulation between the *clpP1* and *clpP3* operons. The most simple explanation may be that ClpP3 and ClpP4 ensure the minimal activity required for cell survival, as the double mutation in *clpP1* and *clpP3* could not be obtained in previous work, suggesting that at least one copy of the *clpP* operons is essential for cell viability (Viala *et al.*, 2000). The wild-type strain overexpressing *popR*-DD displays retarded growth. This may be because the large amounts of ClpP3 ClpP4 present in the wild-type strain overproducing these proteins compete with ClpP1 and ClpP2, thereby affecting growth.

Experimental procedures

Bacterial strains and media

Streptomyces lividans strain 1326 was obtained from the John Innes Culture Collection, and *S. lividans* 1326 *clpP1::Am^r* (de Crecy-Lagard *et al.*, 1999) and *S. lividans* 1326 *clpX::vio^r* (J. Viala and P. Mazodier, submitted) were constructed in this laboratory. YEME medium was used for liquid cultures (Hopwood *et al.*, 1985). Solidified NE medium (Murakami *et al.*, 1989) and R5 medium (Hopwood *et al.*, 1985) were used for *Streptomyces* cultures on plates. The antibiotics apramycin, viomycin, thiostrepton and hygromycin were added to final concentrations of 25, 30, 25 and 200 $\mu\text{g ml}^{-1}$, respectively, to solid medium, and to final concentrations of 20, 10, 10 and 50 $\mu\text{g ml}^{-1}$, respectively, to liquid medium.

Escherichia coli TG1 (Gibson, 1984) was used as the general cloning host. *E. coli* strains were grown in LB medium. Hygromycin and ampicillin were added to final concentrations of 200 and 100 $\mu\text{g ml}^{-1}$ respectively.

Plasmids and plasmid construction

The *E. coli*/*Streptomyces* shuttle vectors used were

Table 1. Primers used in this study.

Ju 41	5'-CATCATATGTCTCCATTACCCGCGGCCCC-3'
Ju 43	5'-GGAGGATCCCTGGCCGCGCCGCGGGGCC-3'
Ju 46	5'-GCTGCGGGGTCCACGACGTC-3'
Ju 62	5'-CATCATATGACGAATCTGATGCCCTCAGCCG-3'
Ju 63	5'-AAGAAGCTTTGCCGGGCCCTCGTCCGGG-3'
Ju 69	5'-TCAGTCGTCCAGGCACATCCCCTCGAACC-3'
Ju 77	5'-AAGAAGCTTTCCAGGCGCCGTCGCCCGCC-3'
Ju 78	5'-CATCATATGAACGACTTCCC CGCAGCGGC-3'
Ju 79	5'-CATCATATGATCCCGCGTTCGTCGAGCGC-3'
Ju 82	5'-CATCATATGGCCGCGAGCCCTCTATCGGT-3'

pUWL219, which contains the replication functions of the *Streptomyces* multicopy plasmid pJ101 (Wehmeier, 1995), and pHM11a, which allows a strong expression from the constitutive *Perm* promoter and contains an integration element directing its insertion into the *Streptomyces* genome at the mini-circle attachment site (Motamedi *et al.*, 1995).

We constructed pJV41 by inserting the 1465 bp fragment corresponding to the *S. lividans* 1326 *clpP3 clpP4* operon between the *NdeI* and *BamHI* sites of pHM11a. This fragment was obtained by polymerase chain reaction (PCR) amplification with oligonucleotides Ju 41 and Ju 43 (for oligonucleotides used, see Table 1). We constructed pJV50 by inserting the 1470 bp fragment corresponding to the *S. lividans* 1326 *clpP1 clpP2* operon between the *NdeI* and *HindIII* sites of pHM11a. This fragment was obtained by PCR amplification with oligonucleotides Ju 62 and Ju 63. pJV51 was constructed by inserting the 620bp fragment corresponding to the *S. lividans* 1326 *clpP1* gene between the *NdeI* and *HindIII* sites of pHM11a. This fragment was obtained by PCR amplification with oligonucleotides Ju 62 and Ju 77. We constructed pJV52 by inserting the 810bp fragment corresponding to the *S. lividans* 1326 *clpP2* gene with the first *clpP2* possible translation initiation codon between the *NdeI* and *HindIII* sites of pHM11a. This fragment was obtained by PCR amplification with oligonucleotides Ju 78 and Ju 63. We constructed pJV53 by inserting the 700 bp fragment encoding the putative mature form of the *S. lividans* 1326 *clpP2* gene between the *NdeI* and *HindIII* sites of pHM11a. This fragment was obtained by PCR amplification with oligonucleotides Ju 79 and Ju 63. pJV54 was constructed by inserting the 594 bp fragment corresponding to the *S. lividans* 1326 *clpP1* gene between the *NdeI* and *HindIII* sites of pHM11a. This fragment was obtained by PCR amplification with oligonucleotides Ju 82 and Ju 77.

pJV100 was constructed as described by Viala *et al.* (2000). pJV110 was constructed in the same way as pJV100, by inserting into the blunted *BamHI* site of pUWL219, the 780bp fragment including the promoter region and the *popR* gene, modified to encode two aspartic acid residues before the stop codon instead of two alanines. This fragment was obtained by PCR amplification with oligonucleotides Ju 46 and Ju 69.

DNA manipulation, transformation and conjugation procedures

Plasmid DNA was extracted from *E. coli* with a Qiagen kit. DNA fragments were purified from agarose gels with

Ultrafree-DA filters (Amicon-Millipore). Restriction enzymes were used as recommended by the manufacturers. DNA fragments were amplified by PCR (Mullis and Faloona, 1987; Saiki *et al.*, 1988). Standard CaCl₂ (Cohen *et al.*, 1972) or electroporation procedures were used for *E. coli* transformation.

Streptomyces protoplasts were prepared and transformed as described by Hopwood *et al.* (1985).

RNA extraction and primer extension experiments

RNA extraction and primer extension experiments were carried out as described previously (Viala *et al.*, 2000).

Protein extraction and Western blotting experiments

Cells were grown at 30°C. We collected 10 ml of culture and added 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM EDTA. Cells were pelleted, resuspended in 500 µl of 20 mM Tris, 5 mM EDTA, 1 mM dithiothreitol (DTT), 2× protease inhibitor cocktail (Roche Boehringer) and lysed by sonication. The resulting suspension was centrifuged for 30 min at 4°C, 12000 r.p.m., and the protein concentration of the supernatant was determined by the Bradford (1976) method. Various quantities of protein extract (10–50 µg protein) were subjected to SDS-PAGE as described by Laemmli (1970). The proteins were transferred to a nitrocellulose membrane (Hybond C), which was then probed with rabbit polyclonal anti-PopR (1:500–1:2000), anti-*Streptomyces* ClpP1 (1:10000) or anti-*Streptomyces* ClpP1 enriched against ClpP2 epitope (1:1000) antibodies, which were detected with the Super Signal detection kit (Pierce).

Glutaraldehyde cross-linking of PopR

We incubated 3 µg of purified PopR (Viala *et al.*, 2000) for 1 h at 37°C with 1 or 10 mM cross-linking reagent (glutaraldehyde) in a 10 µl reaction mixture consisting of 50 mM NaH₂PO₄, 50 mM NaCl and 10% glycerol. The reaction was stopped by adding SDS loading buffer; samples were boiled and subjected to SDS-PAGE. The gel was stained with Coomassie blue.

Immunoprecipitation of ClpP1

To immunoprecipitate ClpP1, anti-*Streptomyces* ClpP1 antibodies were covalently bound to a 1 ml Hi-Trap NHS-activated column (Pharmacia Biotech), as recommended by the manufacturer. The pellet of a 100 ml culture of *S. lividans* 1326 *clpP1* pJV100 pJV50 was resuspended in 5 ml of 20 mM Tris, 5 mM EDTA, 1 mM DTT and subjected to sonication. The resulting suspension was centrifuged. The crude extract (the supernatant) was then loaded onto the prepared column and incubated overnight at 4°C. The column was washed with 20 column volumes of 20 mM Tris, pH 7.5, and 20 column volumes of 20 mM Tris, pH 7.5, 500 mM NaCl. ClpP1 proteins were eluted with 4× 1 ml of 100 mM glycine/HCl, pH 2.5. Eluates were collected in tubes containing 100 µl of 1 M Tris, pH 9. The column was washed with

20 column volumes of 20 mM Tris, pH 8.8. The remaining proteins were eluted in 4× 1 ml of 100 mM triethylamine, pH 11.5. Eluates were collected in tubes containing 100 µl of 2 M Tris, pH 6.8. The column was then washed with 20 column volumes of 20 mM Tris, pH 7.5. The first acid eluate was used for N-terminal sequencing.

Purification of antibodies

Purified ClpP1 or ClpP2 was covalently bound to a 1 ml Hi-Trap NHS-activated column (Pharmacia Biotech), as recommended by the manufacturer. We diluted 2 ml of rabbit serum obtained by immunization with ClpP1 1:5 in 20 mM Tris, pH 7.5, and filtered the resulting solution. This preparation was loaded onto the prepared columns and incubated overnight at 4°C. Washing and elution were performed as described in the previous paragraph.

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