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RamA, which controls expression of the MDR efflux pump AcrAB-TolC, is regulated by the Lon protease

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Objectives: RamA regulates the AcrAB-TolC multidrug efflux system. Using *Salmonella* Typhimurium, we investigated the stability of RamA and its impact on antibiotic resistance.

Methods: To detect RamA, we introduced *ramA*::3XFLAG::*aph* into plasmid pACYC184 and transformed this into *Salmonella* Typhimurium SL1344*ramA::cat* and *lon::aph* mutants. An N-terminus-deleted mutant [pACYC184*ramA*(Δ2-21)::3XFLAG::*aph*] in which the first 20 amino acids of RamA were deleted was also constructed. To determine the abundance and half-life of FLAG-tagged RamA, we induced RamA with chlorpromazine (50 mg/L) and carried out western blotting using anti-FLAG antibody. Susceptibility to antibiotics and phenotypic characterization of the *lon* mutant was also carried out.

Results: We show that on removal of chlorpromazine, a known inducer of *ramA*, the abundance of RamA decreased to pre-induced levels. However, in cells lacking functional Lon, we found that the RamA protein was not degraded. We also demonstrated that the 21 amino acid residues of the RamA N-terminus are required for recognition by the Lon protease. Antimicrobial susceptibility and phenotypic tests showed that the *lon* mutant was more susceptible to fluoroquinolone antibiotics, was filamentous when observed by microscopy and grew poorly, but showed no difference in motility or the ability to form a biofilm. There was also no difference in the ability of the *lon* mutant to invade human intestinal cells (INT-407).

Conclusions: In summary, we show that the ATP-dependent Lon protease plays an important role in regulating the expression of RamA and therefore multidrug resistance via AcrAB-TolC in *Salmonella* Typhimurium.

Keywords: *Salmonella*, transcription factors, proteolysis

Introduction

RamA is an AraC/XylS transcriptional activator that regulates the expression of the genes encoding the AcrAB-TolC resistance-nodulation-division multidrug efflux system in *Salmonella enterica* serovar Typhimurium[1–3] and other Enterobacteriaceae, including *Klebsiella*[4] and *Enterobacter* spp.[5] In *Salmonella* Typhimurium, the expression of *ramA* is repressed by RamR, a member of the TetR family of transcriptional repressors, which usually contain an N-terminal DNA-binding domain and a C-terminal ligand-binding regulatory domain.[6–8] The function of RamR can be ablated by internal point mutations and insertions and/or deletions within the operator target site of RamR at the *ramA* promoter.[8–12] These genetic events confer multidrug resistance (MDR), by preventing RamR binding to its operator target site at the *ramA* promoter, thus relaxing RamR repression.[8,10–12]

In *Escherichia coli*, which does not possess *ramA*, the AraC/XylS transcription factors MarA, SoxS and Rob can each activate expression of the AcrAB-TolC efflux pump.[13–17] Unlike many transcription factors, MarA, SoxS and RamA are not expressed constitutively but are synthesized *de novo* in response to their respective inducers. In the case of SoxS, SoxR is directly activated by redox-cycling agents and in turn activates the expression of *soxS*.[18] With MarA, MarR represses the transcription of *marA*, but in the presence of inducers such as salicylate, the expression of *marA* is increased.[19,20] The exact mechanism by which RamR activity is controlled is as yet still unclear. On removal of the inducer, the first step in re-setting the system is to halt the synthesis of the transcription factor, which is typically via one of four mechanisms: binding of a ligand, covalent modification, partner protein or altering the level of the transcription factor.[21] Following the cessation of transcription, the mechanism by which MarA and SoxS are removed is a reduction

in the level of the protein by an active process of proteolysis involving the Lon protease.[22]

Lon is an ATP-dependent protease belonging to the AAA$^+$ (ATPases associated with a variety of cellular activities) super-family of enzymes.[23] Lon associates into hexameric rings in Gram-negative bacteria and is a homo-oligomer of sub-units each composed of an N-terminal domain, an ATP-binding domain, a substrate sensor and discriminatory domain, and a proteolytically active C-terminal domain. Lon performs a wide range of different cellular functions, and studies with several species have shown that Lon is involved in unfolding misfolded proteins, as well as in their degradation.[23] Lon has been extensively studied in *E. coli* and known Lon targets include RcsA, a transcriptional activator for capsule synthesis[24] and SulA, a regulator of cell division.[25]

The mechanisms by which Lon protease recognizes its substrate are still unclear, but previous work has shown that Lon recognizes certain amino acids or domains of substrates such as the carboxy-terminal histidine of *E. coli* SulA[26] and the amino-terminal domain of *E. coli* SoxS and MarA.[22,27] *E. coli lon* mutants are filamented, sensitive to UV light and DNA damage[28] and hypersusceptible to fluoroquinolone antibiotics.[29] Lon mutants of *Pseudomonas aeruginosa* are also hypersusceptible to ciprofloxacin, filamented[30] and deficient in swarming motility and biofilm formation[31] and exhibit increased haemolytic activity.[32] The Lon protease of *P. aeruginosa* has also been shown to be a negative regulator of quorum sensing.[32] The Lon protease of *Salmonella* Typhimurium negatively controls *Salmonella* pathogenicity island (SPI)-1 expression through degradation of the HilC and HilD transcriptional regulators,[33,34] and Lon, along with ClpAP, is also involved in controlling haem biosynthesis by degrading HemA.[23,35,36]

We hypothesized that the RamRA system is reset after induction by an active process following the removal of chlorpromazine. Using *Salmonella* Typhimurium, we investigated the stability of RamA and its impact on antibiotic resistance.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are shown in Table 1. The widely used representative strain *Salmonella* Typhimurium SL1344 was used throughout. Growth was routinely performed in Luria–Bertani (LB) broth unless indicated otherwise.

Construction of gene-deleted mutants

To establish whether Lon was responsible for the degradation of RamA in *Salmonella* Typhimurium, we constructed a *lon* and *ramA* double mutant in *Salmonella* Typhimurium (SL1344) using the method described by Datsenko and Wanner.[37] To distinguish between the two, we made the *lon* mutant using a kanamycin selectable marker, and the *ramA* mutant using a chloramphenicol marker. For the *lon* mutant, primers were designed to amplify the *aph* cassette from the pKD4 plasmid which contained 40 bases at the 5′ ends that were complementary to the end of the *lon* gene (forward primer 5′-ACCCTGCACGTCTGGCTGACACCATCGCCGCGCATATGCGTG TAGGCTGGAGCTGCTTC-3′ and reverse primer 5′-TACCCCCGGCGGCCCCA CCAGACACAGGATCGGCCCCTTGGGGAATTAGCCATGGTCCAT-3′). For the *ramA* mutant, primers were designed to amplify the *cat* cassette from the pKD3 plasmid which contained 40 bases at the 5′ ends that were complementary to the end of the *ramA* gene (forward primer 5′-GCTCAGGTT ATCGACACGATTGTCGAGTGGATTGATGATAGTGTAGGCTGGAGCTGCTTC-3′ and reverse primer 5′-TTTACGATAAGCGCCTGGCGGCAGGTTGAACGTGCGGGTAGG GAATTAGCCATGGTCCAT-3′). SL1344 containing the pKD46 plasmid was transformed with the PCR fragments, and DNA sequencing was carried out to confirm the deletions. To make the SL1344*lon::aph/ramA::cat* double mutant, we performed P22 transduction.

Western blotting

Bacterial samples required for western blotting were harvested by centrifugation, and cell pellets were resuspended in 50 mM Tris/HCl (pH 8.0). Protein extracts were prepared by sonication on ice with an MSE Soniprep 150 (Sanyo, UK) for four pulses of 30 s with a 30 s pause between each pulse. A Bradford assay was carried out to quantify the protein concentration and 10 μg of protein was run on 4%–12% NuPAGE® Bis-Tris mini gels with NuPAGE® MES SDS running buffer (Life Technologies, UK). Protein was transferred to nitrocellulose transfer membranes (Whatman, UK), and analysed by western blotting using anti-FLAG M2 monoclonal antibody at a 1:1000 dilution (Sigma-Aldrich, UK). Blots were developed using anti-rabbit IgG horseradish peroxidase-linked antibody and analysed using the ECL detection system (GE Healthcare UK).

FLAG-tagged RamA plasmid construction

To make the plasmid construct, a DNA fragment carrying the *ramA*::3XFLAG::*aph* flanked by HindIII and NruI restriction sites was amplified from a previously made chromosomal *ramA*::3XFLAG fusion using the following primers: forward primer 5′-GCTAAGCTTTACACGTTACCCTTATGTCT-3′ and reverse primer 5′-GCGTCGCGATCAGAAGAACTCGTCAAGAA-3′. Following amplification, purification and double-restriction digest with HindIII and NruI, the amplimer was cloned into pACYC184.[38] pACYC184*ramA*::3XFLAG::*aph* was subsequently transformed into the SL1344*ramA::cat* and SL1344*lon::aph/ramA::cat* mutants.

In order to establish whether the N-terminal sequence of RamA is involved in Lon protease recognition, we constructed a deletion mutant in which the first 20 amino acids of RamA were deleted following the initiation codon. To achieve this we designed a pair of primers (5′-GCTAAGCTTAT GCCGTTACGTATTGATGATAT-3′ and 5′-GCGTCGCGATCAGAAGAACTCGTCAA GAA-3′) that amplified the *ramA*::3XFLAG::*aph* flanked by HindIII and NruI restriction sites in which codons 2 to 21 were deleted. Following amplification, purification and double-restriction digest with HindIII and NruI, the amplimer was cloned into pACYC184.[38] pACYC184*ramA*(Δ2-21)::3XFLAG::*aph* was subsequently transformed into the SL1344*ramA::cat* mutant.

Table 1. Strains and plasmids used in this study

		Source
Strain/genotype		
SL1344 wild-type		54
SL1344*ramA::cat*		this study
SL1344*lon::aph*		this study
SL1344*lon::aph/ramA::cat*		this study
SL1344(pACYC-*ramA*::3XFLAG::*aph*)		this study
SL1344*ramA::cat* (pACYC-*ramA*::3XFLAG::*aph*)		this study
SL1344*lon::aph/ramA::cat* (pACYC-*ramA*::3XFLAG::*aph*)		this study
SL1344*ramA::cat* [pACYC-*ramA*(Δ2-21)::3XFLAG::*aph*]		this study
Plasmid		
pSUB11	plasmid containing 3XFLAG sequence (Kan^R)	55
pACYC184	multicopy plasmid (Cm^R, Tc^R)	38

Determination of RamA abundance following removal of inducer

To establish the abundance of the RamA::3XFLAG protein following the removal of the inducer, we grew a culture of SL1344*ramA::cat* (pACYC-*ramA*::3xFLAG) to mid-logarithmic growth phase, and induced it with chlorpromazine (50 mg/L) for 30 min. Aliquots of the initial induced culture were taken every 10 min. Following induction, the remaining culture was split into two and the cells were collected by centrifugation, washed and resuspended in LB medium containing or lacking chlorpromazine (50 mg/L). These cultures were re-incubated at 37°C for a further 30 min with aliquots removed every 10 min. All aliquots taken were subjected to sonication and then SDS-PAGE. For detection of RamA::3XFLAG protein, western blotting was performed as previously described.

RamA half-life experiments

To investigate the absence of Lon on the expression and stability of RamA::3X-FLAG, we performed half-life experiments in which we grew SL1344*ramA::cat* (pACYC184-*ramA*::3XFLAG) and SL1344*lon::aph/ramA::cat* (pACYC184-*ramA*::3XFLAG) in the presence of chlorpromazine, an inducer of RamA. To investigate the effect of the N-terminal 20 amino acid deletion on the expression of RamA::3XFLAG, we grew the SL1344*ramA::cat* [pACYC184*ramA*(Δ2-21)::3XFLAG] mutant in the presence of chlorpromazine. Mutants were grown in 300 mL of LB broth, containing 50 mg/L kanamycin and/or chloramphenicol, at 37 °C with shaking at 180 rpm until an optical density (OD; measured at 600 nm) of 0.5 was attained. Chlorpromazine at a concentration of 50 mg/L was added to the cultures, which were then reincubated at 37°C for a further 60 min. Following treatment with spectinomycin (100 mg/L) to halt protein synthesis, aliquots (25 mL) were transferred every minute for 10 min to centrifuge tubes standing on ice. Each sample was then centrifuged and protein was extracted by sonication. SDS-PAGE was performed followed by western blotting as previously described.

Determination of susceptibility to fluoroquinolones

The MIC of each fluoroquinolone tested was determined by the standardized agar doubling-dilution method as described previously by the BSAC (http://www.bsac.org.uk).[39]

Growth kinetics

The rate of growth in LB broth and minimal medium (Teknova, USA) of SL1344 and SL1344*lon::aph* was determined over 24 h at 37°C using a FLUOstar OPTIMA (BMG Labtech, UK) plate reader as previously described.[12]

Motility assays

The ability of SL1344*lon::aph* to migrate through (swimming) or across (swarming) semi-solid agar was determined by making agar plates based on MOPS minimal medium (Teknova) supplemented with 0.25% and 0.5% (w/v) agar. Plates were inoculated by stabbing them with a sterile loop and incubated at 30°C over 5 days, and zones of migration through the agar were measured daily for each strain. Data were obtained in four separate experiments, each containing two technical replicates. All data were analysed with a Student's *t*-test; P values of <0.05 were taken as significant.

Crystal Violet biofilm assay

Overnight cultures of strains were diluted in fresh, antibiotic-free LB broth without salt to an OD of 0.1 at 600 nm. Ninety-six-well polystyrene microtitre trays (Sterilin) were inoculated with 200 μL of this suspension and incubated at 30°C for 48 h with gentle agitation. After incubation, the liquid was removed from all the wells and the wells were washed with sterile distilled water to remove any unbound cells. Biofilms were stained by adding 200 μL of 1% Crystal Violet to the appropriate wells for 15 min. Crystal Violet was removed and each well was washed with sterile distilled water to remove any unbound dye. The stained biofilm was solubilized by adding 200 μL of 70% ethanol and the OD was measured at 600 nm using a FLUOstar Optima (BMG Labtech). All biofilm assays were carried out three times with two biological and four technical replicates per repeat. A Student's *t*-test was used to compare the statistical significance of the results between the Lon mutant and SL1344 (wild-type).

Measurement of SPI gene expression

SL1344 and SL1344*lon::aph* containing a chromosomal green fluorescent protein (GFP) reporter fused to the promoter of the *prgH* gene were grown overnight in LB broth at 37°C with shaking. A 4% inoculum was added to 10 mL of minimal media and incubated at 37°C with shaking until mid-log phase (an OD_{600} of 0.6). Cells were harvested from 500 μL of culture by centrifugation and resuspended in 1 mL of PBS. An aliquot of 100 μL of each cell suspension was added to a 96-well plate and bacteria were analysed by flow cytometry using an Accuri C6 cytometer (BD Biosciences, USA). Three biological replicates were carried out and 10 000 data points were collected for each sample.

Adherence and invasion of bacteria to human intestinal cells (INT-407) growing in tissue culture

Assays were performed as previously described.[40]

Microscopic morphology

Microscope slides were inoculated with a loopful of bacteria taken from mid-logarithmic growth cultures of SL1344 and SL1344*lon::aph* grown in LB medium. Cells were heat fixed, Gram-stained and examined with a light microscope at ×100 magnification.

Results

Chlorpromazine induces expression of RamA

To date, few inducers of RamA have been reported; however, Nikaido *et al*.[1,41] demonstrated an increased expression of *ramA* in response to the bacterial metabolite indole. Work carried out by Bailey *et al*.[3] using comparative reverse-transcription PCR, and more recently by Lawler *et al*.,[42] with transcriptional GFP reporter fusions, showed that chlorpromazine, a phenothiazine, also induced the expression of *ramA*. For the purposes of our present experiments, we wanted to establish whether the increase in transcription of *ramA* by chlorpromazine translated into increased levels of RamA protein, and in order to do this we carried out western blotting in the absence and presence of chlorpromazine (50 mg/L). Previous studies have had difficulties in detecting chromosomally encoded proteins such as MarA by western blotting.[22,43] To overcome this and produce detectable quantities of protein, we used a multicopy plasmid (pACYC184; Table 1) that harboured a *ramA*::3XFLAG fusion and blotted with an anti-FLAG antibody. In the absence of chlorpromazine, detectable amounts of RamA were produced, presumably due to the use of a multicopy plasmid. In the presence of chlorpromazine, 3-fold more RamA was detected; this showed that induction with chlorpromazine increased RamA production (Figure 1).

Abundance of RamA after removal of inducer is degraded by an active process

To ascertain how the RamRA system is reset and whether this is by an active or a passive process, we determined the abundance of RamA by western blotting following the removal of chlorpromazine. As shown in Figure 2, the relative abundance of RamA was increased following induction and remained constant throughout the initial induced period of 30 min until the cells were harvested and transferred to LB containing or lacking chlorpromazine (50 mg/L). In the LB medium containing chlorpromazine, the amount of RamA remained constant, as was observed in the initial induced culture; however, after 10 min in LB medium with no chlorpromazine, the amount of RamA fell rapidly.

RamA is unstable with a short half-life

Our data (Figure 2) indicate that, in the absence of inducer, the abundance of RamA diminished, suggesting that RamA has a short half-life. As the previous experiment was carried out over a longer time period, and so that we could determine the half-life of RamA more accurately, we induced RamA synthesis using chlorpromazine and determined the abundance of RamA in multiple samples taken every 2 min. Following western blotting, our data revealed that the abundance of RamA rapidly decreased and indicated that the half-life of RamA is ~2 min (Figure 3a).
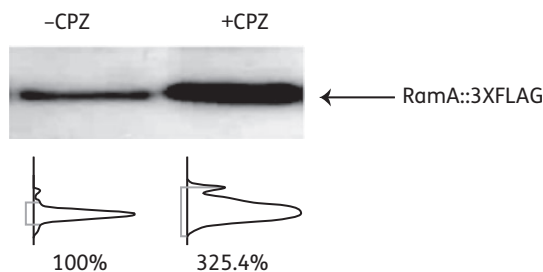
−CPZ   +CPZ

← RamA::3XFLAG

100%   325.4%

Figure 1. Induction of RamA::3XFLAG by chlorpromazine (50 mg/L). Western blot and densitometry plots demonstrating RamA::3XFLAG production in a Δ*ramA* mutant of SL1344 carrying the epitope-tagged ramA *(RamA::3XFLAG)* on pACYC184 in the presence (+CPZ) or absence (−CPZ) of 50 mg/L chlorpromazine.

RamA is more stable in a lon mutant

Having shown that RamA is unstable and has a short half-life, we proceeded to investigate what was responsible for the instability of the RamA. ATP-dependent proteases are known to play an important role in gene regulation by degrading regulatory proteins such as transcription factors[44] and, as previously reported by Griffith *et al.*,[22] the *E. coli* Lon protease is responsible for the rapid turnover of the transcriptional activators SoxS and MarA following induction by their respective inducers. To investigate whether the Lon protease in *Salmonella* Typhimurium carries out a similar role and degrades RamA, we constructed a *lon* deletion mutant in *Salmonella* Typhimurium (SL1344) and carried out half-life experiments. Our data revealed that, over the 10 min period tested and following induction, the abundance of the RamA protein remained high in the *lon*-deleted mutant (Figure 3b) and the half-life increased to >10 min. These data show that RamA is more stable and more abundant in the absence of *lon*.

The N-terminus of RamA is required for Lon protease recognition

Proteases degrade specific proteins in environments occupied by a variety of different proteins, so in order to target the appropriate protein, proteases are able to recognize specific substrates. Protease recognition signals have been found to reside at the N- and C-termini of proteins, which may reflect the accessibility of these ends.[44] Previous work carried out in *E. coli* with the transcriptional activators SoxS and MarA identified that the N-terminus of these proteins play a primary role in Lon protease recognition.[22,27] To determine whether the N-terminus of RamA is required for Lon-mediated degradation, we constructed an N-terminus-deleted RamA plasmid construct and determined the effect of this deletion on the half-life of RamA. We found that the N-terminal deletion increased the half-life of RamA from 2 min to >10 min (Figure 3c), suggesting that the N-terminus of RamA is important for proteolytic degradation by Lon protease.

The lon-disrupted mutant is not multidrug resistant

Previous work with other bacterial species[28–32] has shown that *lon* mutants exhibit certain phenotypic characteristics. Therefore, to see whether the same was true for *Salmonella*, we determined
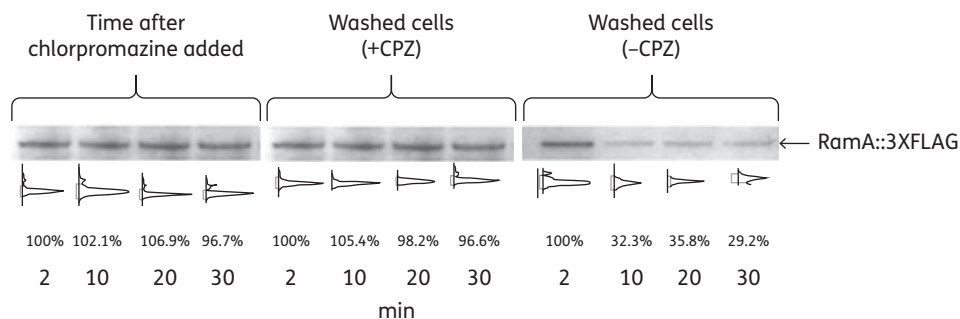
Time after chlorpromazine added				Washed cells (+CPZ)				Washed cells (−CPZ)			
100%	102.1%	106.9%	96.7%	100%	105.4%	98.2%	96.6%	100%	32.3%	35.8%	29.2%
2	10	20	30	2	10	20	30	2	10	20	30

← RamA::3XFLAG

min

Figure 2. Effect of removal of the inducer on the abundance of RamA::3XFLAG. Western blot and densitometry plots demonstrating the abundance of RamA::3XFLAG in a Δ*ramA* mutant of SL1344 carrying the epitope-tagged ramA *(RamA::3XFLAG)* on pACYC184 during induction by chlorpromazine (initial induced culture) over a 30 min time period and following removal of chlorpromazine and resuspension of the cells in medium containing no chlorpromazine (−CPZ) and containing chlorpromazine (+CPZ), over the same time period.
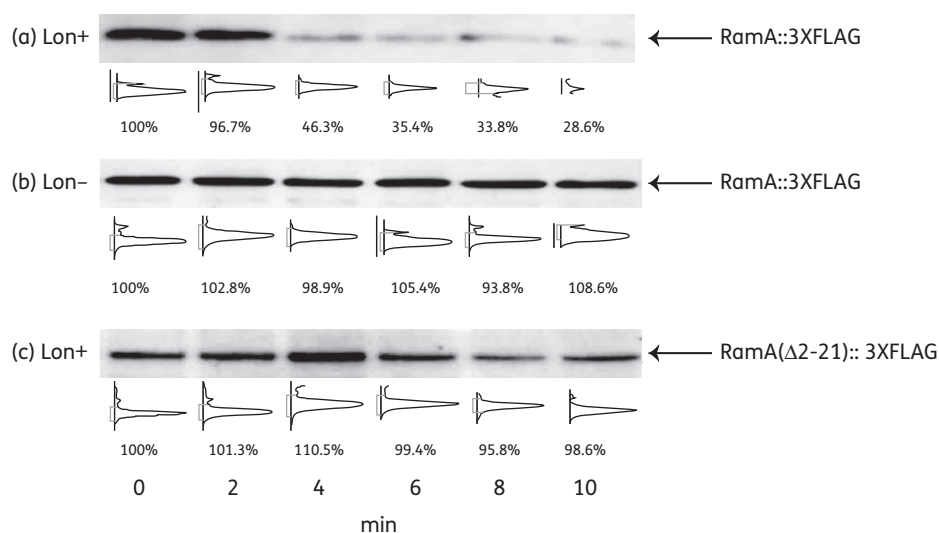
Figure 3. Determination of the half-life of RamA::3XFLAG and RamA(Δ2-21)::3XFLAG. Western blots and densitometry plots demonstrating the abundance of RamA::3XFLAG following removal of inducer in (a) SL1344*ramA::aph* and (b) SL1344*lon::aph/ramA::cat* over a 10 min time period. (c) Abundance of RamA(Δ2-21)::3XFLAG following removal of the inducer in SL1344*ramA::cat* over a 10 min time period.

Table 2. Phenotype of *Salmonella* Typhimurium *lon::aph*

	MIC (mg/L)				Growth in LB and MOPS minimal medium, generation time (min)		Motility, colony diameter (mm)		Percentage of population expressing SPI-1	Filament formation
Strain	CPZ	CIP	NOR	NAL	LB	MOPS	swimming	swarming		
SL1344	512	0.03	0.25	4	47.5 ± 5	100.2 ± 8.9	35 ± 4	13 ± 3	14.12 ± 4.2	−
SL1344 *lon::aph*	256	0.007	0.06	1	92.5 ± 9.5	340.3 ± 12.2	36 ± 5	12 ± 4	70.52 ± 3.7	+

CPZ, chlorpromazine; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid.

the phenotype of the *lon*-disrupted mutant. In agreement with previous work on *P. aeruginosa* and *E. coli*,[29,30] the *lon* mutant was 2- to 4-fold more susceptible to the fluoroquinolone antibiotics ciprofloxacin, norfloxacin and nalidixic acid compared with the wild-type (Table 2). Compared with the wild-type parental strain, SL1344, the SL1344 *lon::aph* mutant also grew poorly in both MOPS minimal medium and LB medium, but showed no difference in swarming or swimming motility or the ability to form a biofilm (although SL1344 forms biofilms poorly due to mutations in *mlrA* and *adrA*)[45] (data not shown). There was also no difference in the ability of the SL1344 *lon::aph* to invade human intestinal cells (INT-407). Despite this, a greater percentage of SL1344 *lon::aph* mutants expressed SPI-1 than did the parental strain (Table 2). When observed by microscopy, the SL1344 *lon::aph* mutant grew as filaments (Table 2).

Discussion

RamA is the primary regulator of expression of AcrAB-TolC in most Enterobacteriaceae[1–5] and understanding how the components of efflux pumps and the factors that regulate them are controlled will provide essential biological information. Furthermore,

knowledge about such mechanisms is essential in the search for new antibacterial compounds.

Transcription factors are regulated in a variety of ways, which ultimately control their activity or their expression. One mechanism is the modification of the DNA-binding affinity of the transcription factor by small ligands, whose concentrations can vary in response to nutrient availability or stress. One example of this is the reduction in the DNA-binding affinity of the Lac repressor by the small molecule allolactose, which is an inducer that binds to the Lac repressor, stopping repression and allowing the transcription of *lacZ* and related genes.[46]

Another mechanism is the modulation in activity of some transcription factors by covalent modification. A good example of this mechanism is that of NarL, which binds to its target DNA only when phosphorylated by its cognate sensor kinases NarX and NarQ.[47]

A third mechanism is where the concentration of a transcription factor controls its activity, either by regulating the expression of the transcription factor or by proteolysis. One example is the transcription of *soxS* that is controlled by SoxR, which is directly activated by redox-cycling agents and in turn activates the expression of *soxS*.[18]

The fourth mechanism by which transcription factors are regulated is sequestration by a regulatory protein to which the

transcription factor binds. An example is that of Mlc, which represses several glucose-related genes, including the phosphotransferase system (PTS) genes *ptsHI* and *ptsG*. Induction of these genes by glucose occurs as a response to the flux of glucose through the PTS and involves the sequestration of Mlc to membranes containing dephosphorylated PtsG.[48] Data arising from this study strongly indicate that the level of RamA is regulated by the Lon protease. Ultimate proof of the involvement of Lon protease in the degradation of RamA could be obtained by performing proteolytic assays.

The proteolysis of transcription factors, in a constitutive or regulated manner, plays a key role in controlling many regulatory networks[22,44,49] and studies of the ATP-dependent Lon protease in different bacteria have shown its involvement in many biological processes.[49] To date, the Lon protease has been shown to regulate two systems in *Salmonella*: the regulation of SPI-1 gene expression and the regulation of haem biosynthesis.[33–36] We have now shown that the Lon protease in *Salmonella* also regulates the expression of RamA, and that it is required for the levels of RamA to be reset to basal level in the absence of induction.

The Lon protease is known to play an important role in protein quality control by degrading misfolded proteins; however, Lon also unfolds and degrades stably folded proteins that have accessible recognition tags. Studies on UmuD, SoxS and MarA[22,27,50] identified an N-terminal degradation tag as being essential for Lon proteolysis. In this study, we demonstrated that the N-terminal region of RamA consisting of the peptide sequence MTISAQVIDTI-VEWDDNLNQ is important for Lon recognition and subsequent degradation of RamA.

The phenotype of the *Salmonella* Lon mutant also suggests that the Lon protease participates in some other pathways, as described for other bacterial species. For instance, the mutant was hypersusceptible to fluoroquinolone antibiotics. This is counterintuitive as RamA is not degraded, so the level of RamA remains high. The same is true for *E. coli* Lon mutants in which the levels of SoxS and MarA presumably also remain undegraded.[22] It has been hypothesized that the lack of MDR in Lon mutants is due to the action of Lon on RecA repressors; in a *lon* mutant, this will lead to hypersusceptibility to fluoroquinolone antibiotics due to the lack of RecA amplification.[51] The lack of MDR in the Lon mutant also suggests that the RamRA regulon is regulated in a similar way to the *E. coli* SoxRS and MarRAB regulons and that RamA is synthesized *de novo* in response to an inducing signal. Lon mutants also formed filamentous cells, similar to the observation by Yamaguchi *et al.*,[29] with *E. coli*. This suggests that the Lon protease in *Salmonella* could have a role in the regulation of SulA, which is induced as part of the SOS system and thereby inhibits cell division, giving rise to filamented cells. The motility of *Salmonella* was unaffected by the inactivation of *lon*. This is not unexpected as it has been shown that FlhD and FlhC in *Salmonella* Typhimurium are degraded by the ClpXP protease and not the Lon protease.[52] The lack of an effect on motility may also explain the lack of effect of the inactivation of the *lon* gene upon biofilm formation as motility is known to be required for the initial stages of biofilm formation in certain bacterial species;[53] however, it should be noted that *Salmonella* Typhimurium SL1344 is poor at forming biofilms due to a mutation in the *mlrA* and *adrA* genes, which are involved in cellulose production.[45] Takaya *et al.*[33,34] showed that Lon negatively regulates SPI-1 expression as disruption of the *lon* gene in *Salmonella* Typhimurium markedly

stimulated the invasion of human intestinal cells (INT-407), with a 10-fold increase in the invasion level of the *lon* mutant compared with that of the parental strain χ3306. Despite observing an increase in SPI-1 expression, we did not see an increased invasion of human intestinal cells (INT-407). One reason for this may be that SPI-1 is not the only determinant of invasion and inactivation of *lon* could alter invasion in other ways. The differences between our study and those of Takaya *et al.*[33,34] may reflect the use of different *Salmonella* strains and hence genotype and phenotype.

In summary, this study has revealed that there is a higher order of regulation of RamA and thus of the AcrAB-TolC MDR efflux system. The involvement of the Lon protease in this process is further evidence that the expression of *acrAB* and *tolC* is under multilevel control and that there could be many ways to prevent the overproduction of AcrAB-TolC and therefore prevent enhanced efflux and multidrug resistance.

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None to declare.

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