A type II secreted RNase of *Legionella pneumophila* facilitates optimal intracellular infection of *Hartmannella vermiformis*

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INTRODUCTION

The Gram-negative bacterium *Legionella pneumophila* is a ubiquitous inhabitant of natural and man-made water systems, where it survives planktonically inside protozoan hosts, and as a part of biofilms (Fields systems, where it survives planktonically inside protozoan ubiquitous inhabitant of natural and man-made water systems). The Gram-negative bacterium *Legionella pneumophila* is a serious form of pneumonia (Diederen, 2008). Following its transmission by the inhalation of contaminated aerosols, *L. pneumophila* enters the lungs, and then invades and grows within alveolar macrophages. Type II protein secretion plays a role in a wide variety of functions that are important for the ecology and pathogenesis of *Legionella pneumophila*. Perhaps most dramatic is the critical role that this secretion pathway has in *L. pneumophila* intracellular infection of aquatic protozoa. Recently, we showed that virulent *L. pneumophila* strain 130b secretes RNase activity through its type II secretion system. We now report the cloning and mutational analysis of the gene (srnA) encoding that novel type of secreted activity. The SrnA protein was defined as being a member of the T2 family of secreted RNases. Supernatants from mutants inactivated for srnA completely lacked RNase activity, indicating that SrnA is the major secreted RNase of *L. pneumophila*. Although srnA mutants grew normally in bacteriological media and human U937 cell macrophages, they were impaired in their ability to grow within *Hartmannella vermiformis* amoebae. This finding represents the second identification of a *L. pneumophila* type II effector being necessary for optimal intracellular infection of amoebae, with the first being the ProA zinc metalloprotease. Newly constructed srnA proA double mutants displayed an even larger infection defect that appeared to be the additive result of losing both SrnA and ProA. Overall, these data represent the first demonstration of a secreted RNase promoting an intracellular infection event, and support our long-standing hypothesis that the infection defects of *L. pneumophila* type II secretion mutants are due to the loss of multiple secreted effectors.
DebRoy et al., 2006b; Flieger et al., 2002; Quinn & Tompkins, 1989; Rossier et al., 2008; Söderberg & Cianciotto, 2008; Szeto & Shuman, 1990). As a result, we have shown that the chitinase promotes persistence in the lung, and that the secreted metalloprotease promotes infection of Hartmannella vermiformis amoebae (DebRoy et al., 2006b; Rossier et al., 2008). We have now characterized the gene (srnA) encoding the L. pneumophila secreted RNAase activity, and here we document that it is necessary for optimal intracellular infection of Hartmannella amoebae.

**METHODS**

**Strains, growth media, and chemicals.** L. pneumophila strain 130b (ATCC strain BAA-74, also known as AA100) served as our wild-type control (Engleberg et al., 1984; Saito et al., 1981). Mutants of 130b containing a kanamycin-resistance (Km) cassette were transformed into ISPF (NU275) or proA (AA200) have been described previously (Moffat et al., 1994; Rossier et al., 2004). Legionellae were cultured in BYE broth or on BCYE agar (Rossier et al., 2004). Growth in broth was assessed by measuring the optical density of cultures at 660 nm. Whereas Escherichia coli DH5α and DH10B (Invitrogen) were hosts for most of the plasmids, C41(DE3) and C43(DE3) (Miroux & Walker, 1996) were used to maintain the plasmid containing intact srnA. E. coli strains were grown on Luria–Bertani agar (Ausubel et al., 1994). Secreted RNase activity was initially assayed by monitoring the release of 100 μl l−1 ampicillin, 100; chloramphenicol (Cm), 6 for L. pneumophila, and 30 for E. coli; gentamicin, 2.5; and kanamycin, 25 for L. pneumophila, and 50 for E. coli. Chemicals were from Sigma.

**RT-PCR analysis of gene expression.** RT-PCR was done as described previously (Allard et al., 2006; Liles et al., 1998; Viswanathan et al., 2002). L. pneumophila RNA was isolated using RNA STAT-60 (TEL-TEST B). The primers (Integrated DNA Tech) used were: OR145srnA (5'-ATCAACTAATCTGTGCTGCGG-3') and OR147srnA (5'-TCTAGGTGTTGTGTTGCGCTG-3') to amplify a 703 bp internal fragment of srnA; OR148lpg2847 (5'-AACACCTCCTCG-AGCGGAAGTTA-3') and OR138srnA (5'-GCCCTCATAGCGAAACC-GTTG-3') to amplify a 289 bp internal fragment of lpg2847; and OR146srnA (5'-AAGCCGACACTACACAC-3') and OR137srnA (5'-AACACCTCC-AGCGGAAGTTA-3') to amplify a 577 bp fragment encompassing the 3' end of srnA, an intragenic region, and the 5' end of lpg2847. Controls in which reverse transcriptase was omitted from the PCR were done to rule out contributions of contaminating DNA in the DNase-treated RNA samples.

**Sequence analysis, gene cloning and mutant constructions.** DNA and protein sequences were analysed using Lasergene (DNASTAR). The CLUSTAL method of Lasergene MEGALIGN was used for protein alignments. Protein homologues were identified in the genome database using programs based on the BLAST algorithm (Altschul et al., 1990). Conserved domains were identified by searching the conserved domain database (Marchler-Bauer et al., 2007). Signal sequences were identified using SignalP (Nielsen et al., 1997), and further predictions concerning the cellular location of proteins were done by using PSORTb (Gardy et al., 2005). DNA was isolated as described previously (Cianciotto et al., 1989). Primers for amplifying DNA from strain 130b were designed based on the L. pneumophila Philadelphia-1 genome database (Chien et al., 2004). RnaA-F-Ndel (5'-GTGAAAGTCCATCTCCACTTGGAAT-3') and RnaA-R-BamHI (5'-TGTTGCTGGAAGTTAATCTCTCCACTTGGAAT-3') were used to obtain a 1 kb fragment containing the srnA ORF only. The fragment was ligated into pGEM-T Easy (Promega), yielding pGsrnA. Plasmid pGsrnA was then digested with Ndel and BamHI, and the released fragment containing srnA was ligated into pET28a (Novagen) to yield pETsrnA, which was used to monitor SrnA activity in E. coli. In order to mutate srnA in strain 130b, Rnsae5' (5'- TGTACCCCTTCTGTGCTGCGG-3') and OR144srnA (5'-GCTTACCCACAAGCTCGCAC-3') were used to obtain a 1139 bp piece containing the first 640 bp of srnA. This fragment was then cloned into pGEM-T Easy, yielding pG5'srnA. To disrupt srnA, pG5'srnA was digested with BstBI, which cuts 110 bp after the start codon. Following Klenow treatment, the resulting fragment was ligated to a Km gene isolated from pMB2190 upon HincII digestion (Grindley & Joyce, 1980), and to a gentamicin resistance (Gm) gene isolated from pX1918GT after HinII and PvuII digestion (Schweizer & Hoang, 1995), to give pGsraN:: Km and pGsrnA:: Gm, respectively. These plasmids were digested with NotI, and following Klenow treatment, disrupted srnA was cloned into Smal-digested pRRE112 (Edwards et al., 1998), yielding pRsraN:: Km and pRsraN:: Gm.

**Detection of enzymic activities.** Cell-free, filter-sterilized supernatants were obtained from L. pneumophila cultures grown in BYE broth to late-exponential phase (Aragon et al., 2000). Secreted protease, phosphatase and lipolytic activities were detected as outlined previously (Aragon et al., 2000, 2001, 2002). Secreted RNase activity was initially assayed by monitoring the release of nucleotides from type III RNA, as previously described (Kar et al., 2000; Rossier et al., 2004). Briefly, a 40 μl volume of L. pneumophila culture supernatant was incubated with 400 μl assay buffer (50 mM Tris, pH 8) containing 10 mg ml−1 Baker’s yeast type III RNA. At the beginning of the reaction, half of the sample was transferred to a fresh tube, and then chilled and precipitated with 2 vols ice-cold 10 % TCA beginning of the reaction, half of the sample was transferred to a fresh tube, and then chilled and precipitated with 2 vols ice-cold 10 % TCA.
Infection assays. H. vermiformis (ATCC 50237) amoebae were infected with L. pneumophila, as previously described (Cianciotto & Fields, 1992; Rossier et al., 2008). Briefly, 10⁶ c.f.u. were used to infect 10⁵ amoebae, and then, at various times, the numbers of bacteria per co-culture were determined by plating. U937 cells were also infected, as described previously (Cianciotto et al., 1989; Rossier & Cianciotto, 2001; Rossier et al., 2004): monolayers containing 10⁶ macrophages were inoculated with 10⁵ c.f.u. L. pneumophila, and, at various times, the numbers of bacteria in the monolayer were determined by plating lysates on BCYE agar, and the numbers of remaining host cells were determined by vital staining. For infection of A/J mice, 6- to 8-week-old females (Jackson Laboratory) were inoculated intratracheally with a 25 μl suspension containing 10⁵ c.f.u. of a 1:1 ratio of wild-type and mutant strains of L. pneumophila (DebRoy et al., 2006b; Rossier et al., 2004). One and 3 days later, infected lungs were homogenized, and the numbers of bacteria, and the ratio of wild-type to mutant, were determined by plating. Animal experiments were approved by the Animal Care and Use Committee of Northwestern University.

RESULTS

Identification and mutation of sRN in L. pneumophila

Using 2D PAGE and proteomic analysis, we identified a protein that is present in wild-type serogroup-1 strain 130b culture supernatants, but not in type II secretion (IspF) mutant culture supernatants; the gene encoding the protein has been annotated in L. pneumophila databases as encoding an RNase (DebRoy et al., 2006b). We hypothesized that the gene, now designated sRN (secreted ribonuclease A), encodes the type-II-dependent RNase activity that we observed in earlier studies (Aragon et al., 2000; Rossier et al., 2004). In the sequenced L. pneumophila serogroup-1 strains Philadelphia-1, Paris, Lens and Corby, sRN is denoted as lpg2848, lpp2906, lpl2760 and lpc3133, respectively (Cazalet et al., 2004; Chien et al., 2004; Glockner et al., 2008). Recent proteomic analysis of culture supernatants of the Philadelphia-1 strain has further confirmed the secreted nature of SrnA (Galka et al., 2008). Compatible with its type II secretion, 38 kDa SrnA contains a Sec-dependent signal sequence (DebRoy et al., 2006b). In the L. pneumophila genomes, sRN exists in a two-gene operon, with the second gene, denoted as lpg2847 in Philadelphia-1, situated 137 bp from sRN. Using RT-PCR, we confirmed that sRN and lpg2847 are co-transcribed in strain 130b (data not shown). The Lpg2847 protein did not exhibit significant similarity to any known protein. In order to determine if SrnA is, in fact, an RNase, we cloned sRN, and used allelic exchange to construct a corresponding set of specific mutants of strain 130b. Three independent sRN mutants were obtained: GmR NU328 and NU329, and KmR NU330. Similar to other Lsp mutants that have been studied (Rossier et al., 2004), these mutants grew normally at 37 °C in BYE broth and on BCYE agar (data not shown), indicating that SrnA is not needed for extracellular growth under standard laboratory conditions. The sRN mutant supernatants had normal levels of acid phosphatase, lipase and protease (data not shown), indicating that the strains do not have general defects in type II secretion. The sRN mutants did not exhibit the altered colony morphology or reduced efficiency of plating at 25–17 °C displayed by lsp mutants (Rossier et al., 2004; Söderberg et al., 2004; Söderberg & Cianciotto, 2008).

Influence of sRN on RNase activity

In order to examine secreted RNase activity, we grew the legionellae in BYE broth to late-exponential phase, and then assayed culture supernatants for the ability to cleave purified yeast RNA. As observed previously (Rossier et al., 2004), strain 130b supernatants contained significant levels of RNase activity, whereas those of its type II secretion mutant NU275 lacked RNase activity (Fig. 1). The sRN mutants NU328, NU329, and NU330 lacked the activity (Fig. 1, and data not shown). That three independently derived mutants behaved identically indicated that this loss of secreted activity was due to the inactivation of sRN rather than spontaneous second site mutation(s). To also rule out the possibility of a polar effect associated with the insertional mutation of sRN, we made mutants with an insertion in lpg2847 (NU331 and NU332) and tested them for secreted activity. Neither of these mutants, which also grew normally in BYE broth and on BCYE agar at 37, 25
and 17°C (data not shown), lacked any secreted RNase activity (Fig. 1, and data not shown). Double mutants inactivated for both srnA and lpg2847 (NU333 and NU334) lacked RNase activity in the same way as did the srnA mutants (Fig. 1, and data not shown). When an in-gel assay for RNase (zymogram) was performed, the supernatant of the wild-type, but not the supernatants of srnA mutants NU328 and NU329, exhibited a single protein band that had activity (Fig. 2a). The protein detected was approximately 38 kDa in size (Fig. 2a), and this indicated that SrnA, predicted to be 38 kDa, is directly responsible for the secreted RNase activity that is in *L. pneumophila* supernatants. That SrnA is an RNase was further confirmed when cloned srnA conferred RNase activity upon recombinant *E. coli* (Fig. 2b). These various data indicate that the Lsp-dependent SrnA represents the major secreted RNase of *L. pneumophila*. When a proA mutant of strain 130b was examined, we did not observe a loss of RNase (data not shown), indicating that SrnA, unlike some of the other *L. pneumophila* type II effectors (Banerji *et al.*, 2005; Flieger *et al.*, 2002; Rossier *et al.*, 2008), is not cleaved by the secreted metalloprotease in order to be active.

**Importance of srnA for infection of mammalian hosts**

Lsp mutants of strain 130b are impaired for infection of human macrophages and murine lungs (Rossier *et al.*, 2004). Thus, to begin to judge the role of the Lsp-dependent RNase in *L. pneumophila* infection, we compared wild-type and srnA mutants for their ability to infect U937 cells. The srnA mutants showed no defect in the ability to grow in the macrophage cell line (Fig. 3a).

![Fig. 2. SrnA in *L. pneumophila* (*Lpn*) supernatants and recombinant *E. coli* lysates. (a) Neat supernatants obtained from wild-type (WT) 130b, and srnA mutants NU328 and NU329, were subjected to SDS-PAGE in the presence of RNA substrate, and then RNase was detected as a clear band following toluidine blue staining of the gel. MW, molecular mass markers (kDa) (BenchMark Protein Ladder; Invitrogen). The results depicted were obtained on two separate occasions using neat supernatants, and a further two times using 40× concentrated supernatants. (b) Lysates obtained from *E. coli* C41 (DE3) containing pET28a (vector) or pETsrnA that had srnA cloned into pET28a (srnA) were subjected to SDS-PAGE, and then RNase staining was done as described above. The two bands in the vector control lane probably represent endogenous *E. coli* RNase(s), whereas the band of approximately 25 kDa that is present in the sample from the srnA clone is probably a breakdown product of 38 kDa SrnA; results similar to each other were obtained on two separate occasions.

![Fig. 3. Infection of macrophages and mouse lungs by srnA mutants of *L. pneumophila*. (a) U937 cells were infected with wild-type strain 130b (□), srnA mutant NU328 (○), and srnA mutant NU329 (●). At various times post-inoculation, the numbers of bacteria per well were determined. The values presented are the means (±SD) obtained from four infected wells, and are representative of at least two independent experiments. (b) A/J mice were inoculated intratracheally with equal numbers of wild-type 130b and srnA mutant NU328, and then the ratio of wild-type to mutant was determined at days 1 and 3 after inoculation. Data are representative of actual values obtained per mouse (n=5), and the horizontal bar indicates the mean value.](http://mic.sgmjournals.org)
Compatible with this result, infection with mutant bacteria ultimately produced death of the macrophage monolayer in a way that was similar to that resulting from wild-type infection (data not shown). When inoculated into the lungs of A/J mice, the srnA mutant grew and exhibited a recoverability from the lungs that was similar to wild-type 130b (Fig. 3b). These data indicate that SrnA is not required for optimal infection of mammalian host cells or tissue.

**Importance of srnA for intracellular infection of protozoan hosts**

In the past, we and others have shown that type II secretion mutants of *L. pneumophila* are severely impaired for infection of protozoa (Hales & Shuman, 1999; Liles *et al.*, 1999; Polesky *et al.*, 2001; Rossier *et al.*, 2004, 2008). Therefore, we next compared strain 130b and its srnA mutants for their ability to infect *H. vermiformis* amoebae. In nine out of nine tests, RNase mutants were significantly impaired for infection of hartmannellae, as evidenced by reduced recovery of the mutants at 48 and 72 h post-inoculation (Fig. 4). The magnitude of the defect ranged from fivefold, as exhibited by the NU328 mutant at 48 h in the experiment depicted in Fig. 4(a), to 17-fold, as shown by the NU329 mutant at 48 h in the experiment depicted in Fig. 4(b). Because both of the independently derived srnA mutants were impaired, this defect was due to the srnA mutation and not a second-site mutation. The NU331 and NU332 mutants inactivated for the lpg2847 gene downstream of srnA did not show any growth defect in the *H. vermiformis* culture (Fig. 4a, and data not shown), and this was a further indication that the defect was due specifically to the loss of srnA. The srnA lpg2847 double mutants displayed a reduced infectivity that was similar to the srnA mutants (Fig. 4a, and data not shown). That srnA mutants displayed reduced recovery only after extended incubation indicates that SrnA does not have a role in the early stages of intracellular infection, but that it is more likely to be involved in later replicative phases. Overall, these results indicate that SrnA is necessary for optimal intracellular infection of *H. vermiformis*.

Recently, we determined that a *L. pneumophila* 130b mutant specifically lacking the Lsp-dependent ProA/Msp metalloprotease is impaired for infection of *H. vermiformis* (Rossier *et al.*, 2008). Thus, we constructed a new double mutant of strain 130b that was lacking both srnA and proA (NU335), and tested it for the ability to infect amoebae. Interestingly, the double mutant had a defect that was significantly greater than that exhibited by the single srnA and proA mutants (Fig. 5). Indeed, the defect for NU335 appeared to be the additive result of losing both SrnA and ProA, indicating that secreted RNase and protease activities contribute separately toward explaining the role of type II secretion in *L. pneumophila* infection of *H. vermiformis*. Since the srnA proA mutant was not as impaired as a lspF mutant (Fig. 5), there are likely to be important Lsp-dependent effectors in addition to SrnA and ProA.

**DISCUSSION**

Here, we report the characterization of SrnA as a type-II-secreted RNase of *L. pneumophila* that promotes bacterial infection of a protozoan host. Current BLASTP results indicate that SrnA belongs to the T2 family of RNases (Deshpande & Shankar, 2002), based on a conserved
domain (cd00374) located between amino acid residues 118 and 294. In the prokaryotic subfamily of T2 RNases (cd01062), SrnA has as its closest homologues proteins within the T2 families (Aravind & Koonin, 2001). These enzymes share the ability to hydrolyse RNA to 3’ mononucleotides via 2’,3’ cyclic nucleotides (Deshpande & Shankar, 2002). The T2 RNases cleave RNA endonucleolytically, and are base non-specific (Deshpande & Shankar, 2002; Irie & Ohgi, 2001). Whereas RNase A proteins are restricted to vertebrates, and T1 RNases are restricted to fungi and Gram-positive bacteria (Dyer & Rosenberg, 2006; Sevcik et al., 2002; Yoshida, 2001), the T2 RNases are widespread across the biological kingdoms, occurring in viruses, bacteria, protozoa, fungi, plants and animals (Deshpande & Shankar, 2002; Irie & Ohgi, 2001). T2 RNases have been most extensively studied in eukaryotes, where they have been implicated in nutritional scavenging of nucleotides and/or phosphate in protozoa, fungi and plants, senescence, self-incompatibility and defence against pathogens in plants, and regulation of membrane permeability in yeast (Deshpande & Shankar, 2002; Irie & Ohgi, 2001; MacIntosh et al., 2001; McGugan et al., 2007). Additionally, some fungal and animal virus T2 RNases have been shown to possess cytotoxic activity, which has potential for anti-carcinogenic and anti-angiogenic therapy (Hulst et al., 1994; Schneider et al., 1993; Schwartz et al., 2007). In contrast to this wealth of information in eukaryotes, there are very little data on the role of T2 RNases in bacteria, even though there are clearly many T2 RNase genes in the prokaryotic genome database. In E. coli, the T2 RNase known as RNase I has been studied biochemically, and it has been defined as being localized to the periplasmic space (Messens et al., 2007; Padmanabhan et al., 2001). A similar situation exists in Aeromonas hydrophila and Shigella sp. (Favre et al., 1993). There are other secreted RNase activities in bacteria, but they represent nucleases that act on both RNA and DNA, e.g. the S1-P1, Staphylococcus nuclease, and colicin families (Desai & Shankar, 2003; Hsia et al., 2005). Secreted RNase activities have been found in some species of Klebsiella, Salmonella, Stenotrophomonas and Vibrio, but it is not clear what type of nucleases they are, or how they are secreted (Arella & Sylvestre, 1979; Favre et al., 1993). Thus, our findings involving SrnA are thought to be the first demonstration of a bacterial T2 RNase clearly localized to the extracellular milieu, and the first documentation of a role for any type of secreted RNase in an intracellular infection process.

The inability of srnA mutants to flourish optimally within H. veriformis cultures indicates that SrnA has a significant role in L. pneumophila environmental persistence and, by extension, transmission to mammalian hosts. Several scenarios can be imagined for how SrnA promotes growth within H. veriformis. First, SrnA might promote nutrient acquisition by degrading host cell RNA in order to obtain (portions of) nucleotides and/or phosphate. Second, SrnA might act to alter host cell function by degrading host RNA. Third, SrnA might possess a second enzymic activity that is relevant for intracellular infection; compatible with such a scenario, the T2 RNase of Aspergillus niger has an actin-binding activity that is separate from its nuclease activity (Schwartz et al., 2007). Finally, although we favour the hypothesis that there is a direct action of SrnA on host cell target, it is also conceivable that the protein acts upon the bacterium itself, and in its absence intracellular legionellae are less able to persist. The location of SrnA in the infected host would influence protein function, and this, in turn, would be dictated by molecular trafficking across the phagosome membrane. Whether SrnA exits the phagosome into the cytoplasm and/or whether host cytoplasmic contents move into the phagosome over time is a key question. Relevant to that question, there are data showing type-II-secreted ProA present within the cytoplasm of infected host cells (Rechnitzer et al., 1992).
Although srrA mutants did not show a defect when grown in media, U937 cells or the A/J mouse lung, we do not conclude that SrrA and secreted RNase activity are only relevant for amoebal interactions. For example, SrrA might have an extracellular role in the environment when the legionellae experience extreme nutrient limitation and/or are faced with heightened competition or predatory microorganisms. Also, as suggested by our in silico analysis (DebRoy et al., 2006b), it is possible that L. pneumophila produces other nucleases that could serve in the absence of SrrA.

With the results presented here, we now have our second demonstration of a type II secreted protein promoting intracellular infection of amoebae. The significance of both SrrA and ProA confirms our long-standing hypothesis that the intracellular growth defects of lsp mutants are due to the loss of secreted effectors versus being solely due to potential cell envelope aberrations. That the srrA and proA mutants exhibited relatively modest defects, and that the srrA proA double mutant was not as impaired as an lsp mutant, imply that the L. pneumophila type II secretion system promotes intracellular infection through the combined action of many secreted effectors. A similar situation occurs with the well-studied type IVB secretion system of many bacteria, for the production of secreted T2 RNases.

It would be worthwhile to examine other bacteria, organisms. Also, as suggested by our in silico analysis, bacteria are faced with heightened competition or predatory microorganisms. For example, SrnA might be required for production of the major acid phosphatase and its role in intracellular infection. The significance of both primarily promote intracellular infection of amoebae. The production of an extracellular acid phosphatase gene potentiates intracellular infection of protozoa and human macrophages. A versatile negative-staining ribonuclease zymogram. Anal Biochem 219, 82–86.


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