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

Vito Ricci, Jessica M. A. Blair and Laura J. V. Piddock*

Antimicrobials Research Group, School of Immunity and Infection and Institute of Microbiology and Infection, University of Birmingham, Birmingham B15 2TT, UK

*Corresponding author. Antimicrobials Research Group, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK. Tel: +44-121-414-6966; Fax: +44-121-414-6819; E-mail: l.j.v.piddock@bham.ac.uk

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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 SL1344ramA::cat and lon::aph mutants. An N-terminus-deleted mutant [pACYC184ramA(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 and other Enterobacteiraeae, including Klebsiella and Enterobacter spp. 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. The function of RamR can be ablated by N-terminal DNA-binding domain and a C-terminal ligand-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

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in the level of the protein by an active process of proteolysis involv-
ing 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 a 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, sen-
sitive to UV light and DNA damage 28 and hypersusceptible to
fluoroquinolones antibiotics. 29 Lon mutants of Pseudomonas aeru-
ginosa are also hypersusceptible to ciprofloxacin, filamented 30 and
deficient in swarming motility and biofilm formation 31,32 and exhibit
increased haemolytic activity. 33,34 The Lon protease of P. aeruginosa
has also been shown to be a negative regulator of quorum sensing. 35
The Lon protease of Salmonella Typhimurium negatively
controls Salmonella pathogenicity island (SPI)-1 expression
through degradation of the HilC and HilD transcriptional regula-
tors, 33,34 and Lon, along with CipAP, 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.

Table 1. Strains and plasmids used in this study

| Strain/genotype   | Source          |
|-------------------|-----------------|
| SL1344 wild-type  | this study      |
| SL1344ramA::cat   | this study      |
| SL1344lon::aph    | this study      |
| SL1344lon::aph/ramA::cat | this study |
| SL1344(pACYC-ramA::3XFLAG::aph) | this study |
| SL1344ramA::cat   | this study      |
| SL1344ramA::cat   | this study      |
| Plasmid           | Source          |
| pSUB11            | plasmid containing 3XFLAG sequence (KanR) |
| pACYC184          | multicopy plasmid (CmR, TeR) |
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 SL1344ramA::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::3XFLAG, we performed half-life experiments in which we grew SL1344ramA::cat (pACYC184-ramA::3XFLAG) and SL1344lon::aph (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 SL1344ramA::cat (pACYC184ramA::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 SL1344lon::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 SL1344lon::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 SL1344lon::aph containing a chromosomal green fluorescent protein (GFP) reporter fused to the promoter of the pgpH 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 OD600 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 10000 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 SL1344lon::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.,62 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 multiplicity 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 multiplicity 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 $\sim 2$ min (Figure 3a).

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

**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 and, as previously reported by Griffith et al., 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. 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. To determine whether the N-terminus of RamA is required for Lon-mediated degradation, we constructed an N-terminal-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 has shown that lon mutants exhibit certain phenotypic characteristics. Therefore, to see whether the same was true for *Salmonella*, we determined...
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 antibacterials 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).

Discussion

RamA is the primary regulator of expression of AcrAB-ToIC 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 reox-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

\begin{table}[h]
\centering
\begin{tabular}{llllllllllll}
\hline
Strain & \multicolumn{3}{c}{MIC (mg/L)} & \multicolumn{2}{c}{Growth in LB and MOPS minimal medium, generation time (min)} & \multicolumn{2}{c}{Motility, colony diameter (mm)} & \multicolumn{2}{c}{Percentage of population expressing SPI-1} & Filament formation \\
& CPZ & CIP & NOR & NAL & LB & MOPS & swimming & swarming & \\
\hline
SL1344 & 512 & 0.03 & 0.25 & 4 & 47.5\(\pm\)5 & 100.2\(\pm\)8.9 & 35\(\pm\)4 & 13\(\pm\)3 & 14.12\(\pm\)4.2 & - \\
SL1344 lon::aph & 256 & 0.007 & 0.06 & 1 & 92.5\(\pm\)9.5 & 340.3\(\pm\)12.2 & 36\(\pm\)5 & 12\(\pm\)4 & 70.52\(\pm\)3.7 & + \\
\hline
\end{tabular}
\caption{Phenotype of Salmonella Typhimurium lon::aph}
\end{table}

CPZ, chlorpromazine; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid.
transcription factor binds. An example is that of Mcr, which represses several glucose-related genes, including the phosphotransferase system (PTS) genes ptsH 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 Mcr to membranes containing dephosphorylated PtSG.68 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 networks22,44,49 and studies of the ATP-dependent Lon protease in different bacteria have shown its involvement in many biological processes.69 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 MarA22,27,50 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 MTSAQVIDTI-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 described for other bacterial species. For instance, the mutant that the Lon protease participates in some other pathways, as forming biofilms due to a mutation in the mlrA gene.83 Should be noted that the expression of multidrug efflux pumps is under multilevel control and that there could be many ways to prevent the overproduction of AcrAB-ToIC and therefore prevent enhanced efflux and multidrug resistance.

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Transparency declarations

None to declare.

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