The Ion gene, encoding an ATP-dependent protease, is a novel member of the HAIR/HspR stress-response regulon in actinomycetes

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Members of a family of ATP-dependent proteases related to Lon from Escherichia coli are present in most prokaryotes and eukaryotes. These proteases are generally reported to be heat induced, and various regulatory systems have been described. The authors cloned and disrupted the lon gene and studied the regulation of its expression in Streptomyces lividans. Ion is negatively regulated by the HspR/HAIR repressor/operator system, suggesting that Lon is produced concomitantly with the other members of this regulon, DnaK and ClpP. The ion mutant grew more slowly than the wild-type and spore germination was impaired at high temperature. Nevertheless its cell cycle was not greatly affected and it sporulated normally.

Keywords: heat shock regulation, Streptomyces, HspR, protease, DnaK

INTRODUCTION

Most bacterial proteins are stable, with a half-life that is longer than the generation time of the cell. However, some proteins with important physiological roles are unstable and are degraded in a few minutes (Gottesman & Maurizi, 1992). In Escherichia coli, these proteins are generally degraded by ATP-dependent intracellular proteases, two families of which have been well characterized: the Lon and Clp serine proteases. The Clp protease is composed of a catalytic subunit (ClpP) and regulatory subunits (ClpA/ClpX). Lon (also called La) is an 87 kDa homo-tetramer. The role of Lon in Es. coli is well known, as several of its substrates have been identified. Lon is involved in cell division, in the regulation of colanic acid production and in the growth of phage lambda. In E. coli, Lon is responsible for the degradation of most abnormal proteins, in particular those produced under stress conditions (Goldberg et al., 1994; Tomoyasu et al., 2001).

The ATP-dependent proteases are central enzymes in the regulation of differentiation in several bacteria. Indeed, in E. coli FtsH degrades the specific heat-shock sigma factor, σ32 (Herman et al., 1995), and ClpXP degrades the specific stationary-phase sigma factor, σ5 (Schweder et al., 1996). In Bacillus subtilis, ClpP degrades ComK, the central regulator of competence (Turgay et al., 1998), and at low pHs Lon degrades the sporulation sigma factor, σH (Liu et al., 1999). The Lon protease also plays an essential role in the formation of asymmetrical flagella in Caulobacter crescentus (Wright et al., 1996).

Streptomyces are model bacteria for the study of differentiation mechanisms. In vitro these soil bacteria follow a differentiation cycle that lasts about a week. The germinated spores form basal or vegetative mycelia. A few days later, aerial mycelia are formed from these structures. These new mycelia partition themselves to form chains of spores that are dispersed following maturation (Hopwood, 1999). The interest of Streptomycetes as a model for studying differentiation is reinforced by the fact that this morphological phenomenon is generally accompanied by the production of secondary metabolites. Currently, 70% of industrially produced antibiotics come from these bacteria.

Regulatory processes associated with spore formation from aerial hyphae and germination take place in cells that do not divide and thus the pre-existing regulatory proteins cannot be diluted. Two types of mechanism can be used to palliate the absence of dilution: the activation/inactivation of central regulators by modification (for example by phosphorylation or methylation), or specific degradation. These considerations led us to study the role of ATP-dependent proteases in the control of the cell cycle (De Crecy-Lagard et al., 1999; Viala et al., 2000).

The ATP-dependent proteases Clp, Lon and FtsH are heat-shock proteins (HSPs) in most bacterial species and
their regulation is well documented in *E. coli* and *B. subtilis*. Although the induction of HSPs is a universal response, a number of mechanisms control HSP synthesis in different organisms. The transcription of heat-shock genes is regulated by both positive and negative mechanisms. In bacteria, the regulation of the heat-shock response was first studied in *E. coli* and shown to rely on the level and activity of specific sigma factors, σE and σ32 (for reviews see Bukau, 1993; Yura et al., 1993). These sigma factors are required for the recognition of specific heat-shock promoters associated with heat-shock genes by the RNA polymerase. The regulation of expression was shown to depend largely on the stability of the sigma factor. Thus, an increase in temperature leads to a rapid increase in the level of active σ32 due to an increase in the synthesis of this molecule and its stabilization. At 30 °C, the DnaK chaperone system destabilizes σ32 and sequesters it in an inactive state that can be degraded by the FtsH protease (Herman et al., 1995). Heat shock causes the denaturation of cellular polypeptides: the DnaK system binds these misfolded polypeptides and releases σ32, in a mechanism allowing positive feedback regulation.

This general dependence on sigma factors for heat-shock regulation is not conserved in prokaryotes. Indeed, in most organisms, important *hsp* genes are controlled exclusively by specific repressors. This is well documented in *Bacillus* and *Streptomyces*. In *Streptomyces*, the synthesis of major HSPs, such as the widespread molecular chaperones DnaK, ClpB, GroEL and HSP18, is negatively controlled at the transcriptional level by at least three different repressors. The control of groE gene expression involves an inverted-repeat element (called CIRCE) that is highly conserved among eubacteria, and the HrcA repressor (Grandvalet et al., 1998). The *dnaK* operon and *clpB* belong to the HspR/HAIR regulon (Bucca et al., 1997; Grandvalet et al., 1999). The HspR repressor-HAIR operator system is used in some bacteria (Spohn & Scarlato, 1999) but is not widespread. In particular, it is not used in low-G+C Gram-positive bacteria.

In this study, the genome sequence of *S. coelicolor* (www.sanger.ac.uk/Projects/S_coelicolor/) was searched for the HAIR motif CTTGAGT-N2-ACCTCAAG. A HAIR sequence was found upstream of a gene closely related to the *lon* gene. We demonstrated that *lon* belongs to the HspR/HAIR regulon.

**METHODS**

**Bacterial strains, media, plasmids.** *S. lividans* TK24 was obtained from the John Innes Culture Collection, Norwich, UK. YEME medium was used for liquid cultures, and R2, R2YE (Hopwood et al., 1985) or NE (Murakami et al., 1989) were used to grow *S. lividans* on plates.

pJ6800 was used for controlled gene expression in *S. lividans* using the thiostrepton-inducible promoter pTipA (Sun et al., 1999), pGM160A (Muth et al., 1989) was used to construct the disruption derivatives. Thiostrepton, viomycin and hygromycin were added to plates at final concentrations of 30 μg ml⁻¹, 25 μg ml⁻¹ and 250 μg ml⁻¹, respectively, as appropriate. Cassettes containing resistance genes (Blondelet-Rouault et al., 1997) were used for gene disruption experiments. *E. coli* TG1 (Gibson, 1984) was used as the general cloning host and *E. coli* strains were grown in LB-Bertani (LB) broth supplemented with 200 μg hygromycin ml⁻¹, 10 μg viomycin ml⁻¹ or 100 μg ampicillin ml⁻¹ when needed. pUC19, pUC18 (Yanisch-Perron et al., 1985) and pBluescript-SK were used as cloning vectors in *E. coli*.

**DNA manipulation and plasmid construction.** Standard cloning procedures were used to produce all plasmids (Sambrook et al., 1989). Restriction and modification enzymes were used according to the manufacturers’ recommendations.

**Cloning of the lon gene of *S. lividans*.** A pair of oligonucleotides, JU74 (5'–GAAGATTTCTACGGGTCTGCTGGAGA-3') and JU76 (5'–AAGAAGCTTTCAAGGCTGACGGCTCTCC-3'), were designed based on the sequence of the *S. coelicolor* *lon* gene (Sanger Centre, Cambridge, UK; http://www.sanger.ac.uk/Projects/S_coelicolor/). These oligonucleotides were used to amplify the *lon* locus from *S. lividans* chromosomal DNA. We cloned a 3 kb PCR-amplified fragment containing the *S. lividans* *lon* locus and its promoter region. The PCR fragment was digested with EcoRI and HindIII and cloned into EcoRI/HindIII-digested pUC19 to generate pJV300. The 3 kb insert was sequenced and found to contain one consensus HAIR motif centred 30 bp upstream of the putative *lon* start codon.

**Expression of lon in *E. coli*.** A 2.4 kb fragment containing the *lon* coding sequence from *S. lividans* was amplified by PCR using oligonucleotides AS58 (5'–ATACCATGCTGCTGATGTCGCCGGCCTTCC-3') and AS59 (5'–ATACCTGAGGGCTGCCAGCCAACCTCACG-3'). The PCR fragment was digested with Ncol/Xbol and cloned into Ncol/Xbol-digested pET28a vector to yield pAS45. This plasmid allowed the overexpression of Lon under the control of the T7 promoter. A translational fusion added six carboxy-terminal histidine residues to Lon, which allowed affinity purification of the protein on a nickel column. Purified Lon was used to obtain antibodies in rabbit (carried out by Eurogentec).

**Overproduction of HspR in *E. coli*.** The DNA fragment containing *S. lividans* *hspR* was amplified by PCR using oligonucleotides AS7 (5'–ATACATATGGACCGTGTCGGGACGCAACCCG-3') and AS11 (5'–ATAGATCTCCTGAGCCAGCCAACCTCACG-3'). These primers introduced Ndel and BglII sites into the resulting 450 bp DNA fragment. The Ndel site replaced the original *hspR* GTF translation start site with an ATG. The Ndel–BglII DNA fragment was cloned into the Ndel and BamHI sites of pET28a (Novagen) to yield pAS16, in which the T7 promoter was used to control gene expression. A translational fusion added 20 residues, including six histidines, to the amino terminus of HspR.

**Purification of Lon protein to homogeneity and production of antibodies.** *E. coli* BL21 (DE3) (pAS45) cells were grown at room temperature in LB medium containing kanamycin and chloramphenicol. When cultures reached an OD600 of 0.6, the production of Lon-His was induced by the addition of 0.1 mM IPTG. After 3 h cells were harvested and washed twice in buffer A (20 mM phosphate buffer pH 7.4, 500 mM NaCl, 20 mM imidazole, 1 mM DTT and 10 %, v/v, glycerol). The cell pellet was resuspended in 20 ml buffer A supplemented with one tablet of Complete protease inhibitor cocktail (Boehringer Mannheim). Cells were disrupted in a French press (9000 p.s.i., 62 MPa). The soluble fraction was obtained by centrifugation at 4°C and 30000 g and was loaded onto a
DNA fragment was cloned into the XbaI to disrupt the replication-thermosensitive plasmid was called pAS49 and pGM160. Thus, we cloned the whole pAS48 plasmid between the hspR sequences from S. lividans to give a 7 kb DNA fragment encompassing the following hspR–disrupting plasmid. Inverse-PCR (I-PCR) was used to disrupt lon-hspR sequences. PCR was performed on AS1 and AS2, 10% DMSO, 200 mM of each dNTP and 1 U Taq polymerase (PerkinElmer) at 93°C, 30 s, 55°C, 40 s, and 72°C, 40 s. The nucleotide sequence upstream and downstream of hspR coding sequence was PCR-amplified with oligonucleotides JU74 and JU75 (5'-ATAGAATTCCGCGACATCGA-3') and JU76 and JU77 (5'-GTCGATGCGCGGGACGAGGA-3') as previously described (Grandvalet et al., 1999).

RESULTS

The genome sequence of S. coelicolor from the Sanger Centre was searched for the HAIR motif by use of the appended ‘DNA motif search’ program. In addition to the HAIR motifs associated with dnaK and clpB, a HAIR sequence was found upstream of a gene closely related to the lon gene, encoding the ubiquitous ATP-dependent protease. The nucleotide sequence upstream of the DNA fragment encompassing 200 bp of lon promoter region and 100 bp of the 5' end of the lon coding sequence was PCR-amplified with oligonucleotides JU74 and JU75 (5'-GCGGGGCTTGCGGAGCTCGGA-3'). The purified fragment was end-labelled with [γ-32P]ATP by the T4 polynucleotide kinase method. Crude extracts with or without overexpressed HspR in E. coli (IPTG induction) were incubated with the labelled probe for 15 min at 25°C. These were incubated in 10 µl gel-shift buffer (10 mM Tris/HCl pH 7.5, 10 mM MgCl2, 1 mM DTT, 0.1% Triton X-100, 50 mM NaCl and 10% glycerol) and 1 µl sonicated herring sperm DNA. Samples were subjected to electrophoresis in 6% polyacrylamide gels containing 50 mM Tris/HCl pH 8, 400 mM glycerol, 1.7 mM EDTA and 2.5% glycerol. Samples were separated for 1 h at 100 V. Finally, the gels were dried and exposed to film.

Western immunoblot analysis. Total protein extracts were prepared from S. lividans wild-type strain TK24 or S. lividans mutants after being grown in YE, supplemented with the appropriate antibiotic if needed. Cells were incubated for 24 h at 30°C, and then samples were subjected to a 40 min heat shock at 40°C. Proteins were separated on polyacrylamide denaturing gels (10% SDS-PAGE) before being electrotransferred to Immobilon membranes (Amersham). Antigens were detected by ECL. Western blotting in the presence of rabbit polyclonal antibodies raised against purified S. lividans Lon-His6.

Transformation procedures and screening of mutants. The lon gene from S. lividans was disrupted after transformation of protoplasts with pAS49. The TK24 protoplasts were prepared and transformed as described by Hopwood et al. (1985). After 24 h incubation thiostrepton was added to the plates. Tsr<sup>R</sup> colonies were tested for the presence of hygromycin resistance. Two doubly resistant clones were incubated in 10 ml YEME medium supplemented with hygromycin and grown for 3 days at 30°C before the crossover selection. To obtain a mutant due to homologous recombination at the lon locus, 10 ml YEME without antibiotics was inoculated with a drop of the pre-culture. The culture was incubated for 3 days with vigorous shaking at 40°C to prevent pAS49 from replicating. Different dilutions (1/10<sup>5</sup>, 1/10<sup>6</sup> and 1/10<sup>7</sup>) of cells were plated onto R5 medium supplemented with hygromycin. Hyg<sup>R</sup> clones were finally patched on both R5 + hygromycin and R5 + thiostrepton plates to select HspR and Tsr<sup>R</sup> clones. Insertion of the hygromycin cassette in the lon gene was controlled by a series of PCR amplifications using oligonucleotides specific to lon and to the cassette.

A S. lividans lon hspR double mutant was obtained by transforming protoplasts of the S. lividans lon mutant with the hspR-disrupting plasmid, pAS25. The mutants were selected as above but with viomycin instead of hygromycin.
from *lon* contains the following sequence: ATTGAGT-N9-ACTCAAC, which is similar to the HAIR consensus motif ATTGAGT-N9-ACTCAAG of the HspR-binding site (Grandvalet et al., 1999). This led us to clone the *lon* gene with oligonucleotides derived from the *S. coelicolor* sequence.

**Cloning of the *lon* gene of *S. lividans***

A pair of oligonucleotides (JU74 and JU76) were designed based on the sequence of the *S. coelicolor* *lon* gene and used to amplify the *lon* locus from *S. lividans* chromosomal DNA. The gene was cloned in pUC19, yielding pJV300. Partial DNA sequence determination confirmed the clone and showed more than 99% identity with *lon* of *S. coelicolor*.

**lon** mutant

The chromosomal *lon* gene was disrupted by a double recombination event using the pAS49 vector containing *lon::hygR*. Candidate Hyg<sup>+</sup> Tsr<sup>-</sup> clones were analysed. The correct integration of *hygR* in chromosomal *lon* was checked by PCR using pairs of oligonucleotides annealing to the *hyg* cassette and to the *lon* chromosomal locus outside the region cloned in pAS49.

The wild-type strain grew faster than the *lon* mutant on all the liquid and solid media tested at 30 °C (NE, R5 YEME). However, the *lon* mutant formed aerial mycelium on plates 30 h after the wild-type and ultimately sporulated. Spores of the *lon* mutant failed to form colonies on NE plates after 1 week at 40 °C, whereas the wild-type produced colonies within 2–3 days, suggesting that the germination process is thermosensitive in the mutant (data not shown).

**Regulation of expression: *lon hspR* double mutant**

To confirm the role of HspR in the regulation of *lon*, a *lon::hygR hspR::vioR* double mutant was constructed. The chromosomal *hspR* was disrupted by a double recombination event using the pAS25 vector containing *hspR::vioR* and the procedure described above used to disrupt *lon* except that candidate Vio<sup>+</sup> Tsr<sup>-</sup> clones were analysed.

Bucca et al. (1997) attempted insertion by a double crossover event of the hygromycin-resistance gene into the 5′ end of *hspR* in *S. lividans*, but without success. Only the entire mutating plasmid could be integrated, leading to a construction containing the mutated *hspR* gene and an intact copy of *hspR*. Our attempts to use pAS25 to integrate a viomycin cassette into the middle of *hspR* in *S. lividans* by a double crossover event failed repeatedly in the wild-type strain; however, as, shown here, the *hspR::vioR* mutant was obtained readily in the *lon* mutant. These results indicate that a high level of *lon* expression in *Streptomyces* could be toxic for the cell (i.e. long-term full induction due to a complete knockout mutation of *hspR*). Toxicity related to *lon* overexpression will be investigated in future work.

**Western blot of Lon**

Proteins extracted from the wild-type strain, the *lon* mutant and the *lon hspR* double mutant grown at 30 °C or subjected to heat shock were analysed by Western blotting using anti-Lon antibodies. In the wild-type a 90 kDa heat-inducible protein was detected (Fig. 1, lanes A and B). In the *lon* mutant the 90 kDa Lon protein was not detected (lanes C and D), but a 30 kDa peptide,
presence of 1 µg crude extract of the strain expressing hspR, most of the labelled fragment was retarded. Competition experiments were conducted to assess the specificity of the binding. The presence of excess unlabelled probe abolished the observed mobility shift of the labelled lon promoter fragment. These results confirm that HspR binds specifically to the lon promoter region. Bucca et al. (2000) have shown that the DnaK protease must be present for HspR to retard a DNA fragment containing the HAIR motif in gel mobility-shift assays. The multiplicity of the bands of the retarded fragment points to the heterogeneity of the HspR/DnaK complex.

**DISCUSSION**

We have identified the first protease in the HAIR/HspR regulon and constructed a viable *S. lividans* lon strain, demonstrating that lon is not essential for the growth and differentiation of *Streptomyces*.

The co-production of the Lon protease and the DnaK and ClpB chaperones may present some advantages. Indeed, the Lon protease and the DnaK system have been reported to act in synergy in *E. coli*, since chaperones detect misfolded proteins that will be either refolded or degraded (Tomoyasu et al., 2001). The co-chaperone DnaJ has also been reported to be necessary for the Lon-dependent degradation of some abnormal proteins by keeping the substrates soluble (Jubete et al., 1996).

The HAIR/HspR regulon is not widespread, but it is found in other actinomycetes. HspR controls expression of the hsp70 and clpB genes in *Mycobacterium tuberculosis* and *Mycobacterium leprae*, but these bacteria do not contain any gene orthologous to *lon*. However, lon orthologues have been found in other mycobacteria, such as *Mycobacterium smegmatis* (Roudiak et al., 1998), and genome analysis revealed HAIR motifs upstream of *lon* in *M. smegmatis*, suggesting that the HAIR/HspR regulation of *lon* may be widespread among actinomycetes.

Although *Streptomyces* spp. consistently display two or more paralogues for many genes, the complete genome sequence surprisingly shows that there is only one copy of *lon* in *S. coelicolor*. In contrast, in bacteria that generally have a lower number of paralogues, such as *B. subtilis* and *C. crescentus*, there are two copies of *lon* and they have different roles in the cell (Serrano et al., 2001). Likewise, *Myxococcus xanthus* also has two copies of the *lon* gene: *lonV*, which is essential for vegetative growth (Tojo et al., 1993a), and *lonD*, which is required for development (Tojo et al., 1993b).

A *lon* mutant has previously been constructed in *Mycobacterium smegmatis* (Knipfer et al., 1999). This mutant displayed wild-type growth rates, whereas we observed that the growth rate of *Streptomyces* was reduced by the *lon* mutation.

*S. lividans* has been used industrially as a host for expression of several heterologous proteins (Pozidis et al., 2000).
al., 2001). Utilization of the lon mutant of S. lividans should be considered when low yield of production points to proteolytic degradation of the protein of interest.

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