Lon protease of the α -proteobacterium *Agrobacterium tumefaciens* is required for normal growth, cellular morphology and full virulence

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The ATP-dependent Lon (La) protease is ubiquitous in nature and regulates a diverse set of physiological responses in bacteria. In this paper a *lon* mutant of the α -proteobacterium *Agrobacterium tumefaciens* C58 has been characterized. Unlike *lon* mutants of *Escherichia coli*, the *lon* mutant of *A. tumefaciens* grows very slowly, is not filamentous and exhibits normal resistance to UV irradiation. The mutant retains motility and chemotaxis, produces apparently normal amounts of exopolysacchride, but displays severe defects in cell morphology, with 80 % of the mutant cells appearing Y-shaped. Lon protease of *A. tumefaciens* shares high homology with its counterparts in *E. coli* and in *Sinorhizobium meliloti*, and functionally complements an *E. coli lon* mutant for defects in morphology and RcsA-mediated regulation of capsular polysaccharide production. Mutations at sites of Lon_{At} corresponding to the ATP-binding site and the active site serine of the *E. coli* Lon protease abolish complementation of phenotypes of the *A. tumefaciens* and *E. coli lon* mutants. The nucleotide sequence upstream of *A. tumefaciens lon* contains an element similar to the consensus σ^{32} heat-shock promoter of *E. coli*. Northern and Western blot analyses indicated that expression of *lon* is induced by elevated temperature, albeit to a much lower level than that of *groEL*. The *lon* mutant is highly attenuated for virulence, suggesting that Lon may be required for the proper expression, assembly or function of the VirB/D4-mediated T-DNA transfer system.

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INTRODUCTION

The ATP-dependent Lon (La) protease is a conserved protein present in diverse organisms, including archaea, bacteria, yeast, plants and animals. Lon from *Escherichia coli* (Lon_{Ec}) is an 87 kDa homo-oligomeric protein and contains highly conserved N-terminal ATPase and C-terminal protease domains (Goldberg *et al.*, 1994). Lon_{Ec} plays a primary role in the degradation of many abnormal proteins as well as unstable regulatory proteins. These latter targets include the λ N protein, the SulA cell division regulator, the positive regulator of capsule synthesis, RcsA, and the F plasmid antidote protein CcdA (Gottesman, 1996). *lon* mutants of *E. coli* exhibit a pleiotropic phenotype that includes increased sensitivity to UV irradiation (Howard-Flanders *et al.*, 1964), cell division defects resulting in filamentation (Howard-Flanders *et al.*, 1964), mucoidy (Markovitz, 1964) and reduced degradation of various abnormal proteins (Gottesman & Zipser, 1978). The phenotypic properties of *lon* mutants are the result of a decreased ability to degrade specific regulatory proteins; SulA in the case of filamentation and RcsA in the case of overproduction of capsular polysaccharide.

Lon plays important roles in the regulation of developmental functions in a number of other bacteria, including normal cell morphogenesis and developmental progression in *Caulobacter crescentus* (Wright *et al.*, 1996), fruiting body formation in *Myxococcus xanthus* (Gill *et al.*, 1993) and lateral flagellar biosynthesis in *Vibrio parahaemolyticus* (Stewart *et al.*, 1997). Recent studies have shown that Lon from *Brucella abortus* functions as a general stress-response protease and is required for wild-type virulence during the initial stages of infection in BALB/c mice (Roberston *et al.*, 2000). In *Sinorhizobium meliloti*, Lon is involved in the regulation of exopolysaccharide synthesis and is required for effective nodulation of alfalfa (Summers *et al.*, 2000).

The phytopathogenic α -proteobacterium *Agrobacterium tumefaciens*, a very close relative of *S. meliloti*, causes crown gall tumours on susceptible plants by transferring T-strand DNA from its tumour-inducing (Ti) plasmid into a susceptible host plant cell. This bacterium has served as a model for studies concerning a number of important biological phenomena, including plasmid conjugation, pathogen–host signalling, Type IV secretion, *trans*-kingdom gene transfer and quorum sensing. Given the requirement

for Lon protease in microbe–host interactions of other α -*Proteobacteria*, we constructed a *lon* null mutant of *A. tumefaciens* and characterized the effect of this mutation on a number of traits, including growth, cellular morphology and pathogenicity.

METHODS

Bacterial strains, media and growth conditions. Strains of *A. tumefaciens* and *E. coli* and the plasmids used in this study are listed in Table 1. Strains of *E. coli* were grown at 37 °C in Luria broth (LB; Invitrogen) or in minimal A glucose medium (Miller, 1972), while strains of *A. tumefaciens* were grown at 28 °C in low salt LB, in MG/L medium (Cangelosi *et al.*, 1991), in AB minimal medium (Chilton *et al.*, 1974) supplemented with 0.2 % mannitol as sole carbon source (ABM) and on nutrient agar (NA; Difco). All liquid cultures were grown with agitation to ensure adequate aeration. Growth in liquid cultures was monitored turbidimetrically using a Klett–Summerson colorimeter fitted with a red filter or with a Spectronic 20 spectrophotometer at 600 nm. Antibiotics were used at the following concentrations in $\mu\text{g ml}^{-1}$: for *E. coli*, kanamycin, 50; tetracycline, 10; ampicillin, 100; and gentamicin, 10; for *A. tumefaciens*, kanamycin, 50; tetracycline, 2; carbenicillin, 100; and gentamicin, 50. Calcofluor (Sigma) was added to LB plates at a final

concentration of $200 \mu\text{g ml}^{-1}$. L-Arabinose was added at a final concentration of 0.1 % to induce expression of the *lon* gene of *A. tumefaciens* from P_{BAD}.

Plasmids. Plasmid pBADlon, a derivative of pBAD22 (Guzman *et al.*, 1995) expressing Lon from *A. tumefaciens* C58 (Lon_{At}) under the P_{BAD} arabinose-controlled promoter, was constructed as follows. The *lon* gene (*lon*_{At}) was amplified from *A. tumefaciens* NTL4 chromosomal DNA with *Pfu* DNA Polymerase (Stratagene) and cloned into the *Xba*I site of pBAD22. A clone containing *lon*_{At} correctly oriented downstream to the BAD promoter was identified and named pBADlon (Table 1). A second PCR-amplified *lon* gene (*lon*_{At}) along with its own promoter region was cloned into the *Xba*I site of pAW50 (Wise *et al.*, 2005), creating plasmid pAWlon. This vector replicates at very low copy number in *A. tumefaciens* (Wise *et al.*, 2005). Site-directed mutations were introduced onto *lon*_{At} using the QuikChange Kit (Stratagene), according to the instructions of the manufacturer. Briefly, for construction of pBADlonK364Q, containing a mutation at lysine-364 in the ATP-binding motif, primers 5'-CTCCGGC-GTCGGCCAGACCTCGCTCGCCAAG-3' and 5'-CTTGGCGAGCG-AGGTCTGGCCGACGCCGGGAGG-3' were used. pBADlonS680A, carrying a mutation at the active site serine-680, was constructed by using primers 5'-CCGAAGGACGACCGGCCGCCGGTGTG-TGCCATG-3' and 5'-CATGGCAACACCGGCCGCCGGTCCGTCC-TTCGG-3'. Similar strategies were used to introduce the two mutations into *lon*_{At} cloned in pAW50. Cloned inserts and the

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>A. tumefaciens</i>		
NTL4	pTiC58-cured derivative of C58, Δ tetRS	Luo <i>et al.</i> (2001)
A136	pTiC58-cured derivative of C58, Rif ^R Nal ^R	Watson <i>et al.</i> (1975)
NTS1	NTL4 <i>lon</i> ::Gm ^R , variable colony size	This study
NTS2	NTL4 <i>lon</i> ::Gm ^R , stable colony size	This study
NTL4 Δ fla	NTL4 in which <i>flaABC</i> is replaced with a <i>tet</i> cassette	This study
<i>E. coli</i>		
DH5 α	F [−] Φ 80dlacZAM15 <i>endA1 recA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44 thi-1 gyrA96</i> Δ (<i>lacZYA-argF</i>)U169	Invitrogen
S17-1	Pro [−] Res [−] Mod ⁺ <i>recA</i> ; integrated RP4-Tet::Mu-Kan::Tn7, Mob ⁺	Simon <i>et al.</i> (1983)
HDB97	MC4100 (<i>ara714 cps</i> :: <i>lacZ</i>)	Bernstein & Hyndman (2001)
HDB98	HDB97 (<i>lon510</i>)	Bernstein & Hyndman (2001)
Plasmids		
pBAD22	ColE1 expression vector that contains P _{BAD}	Guzman <i>et al.</i> (1995)
pBADlon	<i>lon</i> _{At} cloned into pBAD22	This study
pBADlonK364Q	pBADlon expressing Lon _{At} with lysine at position 364 replaced by glutamine	This study
pBADlonS680A	pBADlon expressing Lon _{At} with serine at position 680 replaced by alanine	This study
pAW50	<i>E. coli</i> /Agrobacterium very low-copy-number shuttle vector	Wise <i>et al.</i> (2005)
pAWlon	<i>lon</i> _{At} cloned into pAW50	This study
pAWlonK364Q	pAWlon expressing Lon _{At} with lysine at position 364 replaced by glutamine	This study
pAWlonS680A	pAWlon expressing Lon _{At} with serine at position 680 replaced by alanine	This study
pUC19	Cloning vector	Our collection
pSlon	A 4988 bp PCR fragment containing the <i>lon</i> _{At} locus cloned into pUC19	This study
pMGm	Source of gentamicin resistance cassette	Murillo <i>et al.</i> (1994)
pSlonG	pSlon with a gentamicin resistance cassette inserted into the <i>lon</i> gene	This study
pSR47s	<i>ori</i> T _{RP4} / <i>ori</i> V _{REK} , a mobilizable <i>pir</i> -dependent vector	Merriam <i>et al.</i> (1997)
pSRlonG	pSR47s carrying a <i>Bam</i> HI– <i>Xba</i> I fragment containing the <i>lon</i> gene from pSlonG	This study
pTiR10	Octopine-type Ti plasmid	Our collection
pTiC58	Nopaline-type Ti plasmid	Our collection

mutant alleles were confirmed by automated DNA sequencing performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign.

Genetic manipulations. Plasmids were introduced into *E. coli* by CaCl_2 -mediated transformation and into *A. tumefaciens* strains by electroporation or by biparental mating using *E. coli* S17-1 (Simon *et al.*, 1983).

Disruption of the *A. tumefaciens* chromosomal *lon* gene.

Genomic DNA was prepared from an overnight culture of *A. tumefaciens* NTL4. A 4988 bp chromosomal fragment consisting of the entire coding sequences of *lon* and *clpX* along with the intergenic region between the two genes and an 879 bp sequence downstream from *lon* was amplified using *PfuTurbo* DNA polymerase (Stratagene) (Fig. 1a). The PCR product was cloned directly between the *Bam*HI and *Xba*I

sites of pUC19 to create pSlon (Fig. 1a). The gentamicin resistance cassette from pMGm (Murillo *et al.*, 1994) was excised by *Pst*I, made blunt by T4 DNA polymerase and inserted into the unique *Msc*I site within the *lon* gene in pSlon at a position corresponding to amino acid residue alanine-532, creating pSlonG (Fig. 1a). The *Bam*HI–*Xba*I fragment containing *clpX* and the disrupted *lon* gene was excised from pSlonG and cloned between the *Bam*HI and *Spe*I sites of pSR47s (Merriam *et al.*, 1997). The resulting plasmid, pSRlonG, was transformed into S17-1 λ pir and the resultant strain was mated with NTL4 as described previously (Cook & Farrand, 1992). NTL4 carrying the chromosomal disruption in *lon* was selected by plating on medium containing the appropriate antibiotics and 5% sucrose. Allelic exchange of the mutant *lon* gene for the wild-type allele was confirmed by Southern hybridization (data not shown). A similar strategy was used to construct NTL4 Δ *fla*, a *fla*[−], non-motile strain of *A. tumefaciens* in which the three tandem flagellum genes *flaA*, *flaB* and *flaC* were replaced with a tetracycline resistance cassette.

UV sensitivity. Sensitivity to UV irradiation was assayed as described by Miranda & Kuzminov (2003). Briefly, a fresh overnight culture was diluted 100-fold into 2 ml ABM and grown with shaking at 28 °C to 2×10^8 cells ml^{−1}. Tenfold serial dilutions were made in 0.9% NaCl and 10 μ l volumes of a set of the serial dilutions were spotted in rows of six onto a square Petri dish containing LB agar. The liquid in each spot was allowed to dry down, the plates were partially covered with a screen and exposed to a gradient of doses of UV light in the direction perpendicular to the dilution gradient, so that every dilution column (from 10^{-1} to 10^{-6}) received its own dose. A UV cross-linker (Amersham-Pharmacia), in which all the lamps except the central one were removed and 90% of the remaining lamp was shielded, was used to deliver precise doses of UV (measured by the internal UV sensor). Immediately after exposure, the plate was covered with aluminium foil and incubated at 28 °C for 24 h. The titre of the culture at the zero dose was used to determine the survival at various UV doses.

Motility and chemotaxis assays. Motility assays were conducted in ABM medium solidified with 0.3% agar as described by Ding & Christie (2003). Cell cultures were normalized to an OD₆₀₀ of 0.5 and a 2 μ l volume of each strain was inoculated onto the surface of the motility plates. The plates were examined after 12, 24 and 48 h incubation at 28 °C. Chemotaxis assays were performed on 0.3% soft agar plates without carbon sources. A sterile Whatman paper disk saturated with 15% (w/v) sucrose, glucose or mannitol was placed at the centre of the agar plate. Two microlitres of each cell culture normalized to an OD₆₀₀ of 0.5 was inoculated onto the swarm plate 4 cm from the paper disk. The chemotaxis plates were maintained at 28 °C and examined throughout a 96 h incubation period.

Electron microscopy. *Agrobacterium* strains were grown overnight with shaking at 28 °C in LB containing appropriate antibiotics to an OD₆₀₀ of approximately 0.8. One millilitre of each culture was washed three times and resuspended in sterile PBS buffer. Cells from the washed cultures were absorbed to Formvar-coated nickel grids (EM sciences) for 1 min. Excess culture was blotted with Whatman filter paper and the grid was placed face down on a drop of 1% uranyl acetate (EM Sciences) for 1 min. Excess stain was blotted with Whatman filter paper, the grid was air-dried and viewed with an LEO 906e transmission electron microscope operating at an accelerating voltage of 80 kV.

β -Galactosidase assay. Production of β -galactosidase by *E. coli* strains grown in A medium was quantified using a modification of the Miller method (Miller, 1972). Activity was expressed as units of enzyme per 10⁹ c.f.u. Samples were assayed in triplicate and the experiments were repeated three times. Results from a single representative experiment are shown.

Fig. 1. The *lon* gene of *A. tumefaciens* C58 and its protein product. (a) Genetic organization of the *lon* region from the chromosome of *A. tumefaciens* strain C58. The arrows indicate the direction of transcription. The fragment consisting of the entire coding sequences of *lon* and *clpX* along with the intergenic region between the two genes and an 879 bp sequence downstream from *lon* was cloned between the *Bam*HI and *Xba*I site of pUC19 to create pSlon. A gentamicin resistance cassette was inserted into the unique *Msc*I site to disrupt the coding sequence of the *lon*_{At} gene in pSlon, creating pSlonG. (b) Location of the two major consensus domains in Lon_{At}. The ATPase Walker A motif and the proteolytic active site serine are shown as well as the sites of the two amino acid substitution mutations, LonK364Q and LonS680A. (c) Proteins in total cell lysates of *A. tumefaciens* NTL4 (*lon*⁺) and NTS2 (*lon*[−]) were separated by 7.5% SDS-PAGE and Lon was detected by immunoblotting with polyclonal rabbit antiserum directed against *B. abortus* Lon as described in Methods.

RNA preparation. Cultures of *A. tumefaciens* strains were grown to an OD₆₀₀ of 0.5 in ABM medium at 20 °C or shifted to 37 °C and sampled after 5, 10, 15 and 30 min incubation at the elevated temperature. Total RNA was isolated using Trizol reagent (Invitrogen) as follows. Each 50 ml culture sample was rapidly chilled on ice and the cells were collected by centrifugation. The cell pellet was homogenized in 5 ml hot Trizol reagent (65 °C) and the mixture was shaken at 65 °C for 10 min. Chloroform (1 ml) was added, the tube was shaken vigorously by hand for 15 s and incubated at room temperature for 2–3 min. The phases were separated by centrifugation at 12 000 g for 15 min at 4 °C and the upper phase was removed to a new tube. One volume of isopropyl alcohol (2.5 ml) was added, mixed well and the mixture was incubated at room temperature for 10 min. The precipitated RNA was collected by centrifugation at 12 000 g for 10 min at 4 °C. The supernatant was removed and the pellet was washed once with 5 ml 75 % ethanol and air-dried to near completion. The RNA was dissolved in 300 µl RNase-free water and the preparation was treated with RNase-free DNase [RQ1 DNase (Promega)] to remove contaminating DNA. The preparation was extracted with phenol/chloroform to remove the DNase. The RNA was precipitated with isopropyl alcohol, collected by centrifugation, dried to near completion and dissolved in 100 µl RNase-free water. The RNA concentration was quantified using a Lambda 3B spectrophotometer (Perkin Elmer).

RNA blotting and hybridization. RNA samples (20 µg) were separated on 1.5 % agarose gels containing 2.2 M formaldehyde and transferred to a positively charged nylon membrane (Roche) by capillary action in 20 × SSC (3 M NaCl, 0.3 M tri-sodium citrate). An 887 bp *groEL* DNA probe and a 1.2 kb *lon*_{At} DNA probe were purified from agarose gels and labelled with digoxigenin [(DIG)-11-dUTP] using the DIG-High Prime labelling kit (Roche). DIG labelling, prehybridization, hybridization, posthybridization washes and detection with chemiluminescent substrate were performed according to the instructions of the manufacturer (Roche). DIG-labelled RNA molecular mass marker set II (Roche) was used as the RNA ladder.

Immunoblotting. Cultures of *A. tumefaciens* were grown in ABM at 20 or 37 °C as described above. Proteins in total cell lysates were resolved by 7.5 % SDS-PAGE polyacrylamide gels loaded on a per cell equivalent basis and the separated proteins were transferred to nitrocellulose membrane after electrophoresis. Immunoblots were developed with polyclonal rabbit antiserum directed against *Brucella abortus* Lon obtained from Dr R. Martin Roop II (Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, USA). Primary antibody was detected with horseradish peroxidase (HRP)-conjugated monkey anti-rabbit antiserum. Antibody–antigen interactions were visualized by chemiluminescence with an ECL kit (GE Healthcare). The intensity of protein bands was quantified by digital scanning and analysis of developed X-ray films with NIH Image 1.63 software.

Virulence assays. Tumorigenesis was assessed on leaves of *Kalanchoë diagraphmontiana* using the culture dilution method as described by Nair *et al.* (2003). Strains were grown in MG/L overnight at 28 °C with the appropriate antibiotics. Cells were collected by centrifugation, resuspended in 0.9 % (w/v) NaCl to an OD₆₀₀ of 1.0 and further diluted as necessary in 0.9 % NaCl. Two-centimetre-long wounds made with an 18-gauge needle on the youngest expanded leaves of a 5-week-old *K. diagraphmontiana* plant with three pairs of leaves were inoculated with 3 µl of these dilutions. Tumour formation was monitored over a period of 25 days. Assays were done in triplicate and the experiments were repeated three times.

RESULTS

A. tumefaciens lon locus

Based on the whole-genome sequence of *A. tumefaciens* C58 (Wood *et al.*, 2001), the *lon*_{At} gene (Atu1261), located on the circular chromosome, is 2418 bp in length and encodes a protein of 805 aa with a calculated molecular weight of 88 750. *Lon*_{At} shares 86 % amino acid identity and 92 % similarity with the Lon protease from *S. meliloti* (*Lon*_{Sm}) (Summers *et al.*, 2000), 81 % identity and 90 % similarity with Lon from *B. abortus* (*Lon*_{Ba}) (Roberston *et al.*, 2000), and 61 % identity and 75 % similarity with *Lon*_{Ec} from *E. coli* (Chin *et al.*, 1988). The deduced amino acid sequence of *Lon*_{At} contains a typical ATP-binding motif, GPPGVGK₃₆₄T and KAKKANPLFLD (Chin *et al.*, 1988) at amino acid coordinates 358–365 and 414–425, respectively, and a consensus serine protease active site motif IHVHVPEGATPKDGPS₆₈₀AG (Amerik *et al.*, 1991) at amino acid coordinates 665–682 (Fig. 1b). These features are conserved in all known Lon proteases. The *A. tumefaciens lon* gene is preceded by an ORF encoding a homologue of ClpX, another ATP-dependent protease (Fig. 1a), which is separated from *lon*_{At} by a 414 bp intergenic region predicted to encode the promoter of the *A. tumefaciens lon* gene. Located 275 bp downstream from the *lon* gene, a *hupB* homologue could encode a histone-like protein whose counterpart in *E. coli* is involved in regulating transcription by constraining DNA supercoils and DNA accessibility to regulatory proteins (Drlica & Rouvière-Yaniv, 1987).

The *lon* mutation impairs cell growth

To explore the role of Lon protease in *A. tumefaciens*, we constructed a null mutant by inserting a gentamicin resistance cassette into the *lon* gene by double-crossover homologous recombination as described in Methods (Fig. 1a). On the selection plates, we noted two obviously different-sized colonies, both of which grew slower than the wild-type parent. One such clone, named NTS1, formed very small colonies and grew very slowly. The second, named NTS2, formed relatively larger colonies and grew faster than NTS1. When streaked out for single colony isolation, NTS1 gave two colony sizes, the small one like its parent and larger sized colonies resembling NTS2. When similarly streaked out, NTS2 gave colonies of homogeneous size which grew faster than those of NTS1, but slower than the wild-type parent. Southern blot analysis indicated that the *lon* gene of both NTS1 and NTS2 was disrupted as predicted (data not shown). Strain NTL4 grew with a doubling time of about 2.5 h in ABM minimal medium (Fig. 2). However, strains NTS1 and NTS2 grew with a doubling time of around 8 and 4 h, respectively, in this medium (Fig. 2). To verify that the slow growth phenotype of the *lon* mutants is due to the *lon* mutation, the two strains were complemented with *lon*_{At} cloned with its own promoter in the low-copy-number plasmid pAW50 (Table 1). Derivatives of NTS1 and NTS2 harbouring pAWlon exhibited growth rates similar to that of the wild-type parent (Fig. 2). Viability assays showed

Fig. 2. Growth of *A. tumefaciens* wild-type and *lon* mutant strains in ABM minimal medium. Turbidity values of cultures, expressed as Klett units, were determined at the given times. Data points are from a single culture for each strain and are representative of one of three experiments. ○, Wild-type strain NTL4; ▲, *lon* mutant NTS1; △, NTS1(pAWlon); ●, *lon* mutant NTS2; □, NTS2(pAWlon).

that the mutant gave the same number of c.f.u. as its parent strain at the equivalent levels of turbidity (data not shown). Because of its consistent growth properties, we chose NTS2 for further studies. As assessed by Western analysis, NTS2 failed to produce any protein that interacts with the Lon antiserum (Fig. 1c). The wild-type strain NTL4 yielded a single reactive protein with a mobility corresponding to 89 kDa.

Exopolysacchride production

Succinoglycan is the major acidic exopolysacchride produced by *A. tumefaciens* and can be easily detected by the binding of the optical brightening agent Calcofluor. When streaked out on LB medium containing Calcofluor, colonies of NTS2 fluoresced under UV light with an intensity indistinguishable from those of NTL4 (data not shown).

UV sensitivity and cellular morphology

lon mutants of *E. coli* exhibit enhanced UV sensitivity and filamentation. Strain NTS2 was no more sensitive to UV irradiation at doses ranging from 20 to 60 J m² than its parent, NTL4 (data not shown). Unlike *lon* mutants of *E. coli*, NTS2 did not produce filaments even following UV irradiation (Fig. 3a, panels 1 and 2, and data not shown). However, as seen by phase-contrast microscopy, the *lon* mutant, grown under normal conditions, displayed obvious

Fig. 3. A *lon* mutation affects cell morphology in *A. tumefaciens*. (a) Phase-contrast microscopy; (b) electron microscopy. Cells of wild-type strain NTL4 (a, 1; b, row 1) show a typical short rod shape while most cells of the *lon* mutant NTS2 (a, 2; b, row 2) are branched, appearing as Y shapes. Complementation of the *lon* mutant cells by expression of a wild-type *lon* gene from pAWlon restored the wild-type cell morphology (a, 3; b, row 3). No complementation was observed in the *lon* mutant complemented with the clone expressing Lon_{At}S680A (a, 4). The arrows in (a) 2 and 4 indicate aberrant cell shapes. The bars in (b) indicate 1 μm.

differences in cell morphology (Fig. 3a, panel 2). Electron microscopic examination showed that cells of the wild-type strain NTL4 exhibited a typical short rod shape (Fig. 3b, row 1). However, approximately 80–85 % of the mutant cells were branched, appearing as distinct Y shapes (Fig. 3b, row 2). NTS2 harbouring pAWlon took on a near wild-type shape, although the complemented cells exhibited a somewhat swollen appearance (Fig. 3a, panel 3, and Fig. 3b, row 3). Plasmids expressing the two Lon_{At} mutants, LonK364Q and LonS680A, did not restore normal cell morphology (Fig. 3a, panel 4, and data not shown).

Motility and chemotaxis

Given its aberrant cell morphology, we assessed the influence of the *lon* mutation on motility and chemotaxis using soft agar medium. As shown in Fig. 4(a), the *fla*[−] strain NTL4Δ*fla*, which is deleted for the three flagellum genes *flaA*, *flaB* and *flaC*, is completely non-motile. In contrast, wild-type strain NTL4 is strongly motile. The *lon* mutant NTS2 is also motile, although the zone of spreading is smaller than that of its parent (Fig. 4a). The complemented mutant NTS2(pAWlon) is as motile as the wild-type NTL4. When viewed by phase-contrast microscopy, the percentage of NTS2 cells that were motile appeared to be similar to that of its wild-type parent (data not shown). The wild-type strain NTL4, the mutant NTS2 and the complemented mutant NTS2(pAWlon) all exhibited chemotaxis toward glucose (Fig. 4b), as well as toward sucrose and mannitol (data not shown).

Fig. 4. The *lon* mutant of *A. tumefaciens* is motile and exhibits chemotaxis. (a) Motility was assayed on 0.3% agar plates as described in Methods. (b) Taxis by the strains toward glucose applied to a Whatman filter disk at the centre of plate was assessed as described in Methods.

Genetic complementation of an *E. coli lon* mutant

We determined whether Lon_{At} is equivalent to its *E. coli* homologue using two tests. In the first, we examined whether Lon_{At} could correct the SulA-associated filamentous phenotype exhibited by *E. coli lon* mutants. *E. coli* HDB98 (*lon*510) formed long filaments (Fig. 5a, panel 2), while the strain expressing Lon_{At} exhibited normal cell morphology (Fig. 5a, panel 3). However, neither of the two

Fig. 5. Lon_{At} complements the filamentous and mucoid phenotypes of a *lon* mutant of *E. coli*. (a) Phase-contrast microscopy of strains: 1, HDB97 (*lon*⁺); 2, HDB98 (*lon*); 3, HDB98(pBADlon); 4, HDB98(pBADlonS680A). (b) Cultures of *E. coli* HDB97 (*cpsB*::*lacZ*) and its *lon* mutant HDB98 with or without a *lon*_{At}-expressing plasmid as noted, were grown in minimal A medium with (black bars) or without (white bars) 0.1% arabinose. Cultures were sampled and assayed for β-galactosidase activity.

*Lon*_{At} mutants complemented the cell division defect (Fig. 5a, panel 4, and data not shown). We also assessed the ability of *Lon*_{At} to complement the defect in regulation of *cpsB* by *RcsA*. The *Lon*⁺ *E. coli* strain HDB97, which contains a *cpsB*::*lacZ* fusion, expressed very little β -galactosidase activity, whereas its near isogenic *lon* mutant, HDB98 in which *RcsA* is not turned over, expressed the reporter at a high level (Fig. 5b). Expression of *Lon*_{At} from pBADlon in HDB98 resulted in a strong depression of expression of the *cpsB*::*lacZ* reporter while the same strain expressing either of the two *Lon*_{At} mutants exhibited high levels of β -galactosidase activity characteristic of the host *lon* mutant (Fig. 5b).

Transcription of the *A. tumefaciens lon* gene is induced by growth at elevated temperature

In *E. coli*, *lon* is part of the heat-shock regulon. We assessed the effect of a temperature shift from 20 to 37 °C on the expression of *lon*_{At} in *A. tumefaciens* using two assays. In the first, we examined the level of mRNA transcripts of *lon* accumulated in the cells upon shifting to elevated temperature. In Northern blot analysis, levels of *groEL* mRNA, which appeared as two bands at 2.1 and 1.7 kb, increased to a maximum after 15 min incubation at the elevated temperature (Fig. 6a). In a similar analysis using the *lon* probe, the *lon*-specific mRNA was not detectable in cells grown at 20 °C, but produced a strong signal at 2.5 kb in cells grown for 15 min at the elevated temperature (Fig. 6b). The *lon* mRNA was not detectable in cells grown for 5 and 10 min at 37 °C and disappeared from cells grown for 30 min at the elevated temperature (Fig. 6b). In the second, we evaluated the level of Lon protein after temperature shift using an immunoblot assay. The amount of Lon protein increased slightly but reproducibly after heat shock, reaching its maximum at 15 min followed by a small decrease in level after 30 min (Fig. 6c and d).

The *lon* mutant is highly attenuated for virulence

Given that *lon* mutants of *B. abortus* and *S. meliloti* exhibit defects in interaction with their eukaryotic hosts (Roberston *et al.*, 2000; Summers *et al.*, 2000), we determined if the *lon* mutation influences the virulence of *A. tumefaciens*. Strains NTL4, NTS2 and their derivatives harbouring pTiC58 or pTiR10 were inoculated onto *K. diagrammontiana* leaves at decreasing cell concentrations as described in Methods. Strains NTL4 and NTS2 which lack Ti plasmids were avirulent (Fig. 7). Strains NTL4(pTiC58) and NTL4(pTiR10) induced tumours at all infection doses, although NTL4(pTiC58) yielded less tumour mass than did NTL4(pTiR10) at the lowest inoculation dose ($OD_{600}=0.01$). However, NTS2(pTiC58) and NTS2(pTiR10) failed to induce tumours at the lowest inoculation dose ($OD_{600}=0.01$) and induced much less tumour mass at the higher inoculation doses ($OD_{600}=0.1$ and 1.0 , respectively) as compared to the wild-type parent (Fig. 7). Introducing pAWlon into strains NTS2(pTiC58) and NTS2(pTiR10) restored wild-type levels

Fig. 6. Expression of *lon*_{At} responds to elevated temperature. (a, b) Northern analysis of the *groEL* and *lon* genes. Total RNA of *A. tumefaciens* was isolated before and at different times (5, 10, 15 and 30 min) after raising the incubation temperature from 20 to 37 °C. An 887 bp *groEL* DNA fragment and a 1.2 kb *lon* DNA fragment were labelled with digoxigenin (DIG)-11-dUTP and used as probes for hybridization. (a) Hybridization with the *groEL* probe; (b) hybridization with the *lon* probe. (c) Immunoblot analysis of the levels of Lon protein in cells before and at different times (5, 10, 15 and 30 min) after raising the incubation temperature from 20 to 37 °C. Proteins in total cell lysates were separated by 7.5% SDS-PAGE and Lon was detected by immunoblotting with polyclonal rabbit antiserum directed against *B. abortus* Lon as described in Methods. (d) The intensity of protein bands in (c) was quantified by digital scanning and analysis of developed X-ray films with NIH image 1.63 software. Experiments were repeated two times and data are representative of one of two experiments.

of virulence to the *lon* mutant harbouring either Ti plasmid (Fig. 7). Mutations in the ATPase or the proteolytic domains of Lon expressed from pAWlonK364Q or pAWlonS680A abolished complementation of the attenuated phenotype (Fig. 8b).

We considered the possibility that the decrease in virulence of NTS2 is due to its slow growth. Our isolate of *A. tumefaciens* A136, a Rif^R Nal^R derivative of strain C58 (Watson *et al.*, 1975), grows slower than its parent strain NTL4 and, with a generation time of 3.5 h in minimal medium, grows at a rate similar to that of NTS2 (Fig. 8a).

Fig. 7. The *lon* mutant of *A. tumefaciens* is attenuated for virulence. Strains were grown and inoculated onto *K. diagramontiana* leaves as described in Methods. Wounds in leaves were inoculated with strains diluted to population sizes indicated by the OD_{600} values at the bottom: A, NTL4; B, NTS2; C, NTL4(pTiC58); D, NTL4(pTiR10); E, NTS2(pTiC58); F, NTS2(pTiR10); G, NTS2(pTiC58, pAWlon); H, NTS2(pTiR10, pAWlon). Labels to the left indicate strains inoculated on the left side of each leaf while labels to the right indicate strains inoculated on the right side of each leaf.

The presence of pTiR10 had no effect on their respective growth rates (data not shown). While NTS2 harbouring pTiR10 was highly attenuated, A136 harbouring the same Ti plasmid was strongly tumorigenic (Fig. 8b).

DISCUSSION

Given its strong conservation across phylogenetic lines, it is not surprising that Lon protease from *A. tumefaciens* is closely related to that of *E. coli* and in particular to that of *S. meliloti*, a very close relative in the family *Rhizobiaceae*. It also is not surprising that Lon_{At} requires both the Walker box motif and the active site serine; both domains are required for the activity of the *E. coli* enzyme. Consistent with these genetic, phylogenetic and enzymic relationships, the organization of the entire *lon* locus is conserved; the region flanking *lon* encodes *clpX* and *hupB* (Fig. 1) on the circular chromosome of *A. tumefaciens* C58 as well as on the chromosomes of many prokaryotes (Chin *et al.*, 1988; Roberston *et al.*, 2000; Stewart *et al.*, 1997; Summers *et al.*, 2000). However, despite these similarities, Lon from *A. tumefaciens* targets at least a subset of proteins quite different from those turned over by this protease in *E. coli*.

The influence of Lon on cell shape provides an example of such target diversity. Lon mutants of *A. tumefaciens* and *E. coli* both exhibit altered cell morphologies. However, while *lon* mutants of *E. coli* produce long filaments, cells of the *A. tumefaciens* *lon* mutant exhibit swellings and branching morphologies (Figs 3 and 5). Clearly, at the cellular level Lon is important to the regulation of cell division in both *E. coli* and *A. tumefaciens*. However, the protease contributes to

Fig. 8. The attenuated virulence of the *lon* mutant NTS2 is not due to its slow growth. (a) Growth of Lon⁺ *A. tumefaciens* strains NTL4 and A136 and the *lon* mutant NTS2 in ABM minimal medium. The optical density at 600 nm of the cultures was determined at the indicated times. Data are representative of one of three experiments. ○, NTL4; △, A136; ▲, *lon* mutant NTS2. (b) Virulence assays on *K. diagramontiana* leaves. Wounds in leaves were inoculated with strains: A, NTL4; B, NTS2; C, A136; D, NTS2(pTiR10); E, A136(pTiR10); F, NTS2(pTiR10, pAWlon); G, NTS2(pTiR10, pAWlonK364Q); H, NTS2(pTiR10, pAWlonS680A). All strains were inoculated at a population size corresponding to an OD_{600} of 1.0.

this process by recognizing quite different targets in these two bacteria. In *E. coli*, *lon* mutants fail to degrade Sula, a cell division inhibitor, resulting in filamentation (Mizusawa & Gottesman, 1983; Schoemaker *et al.*, 1984). On the other hand, among the α -*Proteobacteria*, in the most well-studied relative of *A. tumefaciens*, *C. crescentus*, Lon contributes to the regulation of cell division by controlling the intracellular levels of CcrM, a DNA methylase. The levels and thereby the activity of this enzyme signal the initiation of DNA replication (Wright *et al.*, 1996). CcrM is a target of Lon and the unregulated accumulation of this essential methylase in *lon* mutants results in abnormal timing of initiation of DNA replication (Wright *et al.*, 1996). The defect in the timing of initiation most probably results in the altered cellular

morphology through a pathway that couples chromosome replication to cell division. While *A. tumefaciens* lacks Sula, it contains an orthologue of the *C. crescentus* *ccrM* gene (Kahng & Shapiro, 2001). This gene also is essential in *A. tumefaciens* and overexpression of *ccrM* results in altered cellular morphologies very similar to that of the *lon* mutant (Kahng & Shapiro, 2001). Moreover, overexpression of CcrM in *S. meliloti* results in cells with morphologies indistinguishable from that of the *Agrobacterium* *lon* mutant (Wright *et al.*, 1995). We hypothesize that in *A. tumefaciens* CcrM is degraded by Lon and that aberrant accumulation of CcrM in the *lon* mutant causes inappropriate DNA methylation which interferes with the timing of DNA replication and subsequent cell division. CcrM, but not Sula, is present and strongly conserved in all of the α -*Proteobacteria* for which sequence data are available (Wright *et al.*, 1995). We conclude that while Lon plays a central role in controlling cell division in both groups of prokaryotes, the targets of this protease, and therefore the mechanism of regulation, differs between the α - and γ -*Proteobacteria*. Moreover, while the role of Lon in modulating cell morphology is most pronounced during the SOS response within the γ -*Proteobacteria*, the protease is more completely integrated into normal cell division processes in the α -subgroup.

Lon_{At} complements *E. coli* *lon* mutants for filamentation and regulation of expression of *cps*, the operon encoding production of colonic acid capsule (Fig. 5). Thus, while the protease controls cell division in the two groups of bacteria by targeting different regulatory elements, Lon from *Agrobacterium* retains its ability to recognize Sula and also

RcsA, the positive activator of the *cps* regulon from *E. coli*. Lon also is required for proper regulation of extracellular polysaccharide synthesis in the close relative, *S. meliloti* (Summers *et al.*, 2000). However, Lon apparently does not control extracellular polysaccharide in *A. tumefaciens*; as judged by Calcofluor binding, the level of extracellular polysaccharide production by the *lon* mutant is not detectably altered from that of its wild-type parent.

On initial isolation, the *lon* mutants formed two colony morphotypes, one slow-growing and unstable, the other growing somewhat faster but stable (Fig. 2 and data not shown). Given the instability of the NTS1-type and the stability of the NTS2-type colonies, we consider it likely that the stable morphotype contains a second, spontaneous mutation that suppresses some strongly deleterious effect of the *lon* mutation on growth. The nature of this suppressor mutation is not known, but it is unlikely to contribute to the other *lon* phenotypes exhibited by NTS2. All such phenotypes are complementable by wild-type *lon* expressed from its own promoter from a low-copy-number vector.

Two lines of evidence indicate that Lon is a member of the heat-shock regulon in *A. tumefaciens*. First, the transcription of *lon*_{At} was transiently stimulated by elevated temperature (Fig. 6b). Second, the increase in mRNA level was accompanied by a small but reproducibly detectable increase in the intracellular levels of the Lon protein (Fig. 6c and d). Analysis of the nucleotide sequence upstream of *lon*_{At} revealed an element similar to the consensus σ^{32} heat-shock promoter of the γ -proteobacterium, *E. coli* (Fig. 9a). Significantly, a σ^{32} homologue, RpoH, plays a major and global

Fig. 9. Promoter region of *A. tumefaciens* *lon* contains a σ^{32} -like sigma factor recognition site and two inverted repeats. (a) Alignment of promoter sequences of *lon* in α -*Proteobacteria*. Bacteria are abbreviated as follows: Ba, *B. abortus*; Cc, *C. crescentus*; Sm, *S. meliloti*; At, *A. tumefaciens*. The identified and putative start sites of heat-inducible transcripts are underlined and boxed, respectively. N_xatg indicates the number of nucleotide bases between the transcriptional start site and the translation start codon of *lon*. Bases found in at least three of four promoters are indicated in upper case and bold type. Consensus promoter sequences deduced from upstream untranslated regions of *lon* in α -*Proteobacteria* and those of the RpoH-dependent promoters in *A. tumefaciens* (Nakahigashi *et al.*, 1999) and *E. coli* σ^{32} (Yura *et al.*, 1993) are shown for comparison. (b) A 16 bp inverted repeat (IR) located between the putative -10 and -35 regions and a second, 14 bp IR located downstream of the putative transcriptional start site are shown.

role in regulating the heat-shock response in *A. tumefaciens* (Mantis & Winans, 1992; Segal & Ron, 1995; Rosen *et al.*, 2002; Nakahigashi *et al.*, 1999). No sequences upstream of the -35 region of the *lon* promoter were found conserved in the corresponding upstream region of other heat-shock promoters in *Agrobacterium* or *E. coli*. However, a 16 bp inverted repeat (IR) is located between the putative -10 and -35 regions, and a second 14 bp IR is located downstream of the putative transcriptional start site (Fig. 9b). While these IR elements are not conserved in other α -*Proteobacteria*, they may play a role in the transcriptional or posttranscriptional regulation of *lon* in *A. tumefaciens*. Expression of *lon* is also induced by elevated temperature in *B. abortus* (Roberston *et al.*, 2000) and *S. meliloti* (Mitsui *et al.*, 2004), suggesting that, as in the γ -*Proteobacteria*, inclusion of *lon* in the heat-shock regulon may be a common feature in the α -*Proteobacteria*. Interestingly, given that NTS2 does not display increased sensitivity to UV irradiation it is unlikely that Lon is part of the SOS response in *A. tumefaciens*.

Lon in its wild-type form is required for full pathogenicity by *A. tumefaciens* (Fig. 7). Hence, ours joins a growing list of reports regarding the influence of this protease on the interaction of bacteria with their eukaryotic hosts. For example, among the α -*Proteobacteria*, Lon of *B. abortus* is required during the initial stages of infection in BALB/c mice (Roberston *et al.*, 2000) while in *S. meliloti* Lon is required for formation of effective, nitrogen-fixing nodules in alfalfa (Summers *et al.*, 2000). In the γ -*Proteobacteria* Lon is important for systemic infection of mice by *Salmonella enterica* serovar Typhimurium (Takaya *et al.*, 2003). Pathogenesis of *A. tumefaciens* is a complex process involving signalling, gene regulation, assembly and function of a Type IV secretion system (T4SS), and physical interactions between the bacterium and its host plant. At present we do not know how the *lon* mutation affects these processes. Lon might target some protein involved in controlling expression of the *vir* regulon, the functions of which are involved in T-strand processing, transfer and integration into the plant genome. Alternatively, the mutation might interfere mechanistically with T-strand processing or with the assembly or function of the VirB T4SS. Interestingly, a mutant of strain C58 defective in the TAT transport system shows defects in cell morphology and virulence that phenotype those of the *lon* mutant (Ding & Christie, 2003). It will be interesting to determine whether the loss of virulence associated with defects in Lon protease and the TAT secretory system reflect common or different targets.

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