Post-translational control of the *Streptomyces lividans* ClgR regulon by ClpP

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It has been shown previously that expression of the *Streptomyces lividans* *clpP1P2* operon, encoding proteolytic subunits of the Clp complex, the *clpC1* gene, encoding the ATPase subunit, and the *lon* gene, encoding another ATP-dependent protease, are all activated by ClgR. The ClgR regulon also includes the *clgR* gene itself. It is shown here that the degradation of ClgR and Lon is ClpP1/P2-dependent and that the two C-terminal alanines of these new substrates are involved in their stability. The ClpC1 protein, which does not end with two alanines, is also accumulated in a *clpP1P2* mutant. The results presented here support the idea that ClpP1/P2 ensure post-translational control of ClgR regulon members, including ClgR itself.

INTRODUCTION

Proteolysis of intracellular proteins is crucial for all living organisms: protein quality control is required to quickly remove misfolded or damaged proteins and regulation of cellular processes is accomplished by degrading unstable key regulators of stress response, cell cycle or differentiation. In bacteria, different ATP-dependent proteases contribute to intracellular proteolysis: Clp (ClpAP, ClpCP, ClpXP), HslUV (or ClpYQ), Lon and FtsH (Gottesman, 1996). They all contain two distinct domains: an ATPase chaperone domain responsible for substrate recognition and unfolding, and a proteolytic domain. These domains can be either on the same polypeptide chain (Lon and FtsH) or on two different subunits: the ATPase subunits (ClpX, ClpA, ClpC or HslU) and the proteolytic subunits (ClpP or HslV).

Different substrates of Clp proteases in *Escherichia coli* have been identified. For many of them, Clp ATPases recognize the N- or C-terminal region of the substrate. The MuA transposase and the SsrA-tagged polypeptides, which are degraded by ClpXP, have a substrate recognition motif located at their C-terminal sequence (Gottesman *et al.*, 1997; Levchenko *et al.*, 1995). Recently, analysis of trapped ClpXP substrates revealed five distinct classes of ClpX-recognition motifs; one of these includes the C-terminal motif of SsrA-tagged proteins, ending with two alanine residues (Flynn *et al.*, 2003).

Clp proteases play an important role in biological functions of the Gram-positive soil bacteria of the genus *Streptomyces*, a model for bacterial differentiation with regard to its complex life cycle. On solid media, spore germination leads to growth of a basal mycelium which then differentiates into an aerial mycelium and finally septates and differentiates into spores. Two classes of mutants have been characterized: *bld* (bald) mutants which fail to produce aerial hyphae and

whi (white) mutants whose aerial hyphae fail to complete the production of normal, grey-pigmented spores (Chater, 2001). The *Streptomyces lividans* *clpP1clpP2* mutant has a *bld* phenotype and is therefore unable to complete the differentiation cycle (De Crecy-Lagard *et al.*, 1999). This suggests that one or several ClpP1 and/or ClpP2 targets need to be degraded for normal aerial mycelium formation. *clpP1* and *clpP2* form an operon and thus insertion of an apramycin resistance cassette in *clpP1* has a polar effect on *clpP2* expression. This was expected as restoration of differentiation in a *clp1* bald mutant requires the introduction *in trans* of both *clpP1* and *clpP2* (De Crecy-Lagard *et al.*, 1999). Polarity was confirmed by Western blot analysis of *clpP* expression; indeed, no ClpP1 nor ClpP2 can be detected in the *clpP1* mutant (Viala & Mazodier, 2002). Therefore, the *clpP1* mutant is referred to as the *clpP1P2* mutant to underline the absence of the two proteases.

Only one *S. lividans* ClpP1/P2 target has been identified to date: PopR, the transcriptional activator of the *clpP3P4* operon (Viala *et al.*, 2000). PopR is primarily degraded by ClpP1/P2 and the C-terminal two alanine residues play an essential part in the degradation process (Viala & Mazodier, 2002). Recently, we have shown that ClgR, which is encoded by a gene paralogous to *popR*, activates expression of *clpP1*, *clpP2*, *clpC1*, *lon* and *clgR* (Bellier & Mazodier, 2004). ClgR and PopR DNA-binding domain regions share over 50 % amino acid sequence identity and, like PopR, ClgR has two alanine residues at its C terminus. Therefore, ClgR seemed a good candidate as a substrate for ClpP1/P2.

We investigated ClgR stability in wild-type and *clpP1P2* mutant strains. We found that ClpP1/P2 is involved in ClgR degradation and that the two C-terminal alanines are required for degradation. We found that the degradation of Lon is also ClpP1/P2-dependent and that its degradation

also involves the two C-terminal alanine residues. Finally, we found that the product of *clpC1* accumulates in a *clpP1P2* mutant as well. ClgR thus activates *clpP1P2* gene expression, and ClpP1/P2 degrades several proteins encoded by genes of the ClgR regulon. Via specific proteolysis, ClpP1/P2 exerts a negative post-translational control on the ClgR regulon.

METHODS

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 our 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. Antibiotics apramycin, hygromycin and thiostrepton were added to final concentrations of 25, 100 and 25 µg ml⁻¹ to solid medium, and 20, 50 and 10 µg ml⁻¹ to liquid medium, respectively. Stock solutions of antibiotics rifampicin and chloramphenicol were made in 100% methanol at 20 and 10 mg ml⁻¹, respectively. They were added to final concentrations of 100 and 50 µg ml⁻¹, respectively, in solid medium. *E. coli* TG1 (Gibson, 1984) was used as the general cloning host and was grown in LB medium. Antibiotics ampicillin and hygromycin were added to final concentrations of 100 µg ml⁻¹.

DNA manipulation and transformation 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 PCR (Mullis & Faloona, 1987; Saiki *et al.*, 1988). Standard electroporation procedures were used for *E. coli* transformation. *Streptomyces* DNA and protoplasts were prepared and transformed as described (Hopwood *et al.*, 1985).

Plasmids and plasmid constructions. Primer sequences used in this study are given in Table 1. Plasmids pAB54 and pAB55 for *clgR* overexpression in *Streptomyces* have been described previously (Bellier & Mazodier, 2004). To overexpress *clpC1* in *Streptomyces*,

the *clpC1* gene was amplified with primers AB57 and AB58 and was inserted between the *NdeI* and *HindIII* sites of pHM11a. Two M2 Flag epitopes were added by inserting the linker resulting from annealing of primers AB118 and AB119 into the *NdeI* site, yielding pAB59M2. Correct insertion was checked with M2/*clpC1*-specific primer AB133. To overexpress *lon* in *Streptomyces*, pAB70M2 and pAB71M2 were constructed by inserting the 2350 bp fragment including the coding sequence of the *lon* gene obtained by PCR amplification with primers AB124 and AB125, and AB124 and AB95, respectively, inserted between the *NdeI* and *HindIII* sites of pHM11a. The N-terminal fusion with the tandem M2 flag was constructed by cloning the linker resulting from annealing primers AB118 and AB119 at the *NdeI* site. Insertion in the appropriate orientation was checked by hybridization with M2/*lon*-specific primer AB126. The PCR fragment contained in pAB71M2 modified the *lon* gene in such a way that two aspartic acid residues were encoded instead of two alanines before the stop codon. To express this modified *lon* gene in *Streptomyces* under its own promoter, pAB63 was constructed by inserting the 2550 bp fragment including the promoter region and the coding sequence of the *lon* gene obtained by PCR amplification with primers JU74 and AB96 between the *EcoRI* and *XbaI* sites of pSET152. In all the cases, the nucleotide sequences of the cloned DNA fragments were verified by sequencing.

RNA isolation from surface-grown cultures of *S. lividans*. Mycelia of *S. lividans* 1326, with or without pHM11a (control), pAB54 or pAB55, grown on cellophane discs were harvested with a spatula, dispersed in 2 ml water and pelleted. Cells were resuspended in 0.5 ml cold deionized water and added to 0.5 g glass beads (106 µm diam.; Sigma), 0.4 ml 4% Bentone (Rheox) and 0.5 ml phenol/chloroform/isoamyl alcohol, pH 8.0 (Amresco). The cells were disrupted in a Fastprep disintegrator (Bio 101) for 30 s three times at 4°C. After centrifugation for 2 min at 4°C and 20 800 g, the supernatants were collected, treated with phenol/chloroform (1:1, v/v) twice and then with chloroform/isoamyl alcohol (24:1, v/v) twice. Ethanol (250 µl) was added and the sample was applied to an RNeasy mini column (Qiagen) following the RNA clean-up protocol recommended by the manufacturers. The RNA sample was eluted in 35 µl deionized water. RNA concentrations were determined by measuring A₂₆₀.

Table 1. Primer sequences

Primer	Sequence (5'–3')
AB57	AAGCATATGTTTCGAGAGGTTACCGACC
AB58	AAGAAGCTTAGATCTTCACCGCCCTCTTCCCCGACG
AB95	AAGAAGCTTTCAGTCGTCGACCGGAACCTCACGCTCCGC
AB96	TCTTCTAGATCAGTCGTCGACCGGAACCTCACGCTC
AB118	TATGGACTACAAGGACGACGACGACAAGGACTACAAGGACGACGACGACAAGCA
AB119	TATGCTTGTCGTCGTCGTCCTTGAGTCCTTGTCGTCGTCGTCCTTGAGTCCA
AB124	CATCATATGGTCCTGCCCCGGCATGGTCGTC
AB125	AAGAAGCTTTCACGCTGCGACCGGAACCTC
AB126	GACGACGACAAGCATATGGTCCTGCCC
AB133	GACGACGACAAGCATATGTTTCGAGAGGTTTC
JU 74	GAAGAATTCTACGGCGGTGCTGTCCCGAGA
MG8	CAGCAGGTGGAGGAGATCATC
MG9	TCCGTGCCGATGTAGTTGTG
MG12	TGGGACAGTTCCTGCTCAG
MG13	CTCGGCGTGATCTTGATG
MG14	GAAGACCACCGCCAAGAAG
MG15	GTCGTCCTCGTCCTCGTC

Real-time quantitative PCR. Primers were designed with BEACON Designer software. *clpP1*, *clpC1* and *hrdB* expression were detected with primers MG12 and MG13, MG8 and MG9, and MG14 and MG15, respectively. RNA (10 µg) was treated twice with 30 U RNase-free DNase I (Roche) for 30 min at 37 °C. DNase was removed by applying the sample to an RNeasy mini column (Qiagen) following the RNA clean-up protocol. The RNA sample was eluted in 30 µl deionized water. cDNA synthesis was performed with random hexamers (Roche) using SuperScript II RT (Invitrogen) according to the protocol recommended by the manufacturers. Real-time quantitative PCR was performed in a 25 µl reaction volume containing cDNA, 12.5 µl SYBR PCR master mix (Applied Biosystems) and 1 µl gene-specific primers (10 µM). Amplification and detection of specific products were performed with the iCyclerIQ Multi-Colour real-time PCR detection system (Bio-Rad) with the following cycle profile: one cycle at 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 s. The specificity of the amplified product was verified by generating a melting curve with a final step of 80 cycles of 10 s at an initial temperature of 55 °C, increasing 0.5 °C each cycle up to 95 °C. Loss of fluorescence was observed at the denaturing/melting temperature of the product (Ririe *et al.*, 1997). To check whether contaminating chromosomal DNA was present, each sample was tested in control reactions that did not contain reverse transcriptase. For each condition, quadruple assays were done. The analysis gave a threshold cycle (CT) value for each sample, which is defined as the cycle at which a significant increase in amplification product occurs. The CT value was calculated for each quadruple reaction. A Δ CT value was then calculated for each sample by subtracting the mean CT value of the target gene from the mean CT value of the *hrdB* reference gene (*hrdB* encodes an essential and constitutively expressed σ factor; Kelemen *et al.*, 1996). The data were transformed from an exponential to a linear scale by using the formula $x = 2^{-\Delta CT}$ (Livak & Schmittgen, 2001).

Protein extraction and Western blotting experiments. Cultures of *S. lividans* 1326 carrying pHM11a (control), pAB54, pAB55, pAB70M2, pAB71M2 or pAB63 were grown on cellophane discs laid down on the surface of solid NE plates. Proteins were prepared from mycelia of the different strains at different stages of growth (basal mycelium, aerial mycelium, sporulation). Mycelium was resuspended in sonication buffer (20 mM Tris, 5 mM EDTA, 1 mM β -mercaptoethanol, 0.5 mM PMSF) and lysed by sonication. The resulting suspension was centrifuged for 15 min at 4 °C and 20 800 g, and the supernatant was treated with 0.3 % SDS for 5 min at 85 °C. The sample was centrifuged for 15 min at 4 °C and the protein concentration of the supernatant was determined by the method of Bradford (1976). Ten micrograms of protein extract was 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-*Streptomyces* ClgR (1:500) (Agro-Bio), anti-*Streptomyces* ClpP1 (1:10 000), anti-recombinant *Synechococcus* ClpC (1:1000) (Agrisera) (ClpC antibodies were raised against a 13 amino acid epitope of *Synechococcus* ClpC present in ClpC1 but not in other *Streptomyces* ClpC), anti-*Streptomyces* Lon (1:5000) or anti-Flag M2 mAbs (Sigma). Signals were detected with the ECL Western Blotting Detection Kit (Amersham Biosciences).

RESULTS

ClgR, a new target for ClpP1-dependent degradation

Since the ClgR primary sequence ends, like PopR, with two alanines, we assumed that it might be a new target for ClpP1/P2 proteolysis. The constructs expressing *clgR* under the

control of its own promoter on a multicopy plasmid did not allow us to detect the protein (data not shown). Therefore, we used plasmids pAB54 and pAB55, respectively overexpressing *clgR-AA* and *clgR-DD*, where the two C-terminal alanine residues were replaced by aspartate residues, from the strong *erm***p* promoter. Levels of ClgR were examined by Western blotting in the wild-type strain or in the *clpP1P2* mutant harbouring these plasmids (Fig. 1). The level of ClgR-AA is significantly increased in the *clpP1P2* mutant compared to the wild-type strain. Moreover, ClgR-DD accumulates to a very high level in the wild-type strain, to a level that is quite similar to that of ClgR-AA in the *clpP1P2* mutant.

Taken together, these results indicate that ClpP1/P2 is required to degrade the activator of its own synthesis, ClgR, and that the two C-terminal alanine residues are essential for degradation of ClgR.

clpP1 and *clpC1* expression during the cell cycle

In a previous paper (Bellier & Mazodier, 2004), we showed that the ClpP1 level is constant through the differentiation cycle in the wild-type strain, while it is drastically increased in the strain overexpressing *clgR-AA* and even more in the strain overexpressing *clgR-DD*. However, we also showed that the ClpC1 protein is only detectable at the beginning of the life cycle in the wild-type strain, but is overproduced in the strains overexpressing *clgR-AA* and *clgR-DD*, although the signal decreases over time. To understand if these differences between ClpP1 and ClpC1 levels reflect a different expression profile, *clpP1* and *clpC1* expression were measured by RT-PCR using RNAs extracted from plate cultures (Fig. 2). In the pAB54 strain (*clgR-AA* overexpression), expression of *clpP1* and *clpC1* was higher at the basal mycelium stage and then decreased throughout the developmental cycle. In the pAB55 strain (*clgR-DD* overexpression), *clpP1* and *clpC1* were strongly expressed at the basal and aerial mycelium stages and reached a low level of expression during sporulation. Therefore, *clpP1* and *clpC1* show a relatively similar expression profile, contrary to their protein

Fig. 1. Stabilization of ClgR-DD with respect to ClgR-AA in wild-type (wt) and *clpP1*. Crude extracts (10 µg) from liquid cultures of wild-type or *clpP1* carrying pAB54 (*clgR-AA*) or pAB55 (*clgR-DD*) were analysed by Western blotting with polyclonal anti-ClgR antibodies.

Fig. 2. Quantitative real-time RT-PCR analysis of *clpP1* and *clpC1* gene expression in strains carrying pAB54 (*clgR-AA*) or pAB55 (*clgR-DD*). RNA was isolated from surface-grown cultures at different phases: basal mycelium stage (lane 1), aerial mycelium stage (lane 2) and sporulation stage (lane 3). Note that differentiation does not affect 100% of the cells; therefore, some remaining material of earlier phases is still present in the late phases. The RT-PCR experiments were done on two biological replicates (independent RNA purifications) with four experimental RT-PCR analyses each. The two biological replicates gave a constant similar profile. One is shown here. The SD was calculated from the four experimental analyses.

patterns. The differences detected at the protein level might be due to post-translational controls.

ClpP1 and ClpC1 stability

We therefore tested the stability of ClpP1 and ClpC1. For this purpose, cultures were grown on cellophane filters on rich medium until they formed the basal mycelium, a developmental stage where they express both *clpP1* and *clpC1*. The culture discs were then transferred to rifampicin plates to block transcription, or to chloramphenicol plates to block translation, or to control plates containing only antibiotic solvent (0.5% methanol). Proteins were extracted after 0.5, 1 or 2 h incubation and analysed by Western blotting (Fig. 3). While ClpC1 rapidly faded away on rifampicin and chloramphenicol plates, ClpP did not disappear.

Fig. 3. Stabilization of ClpP1 (a) and ClpC1 (b) proteins. Crude extracts from basal mycelium of the pAB55 (*clgR-DD*)-carrying strain were done 0.5, 1 or 2 h after transfer onto NE plates containing only methanol (control, T), chloramphenicol (Cm) or rifampicin (Rif). Western blots were analysed with polyclonal anti-ClpP1 (a) or anti-ClpC1 (b) antibodies.

The two signals detected with anti-ClpP1 antibodies correspond to the unprocessed (upper band) and processed (lower band) forms of ClpP1 (Viala & Mazodier, 2002). Only the processed form is stable. ClpP1 is still detected after 2 h in these conditions.

Our results indicate that ClpP1 is quite stable, whereas ClpC1 appears to be labile.

ClpP1/P2-dependent ClpC1 degradation

As two components of the ClgR regulon accumulated in the *clpP1P2* mutant, we looked at ClpC1 stability in the wild-type and *clpP1P2* strains harbouring pAB59M2, where *clpC1* is under the control of the *erm^{*}p* promoter, so that the ClgR stability in a *clpP1P2* mutant would not interfere with *clpC1* expression. Protein levels in crude extracts from liquid cultures were determined by Western blotting with monoclonal anti-M2 antibodies (Sigma) (Fig. 4). M2-ClpC1 levels were higher in the *clpP1P2* mutant than in the wild-type strain, suggesting that ClpC1 might be a substrate of the Clp proteases.

Presence of the Lon protease during the developmental cycle

Since we had previously shown that the *lon* gene also belongs to the ClgR regulon, Lon protein levels were compared

Fig. 4. Detection of ClpC1 by Western blotting with monoclonal anti-M2 antibodies in wild-type (wt) or in *clpP1* strains carrying pAB59M2 (M2-*clpC1*).

between the wild-type strain and the pAB54 (*clgR-AA*) or pAB55 (*clgR-DD*) strains by Western blotting of crude extracts from plate cultures (Fig. 5). In the wild-type strain, Lon is present at a high level in the basal mycelium stage and then disappears. However, in the pAB54 and pAB55 strains, no obvious effects of *clgR* overexpression on Lon protein levels could be detected.

Since the Lon peptide sequence also ends with two alanines, Lon could also be a target for ClpP1/P2-dependent proteolysis. In this case, the effects of *clgR* overexpression on *lon* expression would be missed due to *clpP1* overexpression and correspondingly increased Lon degradation.

Lon: a new target for ClpP1 proteolysis

To distinguish between transcriptional and post-translational ClpP1-dependent control of Lon levels, the *lon* gene was cloned under the strong *erm***p* promoter. Moreover, to be able to specifically detect the Lon protein, whose expression is controlled by *erm***p*, but not the endogenous Lon protein, two M2 Flag epitopes were added to the N terminus of the Lon protein. Finally, to test if the two alanines could be the substrate degradation motif for Lon, both *lon-AA* (native gene) and *lon-DD* genes were cloned.

Protein levels from crude extracts of liquid cultures were tested by Western blot experiments with monoclonal anti-M2 antibodies (Fig. 6). The level of M2-Lon-AA is higher in the *clpP1P2* mutant than in the wild-type strain. The M2-Lon-DD levels were higher than the M2-Lon-AA levels, but were about the same in the wild-type strain and in the *clpP1P2* mutant.

The Lon protease is thus a new target for ClpP1/P2-dependent proteolysis and the two C-terminal amino acid residues are essential for degradation by the Clp protease.

Effects of ClgR on the stabilized Lon-DD protein

To discriminate the post-translational effects (i.e. degradation of Lon by ClpP1/P2) from the transcriptional effects (i.e. activation of the *lon* promoter by ClgR) of *clgR*

Fig. 5. Detection of Lon by Western blotting during the *S. lividans* cell cycle. Crude extracts from surface-grown cultures of *S. lividans* strains carrying pHM11a (control), pAB54 (*clgR-AA*) or pAB55 (*clgR-DD*) during vegetative growth (lanes 1), aerial hyphae formation (lanes 2) and sporulation (lanes 3) were analysed with polyclonal anti-Lon antibodies.

Fig. 6. Stabilization of Lon-DD with respect to Lon-AA in wild-type and the *clpP1P2* mutant. Crude extracts (10 µg) from liquid cultures of wild-type (wt) or *clpP1* carrying pAB70M2 (M2-*lon-AA*) or pAB71M2 (M2-*lon-DD*) were analysed by Western blotting with monoclonal anti-M2 antibodies.

overexpression on *lon* expression, the *lon-DD* gene was cloned under its own promoter in an integrative vector to give pAB63. Lon protein levels were then tested by Western blotting on plate cultures in wild-type or in pAB55 (*clgR-DD*) strains harbouring pAB63 (Fig. 7). As observed in the wild-type strain, Lon-DD was only present at the beginning of the developmental cycle. However, in the pAB55 strain, the Lon-DD protein was detected throughout the developmental cycle, suggesting that the disappearance of Lon observed previously in the ClgR-DD strain (Fig. 6) was due to its degradation, and that the absence of Lon persistence in the wild-type strain expressing *lon-DD* is due to a shut-off of ClgR-dependent transcription after the basal mycelium stage.

Therefore, as shown for *clpC1* and *clpP1*, *lon* expression is activated throughout the developmental cycle in the pAB55 strain.

DISCUSSION

The bald phenotype of the *clpP1P2* mutant suggests a crucial role for Clp protease targets in *Streptomyces* differentiation. However, to date, only one target of the Clp protease in *Streptomyces* had been identified: PopR, the activator of the

Fig. 7. Detection of Lon by Western blotting in wild-type (wt) or pAB55 (*clgR-DD*) strains both carrying pAB63 (*lon-DD*) during the cell cycle, vegetative growth (lanes 1), aerial hyphae formation (lanes 2) and sporulation (lanes 3) with polyclonal anti-Lon antibodies.

clpP3P4 operon (Viala & Mazodier, 2002). In this study, we identified three new targets of ClpP1/P2-dependent proteolysis: ClgR, ClpC1 and the Lon protease, all encoded by genes that belong to the ClgR regulon. These observations point to the existence of an interactive network within the ClgR regulon members: ClgR activates *clpP1* expression and, in response, ClpP1/P2 degrades ClgR, allowing a negative feedback control of ClgR activation.

Three targets of ClpP proteases in *Streptomyces*, PopR, ClgR and Lon, all end with two alanines preceded by a hydrophobic residue (leucine for PopR and valine for ClgR and Lon) and in each case, the two alanine residues have been shown to be crucial for their degradation by ClpP1. Indeed, replacement of these two C-terminal residues with two aspartates greatly increased the stability of these proteins. Several previously described Clp targets also end with two alanines: the SsrA tag (Gottesman *et al.*, 1998), the LexA autocleavage C-terminal fragment (Neher *et al.*, 2003) and the essential response regulator CtrA in *Caulobacter crescentus* (Domian *et al.*, 1997). It was therefore tempting to speculate that, in *Streptomyces*, the two alanine residues at the C terminus could be sufficient to target proteins for ClpP1- and/or ClpP2-dependent degradation. However, we have shown that the protein encoded by the *Streptomyces coelicolor* SCO3558 gene, homologous to *cicA*, which encodes a phosphotransferase in *Cau. crescentus* and which ends with two alanines preceded by a hydrophobic residue (proline) in *Streptomyces*, does not accumulate in a *clpP1P2* mutant (data not shown). Therefore, in *Streptomyces*, a hydrophobic residue followed by two alanines at the C terminus is not sufficient to target proteins for Clp degradation. We cannot exclude that there may be other features in the sequences, besides these three C-terminal residues, that play an important role for degradation by ClpP1 and ClpP2 in *Streptomyces*. Moreover, the ClpC1 protein, which also accumulates in a *clpP1P2* mutant, does not end with two alanines, confirming that there are probably many different Clp recognition motifs.

Clp-dependent degradation of ClgR might be conserved in all *Actinomycetes*. Indeed, ClgR was recently shown to be stabilized in a *Corynebacterium glutamicum clpP1P2* mutant (Engels *et al.*, 2005). However, the motif responsible for its degradation is probably different since *Cor. glutamicum* ClgR does not end with two alanines. Moreover, the motif is probably not located at the C terminus since the addition of 10 amino acid residues did not interfere with its degradation (Engels *et al.*, 2004).

We have shown that ClpC1 accumulates in a *clpP1P2* mutant. Degradation of Clp subunits by Clp proteases has already been shown. Indeed, in *E. coli*, the ClpA subunit strongly accumulates in a *clpP* mutant (Gottesman *et al.*, 1990), and in *Bacillus subtilis*, ClpE and ClpX are rapidly degraded in wild-type cells during permanent heat stress but remain almost stable in a *clpP* mutant, suggesting ClpP-dependent degradation (Gerth *et al.*, 2004). Moreover, here we show the degradation of Lon by the Clp protease for the first time. Therefore, the levels of ATP-dependent

proteases in the cell seem to require fine tuning, combining transcriptional and post-translational regulation. This is in agreement with the control of *lon* expression by both ClgR and HspR (Sobczyk *et al.*, 2002), probably allowing a flexible response to a variety of signals.

ClgR is not the only example of a regulator that activates expression of proteases responsible for its own degradation. In *S. lividans*, PopR degradation is primarily dependent on ClpP1 and ClpP2, but can also be achieved by ClpP3/P4, whose expression is activated by PopR (Viala & Mazodier, 2002). In *E. coli*, the σ^{32} heat-shock transcriptional sigma factor controls *ftsH* expression and is itself degraded by FtsH (Blaszczak *et al.*, 1999). More generally, degradation of transcriptional activators by ATP-dependent proteases appears to be quite common. In *E. coli*, the RcsA capsular biosynthesis transcriptional activator is degraded by Lon (Torres-Cabassa & Gottesman, 1987) and the σ^S stress sigma factor is degraded by ClpXP (Schweder *et al.*, 1996), and in *Cau. crescentus* the CtrA essential regulator is degraded by ClpXP (Jenal & Fuchs, 1998). For regulatory proteins, control by proteolysis allows a rapid reduction of their cellular levels in response to a specific signal, triggering the corresponding response. For example, the σ^{32} and σ^S sigma factor levels rapidly increase after stress, and the CtrA regulator accumulates and then drastically decreases at specific stages of the *Cau. crescentus* cell cycle. We could assume that ClpP-dependent proteolysis of ClgR is achieved until some stress or specific environmental condition occurs.

ClpP1/P2 may not be the only proteases involved in ClgR and Lon degradation, since in Figs 1 and 6, levels of ClgR-DD and Lon-DD are higher than levels of ClgR-AA and Lon-AA in the *clpP1P2* mutant. Therefore, some proteins ending with -AA are still degraded in the *clpP1P2* mutant. The protease involved in this phenomenon is not known. This protease is not Lon, as ClgR-AA is not stabilized in the *lon* mutant (data not shown). However, several other proteases, such as FtsH, should be tested for their ability to degrade ClgR and/or Lon.

The stress or input signal has not been identified yet. However, the *cinA* gene (encoding a homologue of the *Streptococcus pneumoniae* competence-induced protein) located upstream from *clgR*, belongs to the disulfide stress σ^R regulon (Paget *et al.*, 2001). The intergenic region is 120 bp long and there is no obvious transcription terminator between the two genes, so it is conceivable that they form an operon. *clpP1* expression is not induced by heat shock (Viala *et al.*, 2000), but the ClpP1 complex is likely to be involved in misfolded protein degradation under other stress conditions, which could lead to an increase of ClgR stability, and consequently *clgR* expression. Analogous mechanisms have been described, such as the HspR regulon, where the DnaK chaperone acts as a transcriptional corepressor by binding to the HspR repressor. Under heat-stress conditions, DnaK is recruited by misfolded proteins and the HspR regulon is therefore induced (Bucca *et al.*, 2000).

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