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The *lon* 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*. *lon* 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 ClpB. The *lon* 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.

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 *E. 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, σ^{32} (Herman *et al.*, 1995), and ClpXP degrades the specific stationary-phase sigma factor, σ^{S} (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, σ^{H} (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).

Streptomycetes 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 *Streptomyces* 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

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Abbreviation: HSP, heat-shock protein.

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, σ^{32} and σ^{24} (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 σ^{32} due to an increase in the synthesis of this molecule and its stabilization. At 30 °C, the DnaK chaperone system destabilizes σ^{32} 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 σ^{32}, 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-N$_7$-ACTCAAG. 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.

pIJ8600 was used for controlled gene expression in *S. lividans* using the thiostrepton-inducible promoter *pTipA* (Sun *et al.*, 1999). pGM160Δ (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 Luria-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′-GAAGAATTCTACGGCGGTGCTGTCCC-GAGA-3′) and JU76 (5′-AAGAAGCTTCCAACGGCTGAC-GGCTCCTCC-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 *Eco*RI and *Hin*dIII and cloned into *Eco*RI/*Hin*dIII-digested pUC19 to generate pJV300. The 3 kb insert was sequenced and was 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′-ATACCATGGCTGCTGA-GTCCGCCGCCTTC-3′) and AS59 (5′-ATACTCGAGCGC-TGCGACCGGAACCTCACG-3′). The PCR fragment was digested with *Nco*I/*Xho*I and cloned into *Nco*I/*Xho*I-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′-ATACATATGGACGGTCGGCG-ACGCAACCCG-3′) and AS11 (5′-ATAAGATCTTCAGT-CCGAGGACTGGCCGCG-3′). These primers introduced *Nde*I and *Bgl*II sites into the resulting 450 bp DNA fragment. The *Nde*I site replaced the original *hspR* GTG translation start site with an ATG. The *Nde*I–*Bgl*II DNA fragment was cloned into the *Nde*I and *Bam*HI 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(λDE3)(pAS45) cells were grown at room temperature in LB medium containing kanamycin and chloramphenicol. When cultures reached an OD$_{600}$ of 0·6, the production of Lon-His$_6$ 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

1 ml Ni-NTA column (Qiagen) that had been equilibrated with buffer A. Lon-His$_6$ was eluted with a linear 20–300 mM imidazole gradient. The fractions with the highest protein concentration were pooled, dialysed for 2 h against buffer A and centrifuged at 13 000 g for 5 min. The supernatant (5 ml) was loaded onto the Ni-NTA column for a second identical run. The second protein pool was dialysed against storage buffer C (20 mM phosphate buffer pH 7·4, 50 mM NaCl and 10 % glycerol) and aliquots were stored at −80 °C. Lon-His$_6$ concentration was determined by the Bradford method. This purified protein was used to raise polyclonal antibodies against Lon.

lon-disrupting plasmid. The *Bam*HI cassette containing the hygromycin-resistance gene purified from pHP45ΩHygR (Blondelet-Rouault *et al.*, 1997) was used to disrupt the *S. lividans lon* gene. The *Bam*HI ΩHygR cassette was ligated into the *lon Bam*HI site in pAS45, 878 bp downstream from the *lon* ATG codon. Two plasmids were generated: pAS47 and pAS48. The *hygR* gene was transcribed in opposite direction to *lon* in pAS48. pAS48 was further digested with *Xba*I/*Nco*I. The sites for these enzymes are located close together in pAS48. Thus, we cloned the whole pAS48 plasmid between the *Nco*I and *Xba*I sites of pGM160Δ. The resulting *S. lividans* replication-thermosensitive plasmid was called pAS49 and was used to disrupt *lon*.

hspR-disrupting plasmid. Inverse-PCR (I-PCR) was used to clone *hspR* and large DNA fragments of its surrounding sequences from *S. lividans*. This allowed the region 3′ of *hspR* to be cloned in the absence of sequence data and also created an *Eco*RI site 100 bp downstream of the *hspR* translation start site (ATG) into which a resistance gene could be inserted. We used the pHP45ΩVioR (Blondelet-Rouault *et al.*, 1997) cassette containing the viomycin phosphotransferase gene (*vph*) from *Streptomyces vinaceus* for disruption experiments. *S. lividans* chromosomal DNA (5 μg) was digested with *Pst*I and ligated after dilution to facilitate the intramolecular ligation of the DNA fragments. Two divergent oligonucleotides were designed, AS1 (5′-ATAGAATTCTGGCGCAGCGTCTGC-GGGTG-3′) and AS2 (5′-ATAGAATTCCGCGACATCGA-ACTGCTCCG-3′), based on published *S. coelicolor* (cosmid H35, Sanger Centre sequencing project) and *S. albus* (Grand-valet *et al.*, 1997) *hspR* sequences. PCR was performed on 500 ng ligated *S. lividans* TK24 chromosomal DNA, 20 pmol AS1 and AS2, 10 % DMSO, 200 mM of each dNTP and 1 U *Pfu* DNA polymerase (Promega) as recommended by the manufacturer. PCR generated a 5·5 kb I-PCR fragment with enough DNA sequence upstream and downstream of *hspR* to favour double recombination in *S. lividans*. After purification, the PCR fragment was digested with *Eco*RI and cloned into pBluescript-SK to generate pAS2. The two *hspR* DNA fragments obtained after an *Eco*RI/*Pst*I digestion of pAS2 were purified separately, ligated to the *Eco*RI viomycin cassette and cloned into the *Pst*I site of pBluescript-SK(−) to give pAS3. pAS3 was selected for its ability to confer both ampicillin and viomycin resistance to *E. coli*. Inserts from pAS2 and pAS3 were partly sequenced to confirm the constructs. In pAS3, the *vph* gene is transcribed in the opposite direction to *hspR*. pAS3 was further digested with *Xba*I/*Nco*I to give a 7·6 kb DNA fragment encompassing the following *S. lividans* sequences: the 3′ end of *grpE*, the *dnaJ* gene, disrupted *hspR* and a 3 kb region downstream of *hspR*. This DNA fragment was cloned into the *Xba*I/*Nco*I sites of pGM160Δ to yield the *S. lividans* replication-thermosensitive *hspR*-disrupting plasmid, pAS25.

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^R 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 (1/10^4, 1/10^5 and 1/10^6) of cells were plated onto R5 medium supplemented with hygromycin. Hyg^R clones were finally patched on both R5 + hygromycin and R5 + thiostrepton plates to select Hyg^R and Tsr^s 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.

RNA analysis. RNA was prepared as described previously (Servant & Mazodier, 1996). The transcription start site upstream of *lon* was located by primer extension using oligonucleotide AB10 (5′-GTCGATGCGCGGGACGAGGA-G-3′) as previously described (Grandvalet *et al.*, 1999).

Gel retardation assay. A 300 bp 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′-GCGGGGCTTGCCGGGCTCGGA-3′). The purified fragment was end-labelled with [γ-^{32}P]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 MgCl$_2$, 1 mM DTT, 0·1 % Triton X-100, 50 mM NaCl and 10 % glycerol) and 1 μg sonicated herring sperm DNA. Samples were subjected to electrophoresis in 6 % polyacrylamide gels containing 50 mM Tris/HCl pH 8, 400 mM glycine, 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 YEME, 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 electrotrans-ferred to Immobilon membranes (Amersham). Antigens were detected by ECL Western blotting in the presence of rabbit polyclonal antibodies raised against purified *S. lividans* Lon-His$_6$.

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

from *lon* contains the following sequence: ATTGAGT-N$_7$-ACTCAAC, which is similar to the HAIR consensus motif CTTGAGT-N$_7$-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*.
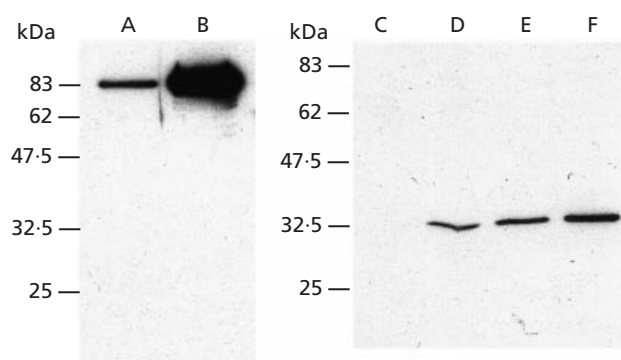
lon mutant

The chromosomal *lon* gene was disrupted by a double recombination event using the pAS49 vector containing *lon*::*hygR*. Candidate Hyg^R Tsr^S 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^R Tsr^S 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* may 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.

Fig. 1. Western blot analysis of *lon* expression. Total cell extracts were prepared from the wild-type strain (lanes A and B), the *lon* mutant (lanes C and D), or the *hspR lon* double mutant (lanes E and F). Cultures were grown at 30 °C (lanes A, C and E) or were subjected to a 40 min heat shock at 40 °C (lanes B, D and F). Different amounts of total protein were loaded: 5 μg in lanes A and B, 10 μg in lanes C–F. Blots were probed with polyclonal antibodies directed against the *S. lividans* Lon protein. These antibodies detect the bands corresponding to the 90 kDa Lon protein, and a 30 kDa peptide which corresponds to the predicted (29·5 kDa) truncated Lon protein.
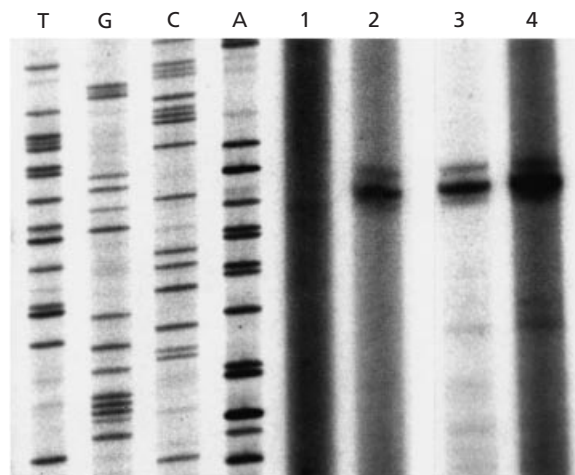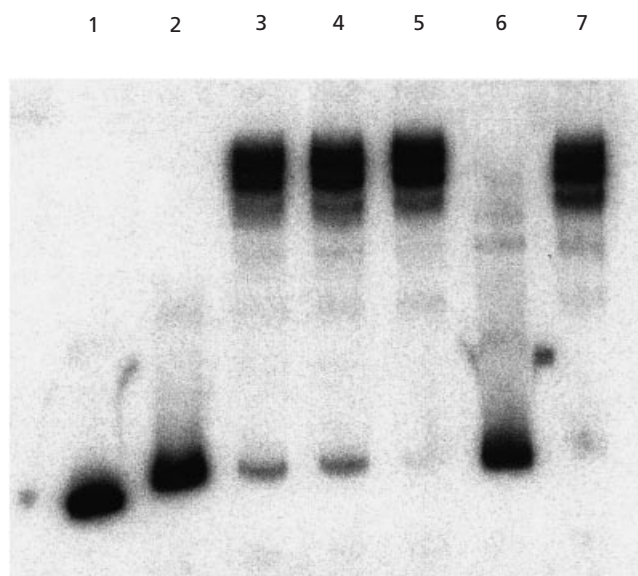

Fig. 2. Mapping of the *S. lividans lon* transcription start site by primer extension. Dideoxy-terminated DNA sequencing reaction products (lanes A, C, G and T) and primer extension reaction products are shown. RNA was isolated from the wild-type strain (lanes 1 and 2) or the *hspR lon* mutant (lanes 3 and 4). Cells were grown at 30 °C (lanes 1 and 3) or subjected to heat shock for 40 min at 40 °C (lanes 2 and 4).

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,

corresponding to the predicted (29·5 kDa) truncated Lon peptide, was present. In the *lon* mutant, this 30 kDa protein was heat induced, whereas in the *lon hspR* double mutant it was constitutively synthesized at 30 °C and 40 °C (lanes E and F).

Mapping the transcription start site of *lon*

The transcription initiation site of the *lon* gene was determined by primer extension. Primer extension was done on mRNA prepared from cultures grown at 30 °C or subjected to a 40 min heat shock at 40 °C. Cultures of wild-type, *lon* mutant and *lon hspR* double mutant were analysed. In the wild-type and in the *lon* mutant a heat-inducible transcript was detected at 40 °C; it started at an adenine in the loop part of the HAIR motif, ATTGAGT(cg**a**tgta)ACTCAAC. In the *lon hspR* this transcript was detected at 30 °C and was constitutively expressed independently of the temperature (Fig. 2).

Gel retardation assay with HspR

Gel mobility-shift assays were performed to determine whether HspR interacts directly with the *lon* promoter (Fig. 3). The 300 bp *lon* promoter region was amplified by PCR and labelled using oligonucleotides JU74 and JU75. Cell extracts of the control strain, without HspR, did not affect the mobility of the 300 bp fragment. In

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 protein 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., 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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