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

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SUMMARY

In *Streptomyces*, the *clpP* genes are organized as two bicistronic operons, *clpP1P2* and *clpP3P4,* and a monocistronic *clpP5* gene. Previous studies have shown that expression of the *clpP3P4* operon is activated by PopR, which is degraded by ClpP1 ClpP2 and that the 2 carboxy-terminal alanine residues of PopR play an essential part in the degradation process. We had shown that expression of the *clpP1P2* operon, which is required for the differentiation cycle, is activated by ClgR, a regulator encoded by a gene paralogous to *popR*. The ClgR regulon also includes *lon* encoding an ATP-dependent protease, *clpC1*, encoding the ATPase subunit of the Clp complex and the *clgR* gene itself. We show here that ClgR and Lon, which have 2 carboxy-terminal alanine residues, are degraded by ClpP1/ClpP2 and that the 2 C-terminal alanines are involved in ClgR and Lon stability. The results presented here support the idea that ClgR activation is specific to the first step of *Streptomyces* differentiation, and that by degrading products of the genes activated by ClgR, including ClgR itself, ClpP1 maintains a low basal level of the ClgR regulon through a negative feedback control of ClgR activation.

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 eukaryotic cells, these functions are served by the energy-dependent 26S proteasome complex (Baumeister *et al.*, 1998). 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 2 distinct domains : an ATPase chaperone domain responsible for substrate recognition and unfolding, and a proteolytic domain. These domains can either be on the same polypeptide chain (Lon and FtsH) or on 2 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 NH2- or COOH-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). For other substrates, like RepA, the P1 plasmid replication protein (Hoskins *et al.*, 2000), or the λO phage replication protein (Gonciarz-Swiatek *et al.*, 1999), recognition sites are near the NH2 terminus. Recently, ClpXP substrates have been trapped *in vivo* using an *E. coli* inactive variant of ClpP. Analysis of more than 50 trapped proteins revealed five distinct classes of ClpX-recognition motifs : 3 N-terminal and 2 C-terminal motifs (Flynn *et al.*, 2003). One of these classes includes the C-terminal motif of SsrA-tagged proteins, ending with 2 alanine residues.

Identifying the proteolytic targets of specific proteases is critical for understanding their diverse physiological roles. In *Bacillus subtilis*, the ComK competence transcriptional factor

is specifically degraded by ClpCP until the competence is induced (Turgay *et al.*, 1998) ; the anti-sporulation σ^F factor is degraded by ClpCP during sporulation (Pan *et al.*, 2001) and the CtsR class III heat shock gene repressor is degraded by ClpXP at 37°C (Derre *et al.*, 2000) and by ClpCP under stress conditions (Kruger *et al.*, 2001). Clp proteases thus play crucial roles in competence, sporulation and heat shock response in *B. subtilis*. In *Caulobacter crescentus*, the CtrA response regulator protein that directly controls more than 50 genes during the cell cycle is eliminated by ClpXP before cells enter the DNA replication phase (Jenal and Fuchs, 1998) and the flagellar and chemotaxis machineries are regulated by ClpXP proteolysis (Alley *et al.*, 1992; Grunenfelder *et al.*, 2004; Potocka *et al.*, 2002).

Clp proteases also play an important role in biological functions of the Gram-positive soil bacterium *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 : the *bld* (bald) mutants that fail to produce aerial hyphae and the *whi* (white) mutants whose aerial hyphae fail to complete the production of normal, grey-pigmented spores (Chater, 2001). The *S. 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 targets need to be degraded for normal aerial mycelium formation.

However, only one *S. lividans* ClpP1 target has been identified to date : PopR, the transcriptional activator of the *clpP3clpP4* operon (Viala *et al.*, 2000). PopR is primarly degraded by ClpP1 and the carboxy-terminal residues Ala-Ala play an essential part in the degradation process (Viala and Mazodier, 2002). Recently, we have shown that ClgR, which is encoded by a gene paralogous to *popR*, activates expression of *clpP1*, *clpC1*, *lon* and *clgR* (Bellier and Mazodier, 2004). ClgR and PopR DNA-binding domain regions share over 50 %

amino-acid sequence identity, and like PopR, ClgR has 2 alanine residues at its C-terminus. Therefore, ClgR seemed a good candidate as a substrate for ClpP1. Moreover, we had observed that *clgR* overexpression led to a strong differentiation delay, suggesting that ClgR or members of its regulon could be involved in aerial mycelium formation.

We investigated ClgR stability in wild-type and *clpP1* mutant strains. We found that ClpP1 degrades ClgR and that the 2 C-terminal alanines are required for degradation. We also found that Lon, whose synthesis is activated by ClgR, is also degraded by ClpP1 and that its degradation also involves the 2 C-terminal alanine residues. ClgR thus activates *clpP1* gene expression, and ClpP1 degrades several proteins encoded by genes of the ClgR regulon. Via specific proteolysis, ClpP1 ensures a negative post-translational control of the ClgR regulon.

Results

ClgR a new target for ClpP1-dependent degradation

Since the ClgR primary sequence ends, like PopR, with 2 alanine, we assumed that it might be a new target for ClpP1 proteolysis. We used pAB54 and pAB55 plasmids, respectively overexpressing the native *clgR*, *clgR-AA*, or *clgR-DD*, where the two carboxy-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 *clpP1* mutant harbouring these plasmids (Fig. 1). The level of ClgR-AA is significantly increased in the *clpP1* 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 *clpP1* mutant.

Taken together, these results indicate that ClpP1 is required to degrade the activator of its own synthesis, ClgR, and that the 2 C-terminal alanine residues are essential for degradation of ClgR.

Presence of ClgR during S. lividans *cell cycle*

In order to determine if *clgR* is differentially expressed during the cell cycle, protein extracts were prepared from plate cultures at the 3 different stages of differentiation : basal mycelium, aerial mycelium and sporulation ; and analyzed by Western blot experiments.

We first used pAB50 and pAB51 strains, overexpressing the native *clgR*, *clgR-AA*, or *clgR-DD*, under the control of its own promoter on a multicopy plasmid. However, no obvious

effects on expression of the ClgR regulon were observed and the ClgR protein was only weakly detectable at the basal mycelium stage in these strains (data not shown).

We then used pAB54M2 and pAB55M2 strains, overexpressing *clgR-AA* or *clgR-DD*, cloned in N-terminal translational fusion with two M2 Flag epitopes and from the strong *erm*p* promoter. Using polyclonal anti- *S. lividans* ClgR antibodies or anti-M2 monoclonal antibodies to analyze the crude extracts, signals were detected only at the basal mycelium stage in both strains (Fig. 2), with a stronger intensity in the pAB55M2 strain (*clgR*-DD), in agreement with an increased stability of the ClgR-DD protein. We then decided to use these strains for the rest of the study presented here.

Expression of clgR *during the cell cycle*

The *erm*p* promoter had been described as a constitutive promoter (Bibb, 1994). Therefore, the results described above showing that the ClgR protein is only present at the basal mycelium stage were unexpected, especially in the pAB55 strain (expressing *clgR*-DD), where ClgR proteolysis by ClpP1 is, if not abolished, at least strongly decreased. This led us to check *clgR* expression from the plasmid *erm*p* promoter or from the chromosomal *clgR* native promoter in strains harbouring pAB54 and pAB55. RNA was extracted from plate cultures at the 3 different stages of differentiation and *clgR* expression was determined by RT-PCR experiments.

In pAB54 and pAB55 strains, the *erm*p* promoter expression is stronger during the basal mycelium stage and then decreases throughout the cell cycle (Fig. 3a). The endogenous *clgR* promoter activity is different (Fig. 3b). Indeed, in the pAB54 strain, *clgR* expression from its native promoter remains low during the cell cycle. In the pAB55 strain, expression from the *clgR* native promoter is much more important at the basal mycelium stage than in the pAB54

strain (increasing more than ten times). Its expression remains strong at the aerial mycelium stage, and finally decreases during sporulation.

We finally tested the global *clgR* expression in the cell, reflecting *clgR* expression from both the *erm*p* promoter and its native promoter (Fig. 3c). While global *clgR* expression remains low and constant in the pAB54 strain, its expression is strongly increased at the basal and aerial mycelium stages in the pAB55 strain (about three-fold) and finally decreases at the sporulation stage to reach a level of expression that is comparable to that in the pAB54 strain.

Correlation between clgR *expression and activation of* clpP1 *and* clpC1 *expression*

In order to confirm the activation of the ClgR regulon at a specific time within the cell cycle, as observed at the protein level (Bellier and Mazodier, 2004), *clpP1* and *clpC1* expression were measured by RT-PCR using RNAs extracted from plate cultures (Fig. 4). In the pAB54 strain (*clgR*-AA), expression of *clpP1* and *clpC1* was higher at the basal mycelium stage and then decreased throughout the cell cycle. In the ClgR-DD strain, *clpP1* and *clpC1* were strongly expressed at the basal and aerial mycelium stages, and reached a low level of expression during sporulation. Therefore, activation of *clpP1* and *clpC1* expression is directly correlated to global *clgR* expression in the cell.

Moreover the RNA expression profile of *clpP1* and *clpC1* is consistent with the protein levels shown previously (Bellier and Mazodier, 2004). However, in the Western blot experiments in the pAB54 strain, ClpP1 levels were constant, while ClpC1 protein levels actually decreased, in agreement with the RNA expression profile. In order to explain this difference between the RNA and protein levels, the stability of ClpP1 and ClpC1 was tested. For this purpose, cultures were grown on filters on rich medium until they formed the basal mycelium, a developmental stage where they express both *clpP1* and *clpC1*. The culture disks were then

transferred to rifampicin plates in order to block transcription, or to chloramphenicol plates in order to block translation. Proteins were extracted after 2 or 6 hours incubation on these plates and detected by Western blotting (Fig. 5). While ClpC1 rapidly disappears on rifampicin and chloramphenicol plates, ClpP1 is still detected even after 6 hours in both conditions.
These results indicate that ClpP1 is quite stable, whereas ClpC1 appears to be labile.

Presence of the Lon protease during the cell cycle

Since we had previously shown that the *lon* gene also belongs to the ClgR regulon, Lon protein levels were compared 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. 6). In the wild-type strain, Lon is present at a high level in the basal mycelium stage and then disappears. However in the pAB54 or in the pAB55 strains, no obvious effects of *clgR* overexpression on Lon protein levels could be detected.
Since the Lon peptide sequence also ends by 2 alanines, Lon could also be a target for ClpP1-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 is a new target for ClpP1 proteolysis

In order 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, in order to be able to specifically detect the Lon protein, whose expression is controlled by *erm*p*, but not the endogenous Lon protein, 2 M2 Flag epitopes were added to the N-terminal of the Lon

protein. Finally, to test if the 2 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 from liquid cultures were tested by Western blot experiments with monoclonal anti-M2 antibodies (Fig. 7). The level of M2-Lon-AA is higher in the *clpP1* mutant than in the wild-type strain. The M2-Lon-DD levels are higher than the M2-Lon-AA levels, and they are about the same in the wild-type strain and in the *clpP1* mutant.

The Lon protease is thus a new target for ClpP1-dependent proteolysis and the 2 C-terminal amino residues are essential for degradation by the Clp protease.

Effects of ClgR on the stabilized Lon-DD protein

In order to discrimate the post-translational effects (ie degradation of Lon by ClpP1) from the transcriptional effects (ie activation of *lon* promoter by ClgR) of *clgR* overexpression on *lon* expression, *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. 8). As observed in the wild-type strain, Lon-DD is only present at the beginning of the cell cycle. However, in the pAB55 strain, the Lon-DD protein is now detected throughout the cell cycle, suggesting that the Lon disappearance observed previously in the ClgR-DD strain (Fig. 7) 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 cell cycle in the pAB55 strain.

Discussion

Previous studies have shown that the *clpP1P2* operon, *clpC1*, *clgR* and *lon* genes are activated by ClgR. PopR, the activator of the *clpP3* operon, is the only one identified target of the Clp protease in *Streptomyces*. In this study, we identify 2 new targets of ClpP1-dependent proteolysis : ClgR and the Lon protease, encoded by a gene that belongs 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 degrades ClgR, allowing a negative feedback control of ClgR activation. The identification of these new targets of ClpP1 proteolysis, ClgR and Lon, which play important roles in the *Streptomyces* cell cycle, emphasize on the crucial role of ClpP1 in *Streptomyce*s differentiation.

The 3 identified targets of ClpP proteases in *Streptomyces*, PopR, ClgR and Lon, all end with 2 alanines preceded by a hydrophobic residue (leucine for PopR and valine for ClgR and Lon) and in each case, the 2 alanine residues have been shown to be crucial for their degradation by ClpP1. Indeed, replacement of these 2 carboxy-terminal residues by 2 aspartates greatly increased the stability of these proteins. Several previously described Clp targets also end by 2 alanines : the SsrA tag that is added to the C-terminus of truncated proteins to target them for ClpXP degradation (Gottesman *et al.*, 1998) ; the LexA autocleavage C-terminal fragment which is degraded by ClpXP in *E. coli* (Neher *et al.*, 2003) and the essential response regulator CtrA in *C. crescentus*, whose proteolysis is prevented when the last 2 alanines are replaced by 2 aspartates (Domian *et al.*, 1997).

It was then tempting to speculate that in *Streptomyces*, the 2 alanine residues at the carboxy-terminus could be sufficient to target proteins for ClpP1 and ClpP2-dependent degradation. However, we have shown that the protein encoded by the *S. coelicolor* SCO3558 gene, homologous to *cicA*, which encodes a phosphotransferase in *C. crescentus*, and which ends

with 2 alanines preceded by a hydrophobic residue (proline) in *Streptomyces* does not accumulate in a *clpP1* mutant (data not shown). This result was unexpected since the recent analysis of the sequences of 50 trapped proteins using an inactive variant of ClpP in *E. coli* has revealed 5 classes of recognition signals, one of which is a C-terminal motif similar to the SsrA terminus (Leu-Ala-Ala) (Flynn *et al.*, 2003). Therefore, in *Streptomyces*, a hydrophobic residue followed by 2 alanines at the C-terminus is not sufficient to target proteins for Clp degradation. Therefore, we cannot exclude that there may be other features in the sequences, besides these 3 carboxy-residues, that play an important role for degradation by ClpP1 and ClpP2 in *Streptomyces*.

Degradation of Lon by the Clp protease is shown here for the first time. However, ATP-dependent protease degradation by other ATP-dependent proteases has already been shown. Indeed, in *E. coli*, the ClpA subunit strongly accumulates in a *clpP* mutant (Gottesman *et al.*, 1990) and in *B. subtilis*, ClpE and ClpX are rapidly degraded in wild-type cells during permanent heat stress but remained almost stable in a *clpP* mutant, suggesting ClpP-dependent degradation (Gerth *et al.*, 2004). Therefore, amounts of ATP-dependent proteases in the cell seem to require fine tunings, combining transcriptional and post-translational regulation.

Clp-dependent degradation of ClgR is probably conserved in all Actinomycetes. Indeed, in *C. glutamicum*, ClgR is only present in Δ*clpC* but not in wild-type cells, whereas the levels of *clgR* mRNAs are comparable in both strains (Engels *et al.*, 2004). However, the motif responsible for its degradation is probably different since *C. glutamicum* ClgR does not end with 2 alanines. Moreover, the motif is probably not located at the C-terminus since the addition of 10 amino-acid residues does not interfere with its degradation (Engels *et al.*, 2004). ClgR is not the only example of regulators that activate expression of proteases responsible for their own degradation. In *S. lividans*, PopR degradation is primarily dependent

on ClpP1 and ClpP2, but can also be achieved by ClpP3 ClpP4, whose expression is activated by PopR (Viala and 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 and Gottesman, 1987) and the σ^{S} stress sigma factor is degraded by ClpXP (Schweder *et al.*, 1996), and in *C. crescentus* the CtrA essential regulator is degraded by ClpXP (Jenal and Fuchs, 1998). For regulatory proteins, control by proteolysis can allow 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 factors levels rapidly increase after a stress, and the CtrA regulator accumulates and then drastically decreases at specific stages of the *C. crescentus* cell cycle. We could then assume that ClpP-dependent proteolysis of ClgR is achieved until some stress or specific environmental conditions occur.

In the wild-type strain, the ClgR protein is almost not detected. In order to get a strong protein signal and to have effects on the ClgR regulon members expression, we had to use a strain overexpressing *clgR* under a strong promoter, and to modify the protein in order to increase its stability. However, the fact that we have not been able to inactivate *clgR*, presumably because it is required for *clpC1* expression, which we think is essential, suggests that control by ClgR is crucial for the cell, probably under specific conditions.

The stress or input signal has not been identified yet. However, the *cinA* gene (coding for a homolog of *Streptococcus pneumoniae* competence induced protein) located upstream from *clgR*, belongs to the disulphide 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 its 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 co-repressor by binding to the HspR repressor. In heat stress conditions, DnaK is recruited by misfolded proteins and the HspR regulon is therefore not induced (Bucca *et al.*, 2000).

It is interesting to note that *lon* expression is controlled by both ClgR and HspR. This suggests that ATP-dependent proteases are tightly regulated in *Streptomyces*, allowing a flexible response to a variety of signals.

According to Western blot experiments, it seems that ClgR is specifically present during the basal mycelium stage, suggesting that ClgR could be degraded during the aerial mycelium and spores stages, and that proteolysis would stop once the spore germinates. Arrest of degradation combined with *clgR* positive autoregulation leads to a very rapid increase of ClgR level. Similarly, CtrA is degraded by ClpXP and one of the *ctrA* promoters is under positive feedback control (Domian *et al.*, 1999). Together with CtrA phosphorylation, the coupling of these mecanisms results in the specific pattern of CtrA activity required for the temporal and spatial control of the *Caulobacter* cell cycle.

The fact that in the wild-type strain, ClgR, ClpC1 and Lon are specifically present at the basal mycelium stage of differentiation, while the ClpP1 protein is present throughout the cell cycle led us to suggest a negative feedback control of the ClgR regulon by ClpP1. ClgR expression is specifically activated during the basal mycelium stage, leading to activation of *clgR* itself and of the ClgR regulon genes, including those encoding the ClpP1, ClpP2, ClpC1 subunits and the Lon protease. ClpP1, in association with an ATPase that is still unidentified, specifically degrades ClgR and Lon. ClgR degradation will consequently end ClgR regulon activation and Lon degradation will allow a rapid elimination of Lon from the cell. Therefore, ClpP1 is responsible for a negative feedback on ClgR activation. ClpP1, which is a very

stable protein, is the only protein so far encoded by the ClgR regulon that is present throughout the cell cycle.

The *clpP1P2* mutant fails to form aerial mycelium, suggesting that Clp protease targets must be degraded for normal differentiation. In other words, Clp target protein accumulation will block the cell cycle at the basal mycelium stage. We have shown that ClgR, ClpC1 and Lon are only present at this stage, suggesting that their functions are required before aerial mycelium formation. Therefore, we could assume that overproduction of these 3 proteins in the *clpP1P2* mutant could be responsible for the *bld* phenotype. This is not confirmed by the pAB55 strain (overexpressing *clgR*-DD) phenotype, which is not bald but only strongly delayed in its cell cycle. However, the ClgR protein level in the pAB55 strain might be lower than the level in the *clpP1* mutant, since we could assume that the –DD motif is not sufficient to completely stabilize the ClgR protein. Indeed, it has been shown that even if the global regulator CtrA is greatly stabilized when the 2 alanines are modified to 2 aspartates, the 56 N-amino-terminal residues are also part of the degradation signal (Ryan *et al.*, 2002). Moreover, the strong differentiation delay observed in the pAB55 strain is in agreement with the necessity for clearance from the cell of the ClgR protein and/or proteins encoded by genes that belong to the ClgR regulon, at the end of the basal mycelium stage, in order to successfully complete the differentiation cycle.

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 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. Antibiotic apramycin, hygromycin and thiostrepton were added to final concentrations of 25, 100 and 25 μg ml^{-1} to solid medium, and to 20, 50 and 10 μg ml^{-1} to liquid medium respectively ; and antibiotic rifampicin and chloramphenicol were added to final concentrations of 100 and 50 μg ml^{-1} to solid medium

E. coli TG1 (Gibson, 1984) was used as the general cloning host and *E. coli* BL21λDE3, containing the pREP4 plasmid (Amrein *et al*., 1989) for protein production and purification. *E. coli* strains were 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 extracted was from *E. coli* using a Qiagen Kit. DNA fragments were purified from agarose gels with Ultrafree-DA (Amicon-Millipore). Restriction enzymes were used as recomended by the manufacturers. DNA fragments were amplified by the PCR technique (Mullis and 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 by Hopwood *et al*.(Hopwood *et al.*, 1985).

Plasmids and plasmid constructions

Plasmids pAB54 and pAB55 for *clgR* overexpression in *Streptomyces* have previously been described (Bellier and Mazodier, 2004). To overexpress *clgR* or *clgR-DD* under *clgR* promoter, PCR amplifications from AB71 and AB72 and from AB71 and AB73 primers were cloned in *Bam*HI and *Hind*III sites of the multicopy pUWL219 plasmid, yielding pAB50 and pAB51, respectively. To overexpress *lon* in *Streptomyces*, pAB70M2 and pAB71M2 were constructed by inserting the 2350 bp fragment including the coding sequence of *lon* gene obtained by PCR amplification with primers AB124 and AB125, and AB124 and AB95 respectively inserted between the *Nde*I and *Hind*III sites of pHM11a. The N-terminal fusion with the tandem M2 flag was constructed by cloning at the NdeI site the linler resulting from annealing AB118 and AB119 oligonucleotides. The PCR fragment contained in pAB71M2 modified the *lon* gene in order to encode two aspartic acid residues 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 promoter region and the coding sequence of *lon* gene obtained by PCR amplification with primers JU74 and AB96 inserted between the *Eco*RI and *Xba*I sites of pSET152.

RNA isolation from surface-grown cultures of *S. lividans*

Mycelia of *S. lividans* 1326 with or without the pHM11a (control), pAB54 or pAB55 grown on cellophane discs were harvested with a spatula, dispersed in 2 ml of water and pelleted.

Cells were resuspended in 0.5 ml of cold deionized water and added to 0.5 g of glass beads (106-μm diameter ; Sigma), 0.4 ml of 4 % Bentone (Rheox) and 0.5 ml of phenol-chloroform-isoamyl alcohol, pH8.0 (Amresco). The cells were disrupted in a Fastprep desintegrator (Bio101, Inc.) for 30 s 3 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. 250 μl of ethanol was added and the sample was applied to an RNeasy mini column (QIAGEN) following the RNA cleanup protocol recomended by the manufacturers. RNA sample was eluted in 35 μl of deionized water. RNA concentrations were determined by measuring absorbance at 260 nm.

Real-time quantitative PCR

Primers (Table 1) were designed with BEACON Designer software. RNA (10 μg) was treated twice with 30 U of 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 cleanup protocol. RNA sample was eluted in 30 μl of 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 of SYBR PCR master mix (Applied Biosystems) and 1 μl of gene-specific primers (10 μM). Amplification and detection of specific products were performed with the iCyclerIQ Multi-Color real-time PCR detection system (Biorad) with the following cycle profile : one cycle at 95°C for 3 min, 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 is verified by generating a melting curve with a final step of 80 cycles of 10 s at an intitial temperature of 55°C, then increased of 0.5°C each cycle until 95°C. Loss of fluorescence is

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, quadriplate 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 occcurs. The CT value was calculated for each quadriplate reactions. A ΔCT value was then calculated for each sample by substracting the mean CT value of the target gene from the mean CT value of the *hrdB* reference gene (*hrdB* is encoding for an essential and constitutively expressed σ factor). The data was transformed from a logarithmic to linear scale by using the formula $x=2^{-\Delta CT}$ (Livak and 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 the 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 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 (Bradford, 1976). Ten μg of protein extract were subjected to SDS-PAGE as described by Laemmli (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* Lon (1 :5,000) or anti-Flag M2 monoclonal

antibodies (Sigma). Signals were detected with the ECL Western blotting Detection Kit (Amersham Biosciences).

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LEGENDS

Fig. 1 : Stabilization of ClgR-DD with respect to ClgR-AA in wild-type 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 : Detection of ClgR by Western blotting during *S. lividans* cell cycle. Crude extracts from wild-type carrying pHM11a (control), pAB54M2 (M2-*clgR*-AA) or pAB55M2 (M2-*clgR*-DD) at 3 time points corresponding to vegetative growth, aerial hyphae formation and sporulation were analysed with monoclonal anti-M2 antibodies.

Fig. 3 : Quantitative real-time RT-PCR analysis. Expression of *clgR* gene under *Erm*p* promoter, under its own promoter, or global *clgR* expression were analysed at 3 time points (vegetative growth, aerial hyphae formation and sporulation) in strains carrying pAB54 (*clgR*-AA) or pAB55 (*clgR*-DD). The ratio between the target gene expression and the *hrdB* reference gene expression at the vegetative growth in the pAB54 strain is standardized to a value of 1. The expression of the target gene in other conditions was then relative to this ratio of 1. Values represent the means from 2 independent RT-PCR experiments from independent RNA preparations with error bars representing standard deviation.

Fig. 4 : Quantitative real-time RT-PCR analysis of *clpP1* and *clpC1* genes expression during cell cycle in strains carrying pAB54 (*clgR*-AA) or pAB55 (*clgR*-DD).

Fig. 5 : Stabilization of ClpP1 and ClpC1 proteins. Crude extracts from wild-type carrying pAB55 (*clgR*-DD) during vegetative growth from NE plate (t0), after 2 or 6 hours on NE plates with methanol (control), rifampicine (Rif) or chloramphenicol (Cm) were analysed by western blotting with polyclonal anti-ClpP1 or anti-ClpC1 antibodies.

Fig. 6 : Detection of Lon by Western blotting during *S. lividans* cell cycle. Crude extracts from wild-type carrying pHM11a (control), pAB54 (*clgR*-AA) or pAB55 (*clgR*-DD) during

vegetative growth, aerial hyphae formation and sporulation were analysed with polyclonal anti-Lon antibodies.

Fig. 7 : Stabilization of Lon-DD with respect to Lon-AA in wild-type and *clpP1* mutant. Crude extracts (10 μg) from liquid cultures of wild-type or *clpP1* carrying pAB70M2 (M2-*lon*-AA) or pAB71M2 (M2-*lon*-DD) were analysed by western blotting with monoclonal anti-M2 antibodies.

Fig. 8 : Detection of Lon by Western blotting in wild-type or in pAB55 (*clgR*-DD) strain carrying pSET152 (control) or pAB63 (*lon*-DD) during the cell cycle with polyclonal anti-Lon antibodies.

Figures

Fig. 1

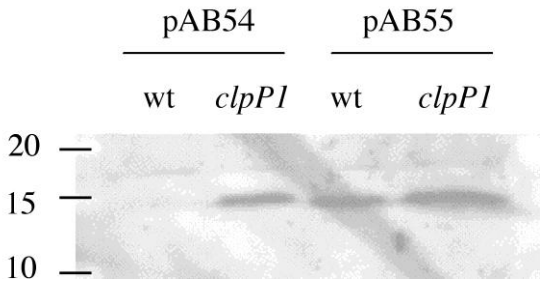
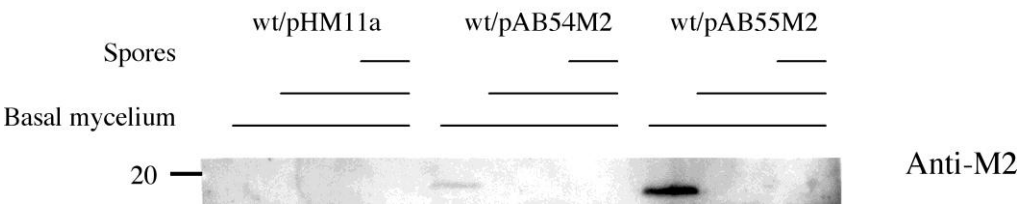


Fig. 2



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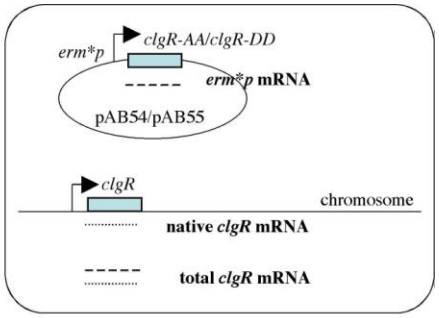
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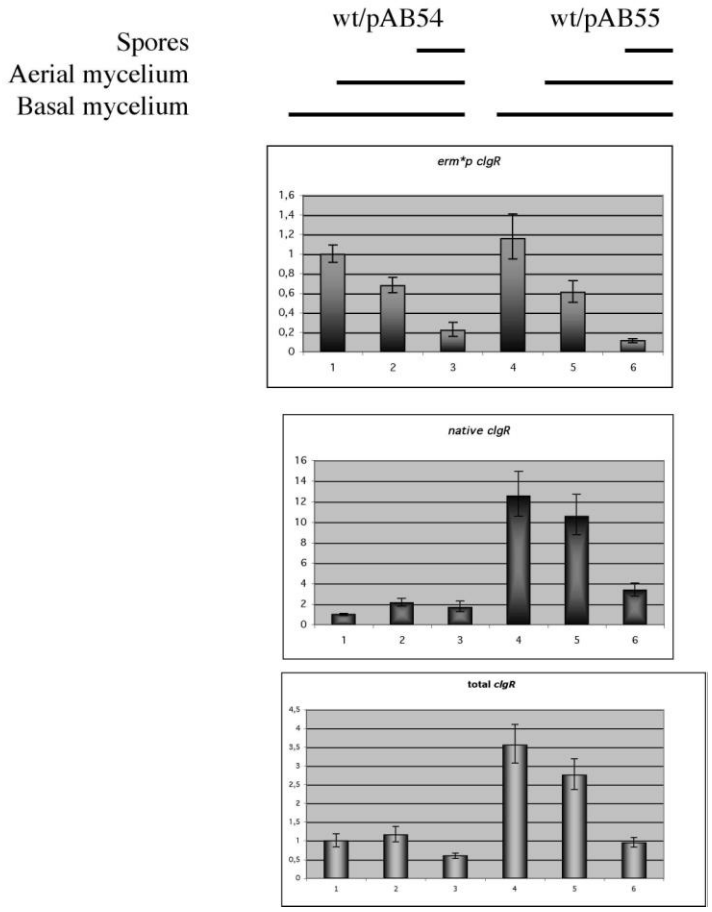
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wt/pAB55
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Basal mycelium
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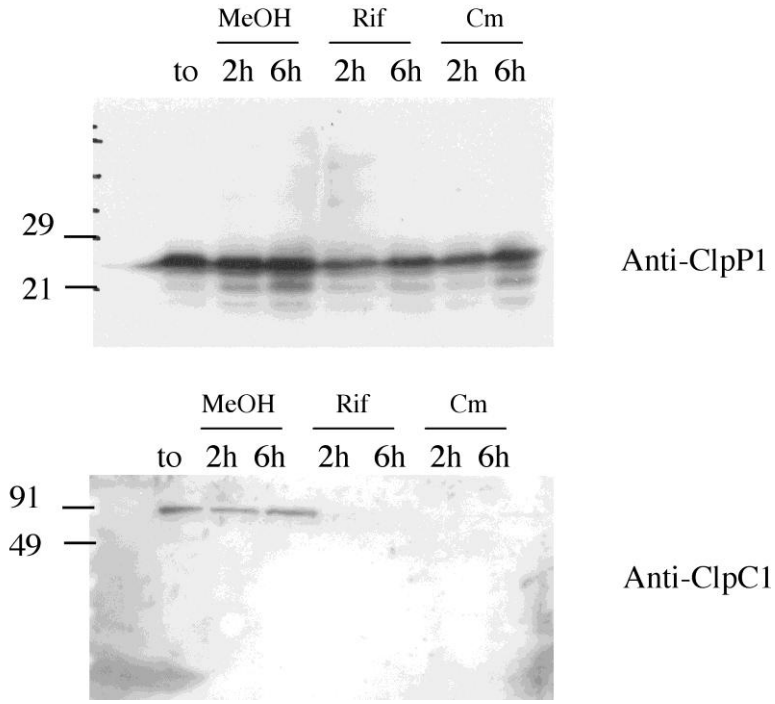
total clgR

Fig. 5



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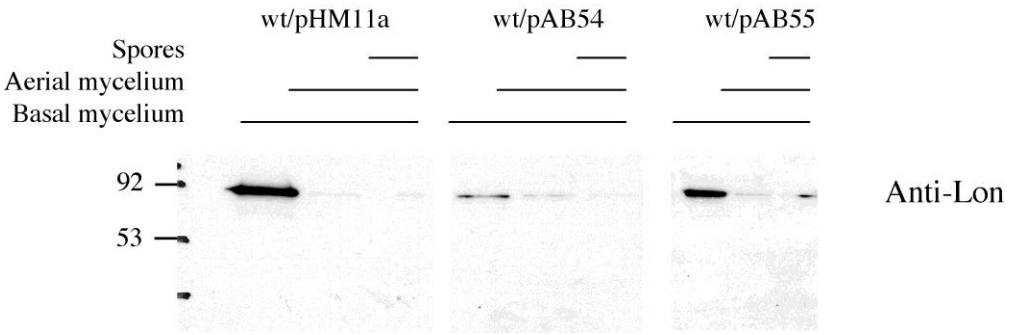

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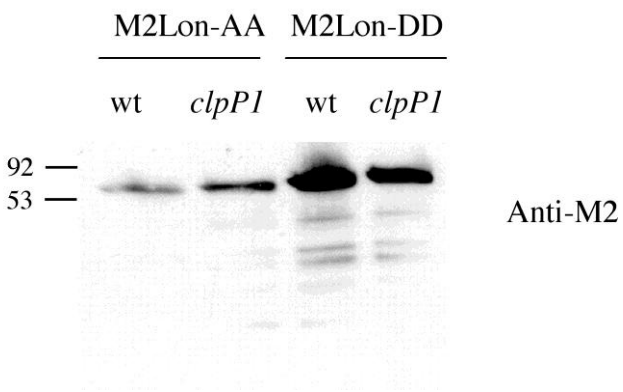

Fig. 8

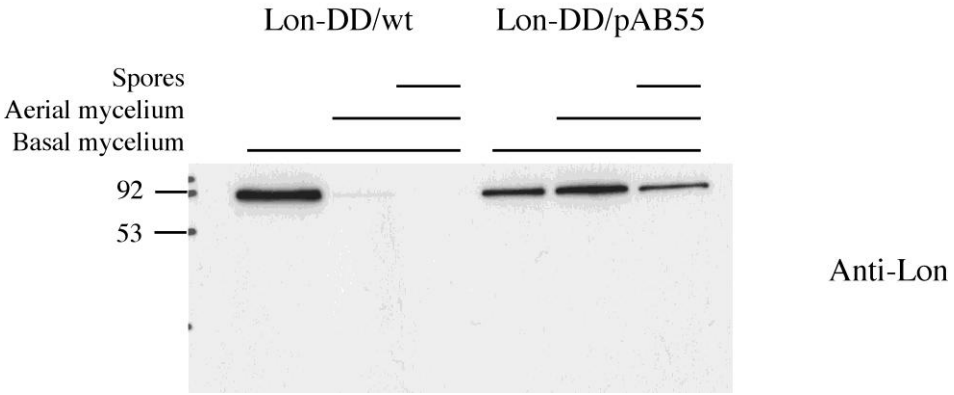

Table 1 : Primers used in this study

Primer	Sequence
AB71	GGAGGATCCGGCCCTTCAGGGCCGAGCAGG
AB72	AAGAAGCTTGGGCGCTCAGGCGGCGACGAC
AB73	AAGAAGCTTTCAGTCGTCGACGACGTCCAC
AB95	AAGAAGCTTTCAGTCGTCGACCGGAACCTCACGCTCCGC
AB96	TCTTCTAGATCAGTCGTCGACCGGAACCTCACGCTC
AB118	TATGGACTACAAGGACGACGACGACAAGGACTACAAGGACGACGACGACAAGCA
AB119	TATGCTTGTCGTCGTCGTCCTTGTAGTCCTTGTCGTCGTCGTCCTTGTAGTCCA
AB124	CATCATATGGTCCTGCCCGGCATGGTCGTC
AB125	AAGAAGCTTTCACGCTGCGACCGGAACCTC
AB127	CACAGGAGGACCCATATGATTC
AB128	GGCGGACGAGGAGACTTC
AB129	GAGCCACCGATGATTCTG
AB130	AGAGATAGCCGAGTGAGAC
Ju 74	GAAGAATTCTACGGCGGTGCTGTCCCGAGA
MG8	CAGCAGGTGGAGGAGATCATC
MG9	TCCGTGCCGATGTAGTTGTG
MG10	CGCTCCACGGTCTACTTC

MG11 GCCGCCGAACTTGTACTC

MG12 TGGGACAGTTCCTGCTCAG

MG13 CTCGGCGTGGATCTTGATG

MG14 GAAGACCACCGCCAAGAAG

MG15 GTCGTCCTCGTCCTCGTC