Stenotrophomonas maltophilia strains replicate and persist in the murine lung, but to significantly different degrees

Ruela Rouf, Sara M. Karaba, Jenny Dao and Nicholas P. Cianciotto

Department of Microbiology and Immunology, Northwestern University Medical School, Chicago, IL 60611, USA

INTRODUCTION

Stenotrophomonas maltophilia is an environmental, Gram-negative bacterium that is increasingly identified as an opportunistic and nosocomial pathogen (Falgas et al., 2009; Looney et al., 2009). Originally classified as Pseudomonas maltophilia and then Xanthomonas maltophilia, S. maltophilia belongs to the γ-Proteobacteria and is the best known of the 11 species that form the genus Stenotrophomonas (Kim et al., 2010; Lee et al., 2011; Ryan et al., 2009; Yi et al., 2010). In nature, S. maltophilia exists in a wide variety of environments, being found in water, soil and plant material (Denton & Kerr, 1998; Ryan et al., 2009). But, S. maltophilia also occurs in man-made water systems, and most problematic is its presence in devices and reagents in hospital settings (Denton & Kerr, 1998; Looney et al., 2009). Increasingly isolated by clinical microbiology laboratories, S. maltophilia is associated with a spectrum of diseases, including infections of the lung, blood, heart, urinary tract, eyes, CNS, skin and soft tissue (Denton & Kerr, 1998; Falagas et al., 2009; Looney et al., 2009). The respiratory tract is the most common locale for S. maltophilia infection, with surveys indicating that ~5% of nosocomial pneumonias are associated with this organism (Denton & Kerr, 1998; Looney et al., 2009). Some of the factors that predispose patients to S. maltophilia are immunosuppression, cancer, indwelling devices, mechanical ventilation, and broad-spectrum antimicrobial therapy (Ansari et al., 2007; Denton & Kerr, 1998; Falagas et al., 2009; Paez et al., 2008; Looney et al., 2009; Safdar & Rolston, 2007). Investigators throughout the world also report an increasing prevalence of S. maltophilia in cystic fibrosis (CF) patients; in the US, S. maltophilia is linked to ~10% of CF patients, and in Europe, the linkage is as high as 30% (Davies & Rubin, 2007; Lambiasi et al., 2006; Nicoletti et al., 2011; Paschoal et al., 2007; Spicuzza et al., 2009; Waters et al., 2011). A recent study has also determined that chronic S. maltophilia infection is an independent risk factor for lung exacerbations in CF patients (Waters et al., 2011). The prevalence of S. maltophilia in patients is partly explained by the marked resistance of the organism to antibiotics,
which involves β-lactamases, enzymes that act on aminoglycosides, and multidrug efflux pumps (Alonso & Martínez, 2000; Avison et al., 2002; Gordon & Wareham, 2010; Gould & Avison, 2006; Hernández et al., 2009; Huang et al., 2010; Looney et al., 2009; Okazaki & Avison, 2007; Sánchez et al., 2008, 2009; Sánchez & Martínez, 2010; Shimizu et al., 2008).

Despite the increasing association of *S. maltophilia* with disease, our understanding of the pathogenicity and virulence of this bacterium is still quite minimal. Limited phenotypic analysis of *S. maltophilia* strains in vitro and the recent sequencing of the *S. maltophilia* genome both suggest that the organism has traits that have been linked to the virulence of other bacteria (Crossman et al., 2008; Denton & Kerr, 1998; Looney et al., 2009; Nicoletti et al., 2011; Rocco et al., 2009; Ryan et al., 2009). Also, inoculation of *S. maltophilia* into the lungs of mice results in a marked inflammatory response within 24 h that involves, among other things, elevated cytokines (Di Bonaventura et al., 2010; Waters et al., 2007; Zgair & Chhibber, 2010a). However, the degree to which *S. maltophilia* is capable of replicating on its own within a mammalian host has remained a point of some controversy for several reasons. First, *S. maltophilia* is often isolated from patients along with other bacteria, especially in cases of pneumonia (Ryan et al., 2009). Second, several studies which used a pure culture of *S. maltophilia* to infect the lungs of mice or rats did not document significant increases in bacterial numbers post-inoculation; rather, bacterial numbers were only reported as declining between days 1 and 7 post-inoculation (Di Bonaventura et al., 2010; George et al., 1993; McKay et al., 2003; Waters et al., 2007). Thus, we sought to more thoroughly address the issue of *S. maltophilia* infectivity by examining six different strains of *S. maltophilia* for their ability to replicate and persist in the lungs of four different strains of inbred mice, utilizing various inoculating doses and assaying bacterial numbers at earlier times post-inoculation. We now document that *S. maltophilia* strains can indeed replicate as much as 10-fold following inoculation into the lungs of some but not all strains of mice. We also report that clinical isolates of *S. maltophilia* differ significantly in their ability to grow and persist in the murine lung.

**METHODS**

_Bacterial strains and media._ The primary *S. maltophilia* strain used in this study was K279a, obtained from Matthew Avison at the University of Bristol. K279a is a multidrug-resistant strain that was originally isolated from the blood of a cancer patient (Avison et al., 2000). The five additional strains of *S. maltophilia* that were included in the study were obtained from Paul Edelstein at the University of Pennsylvania. These isolates, which were obtained from the respiratory tracts of patients at the University of Pennsylvania Hospital, were designated UPSm1, UPSm2, UPSm3, UPSm4, and UP Sm5. Strains of *S. maltophilia* were routinely cultured on standard Luria–Bertani (LB) agar (Ausubel et al., 1989). Growth of these strains in bacteriological media was also assessed by incubating LB broth cultures (25 ml in a 125 ml flask) at 37 °C with agitation and then monitoring the OD_{600} using a Beckman Coulter DU 720 spectrophotometer. Inocula for these cultures were derived from exponential-phase pre-cultures.

_Mouse lung infection_. Female A/J, DBA/2, BALB/c and C57BL6 mice were obtained from Jackson Laboratories. After their arrival, mice were acclimated for 1 week and fed a standard diet. At the age of 6–7 weeks, mice were infected with *S. maltophilia* administered via standard intranasal inoculation. Just prior to their infection, the animals were anaesthetized by intraperitoneal injection of ketamine and xylazine as per veterinary guidelines. The bacteria used for the inoculum were grown for 15 h on LB agar and then suspended and diluted in sterile PBS. Differing amounts of bacteria (as detailed below) were inoculated in a 20 μl aliquot, half of which was delivered into each nostril. At various times post-inoculation, total lung homogenates were obtained as previously described (DeRoy et al., 2006; Rossier et al., 2004), and then the c.f.u. of bacteria within the lungs were determined by plating on LB agar. The c.f.u. recovered immediately after inoculation (t = 0) represented the initial lung deposition. The amount deposited in the lungs was ~30% of the bacteria administered into the nose. In one set of experiments, animals were also inoculated with heat-killed bacteria, which had been obtained by heating the bacterial suspension for 10 min at 65 °C. All animal experiments were approved by the Animal Care and Use Committee of Northwestern University.

_Cytokine assays._ To monitor cytokine levels in infected lungs, A/J mice were infected as described above. At various times post-inoculation, lung homogenates, which had been cleared by centrifugation at 1000 g for 10 min to remove cellular debris, were examined for tumour necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 using murine ELISA kits (Ready-SET-Go! kits, eBioscience) according to the manufacturer’s instructions.

**RESULTS AND DISCUSSION**

*S. maltophilia* K279a replicates within the lungs of A/J mice

As a first step toward assessing _in vivo_ growth of *S. maltophilia*, we examined strain K279a for its ability to replicate in the lungs of 6–7 week-old female A/J mice following intranasal inoculation. Strain K279a is the clinical isolate of *S. maltophilia* for which the genome was recently entirely sequenced (Crossman et al., 2008). We and others have used inbred A/J mice to examine lung infection by _Legionella pneumophila_ (Brieland et al., 1994; DeRoy et al., 2006; Rossier et al., 2004). This inbred strain of mouse has also been used to study infection by a variety of other microbial pathogens, including _Acinetobacter baumanii_, _Bacillus anthracis_, _Candida albicans_, _Listeria monocytogenes_, _Mycobacterium tuberculosis_, _Pseudomonas aeruginosa_ and _Staphylococcus aureus_, some of which are also major causes of lung infection (Ahn et al., 2010; Glomski et al., 2008; Qiu et al., 2009). In an initial infection experiment which began with a K279a deposition count in the lungs of 1.6 × 10^⁸ c.f.u., we observed a fivefold increase in c.f.u. at 4 and 8 h post-inoculation (Student’s _t_ test, _P_ < 0.05), and then a relatively modest reduction in bacterial recovery at 12 h followed by a larger drop in c.f.u. at 24 h (Fig. 1a). Significant increases in c.f.u. at the
two early times points were also observed in four more experiments that utilized the same inoculation dose (data not shown). That 1 × 10^6 c.f.u. of K279a deposited into the lungs represents a sublethal dose was confirmed in another trial, when all 20 animals inoculated with this dose survived over the next 21 days of observation. When the assay was next performed with a lower deposition of 8.0 × 10^5 c.f.u., significant growth was still observed at 4 and 8 h (P<0.05), although the peak in c.f.u. recovered was shifted to the later time point (Fig. 1b). With this inoculating dose, the overall increase in c.f.u. above the deposition count was now 10-fold. In two additional experiments, when the deposition count was lowered even further to 2.0 × 10^4, we still observed a significant increase in c.f.u. (P<0.05) at 8 and 12 h post-inoculation (Fig. 1c and data not shown).

To determine whether infection of the A/J lung with S. maltophilia results in an inflammatory response, we infected the mice with a deposition of ~1 × 10^6 c.f.u. of strain K279a as above, and then examined the infected lung for elevations in the pro-inflammatory cytokines IL-6, TNF-α and IL-1β at 24 h post-inoculation. We observed significant increases (P<0.05) in all cytokines compared with uninfected controls (Fig. 2a). An additional infection confirmed the presence of elevated cytokine levels at 24 h in mice infected with viable bacteria compared with uninfected animals (data not shown). In contrast, inoculation with heat-killed K279a did not result in elevated cytokines (Fig. 2a). Further assessments of TNF-α levels indicated that the pro-inflammatory response in the A/J mice was evident as early as 8 h post-inoculation (Fig. 2b). Next, to ascertain whether infection of the A/J lung with strain K279a also results in a functional adaptive immune response, we sought to determine whether infection with strain K279a afforded any protection against a subsequent challenge. To that end, one group of mice (n=20) was inoculated with a deposition count of 1.1 × 10^6 c.f.u. of K279a, and another group (n=19) was inoculated with PBS alone. Twenty-one days later, we infected the two groups as above and then determined the numbers of c.f.u. in the lungs at 0, 4, 8 and 12 h post-inoculation (Fig. 3). The animals that had not been previously exposed to K279a exhibited a pattern of bacterial growth and survival that was comparable with that of earlier experiments; e.g. compare Fig. 3 with Fig. 1(b). In contrast, those animals that had been previously exposed showed a more rapid clearance (P<0.01) of bacteria that was evident as early as 8 h post-inoculation (Fig. 3).

Taken together, these data demonstrated that S. maltophilia is fully capable of replicating at least five- to 10-fold within the A/J mouse lung and without the ‘assistance’ of another...
bacterial species. Furthermore, *Stenotrophomonas* replication was observed after an initial lung deposition count as low as 10⁴ c.f.u. Finally, these experiments established that the sequenced clinical isolate of *S. maltophilia* is capable of both replicating and triggering an innate and adaptive immune response in a mammalian model of disease. The K279a genome sequence reveals a number of potential virulence factors and immune modulators, based upon the in vivo importance of their homologues in other bacteria (Crossman et al., 2008). These factors include type I, II, IV and V protein secretion systems, tissue-degradative exoenzymes, various pili, putative adhesins, flagella, LPS, putative exopolysaccharide, siderophores and quorum sensing (Crossman et al., 2008; Denton & Kerr, 1998; Fouhy et al., 2007; Huang & Wong, 2007; Looney et al., 2009; Minkwitz & Berg, 2001; Rocco et al., 2009; Travassos et al., 2004; Waters et al., 2007; Zgair & Chhibber, 2010b). Thus, the A/J mouse model can be used to compare wild-type K279a with its mutant derivatives in order to formally define the virulence factors of *S. maltophilia*.

*S. maltophilia* K279a replicates in the lungs of DBA/2 mice but not BALB/c and C57BL/6 mice

To discern whether or not the growth that we had observed for K279a was specific to A/J mice, we performed intranasal inoculations and lung c.f.u. determinations in three more inbred strains of mice; i.e. 6–7-week-old female DBA/2, BALB/c and C57BL/6 mice. We included the DBA/2 strain in particular because it shares with the A/J mouse a genetic deficiency in complement factor C5 (http://jaxmice.jax.org/strain/000646.html), which could potentially influence susceptibility to *S. maltophilia*. Although the initial lung deposition of K279a in these strains of mice was comparable with that which had been obtained with the A/J mouse infections, a spectrum of growth and persistence was subsequently observed (Fig. 4). In the DBA/2 host, strain K279a behaved in a manner that was similar but not identical to that which had been observed in the A/J mice; i.e. five- to 10-fold increases (*P*<0.05) in c.f.u. were detected at 4 h post-inoculation, but then there was a
di Bonaventura et al. however, as noted above, this study did not report bacterial replication of some bacteria, including P. aeruginosa, for which the primary reason for A/J susceptibility is not linked to the absence of C5 (Qiu et al., 2009); e.g. in S. aureus, the key factors are encoded by the Tnfaip8 and Seh1l loci (Ahn et al., 2010; Qiu et al., 2009), and in L. pneumophila, the critical marker is Naip5/Bircle (Zamboni et al., 2006). Third, the susceptibility of DBA/2 mice to the replication of some bacteria, including P. aeruginosa, is also not linked to the C5 deficiency (Wilson et al., 2007). Based upon our data, genetic crosses between the permissive and non-permissive mice can map and then help define host factors that govern susceptibility to S. maltophilia, with the potential for discovering new attributes of host susceptibility that have implications for explaining human cases of S. maltophilia disease.

Strains of S. maltophilia replicate and persist to differing degrees within the lungs of A/J mice

Utilizing the A/J mouse model of infection, we next sought to determine whether strains of S. maltophilia differ in their ability to replicate or persist in the lung. To that end, we employed five clinical isolates obtained from the respiratory tract. The first strain tested, UPSm1, which was obtained from a tracheal aspirate, grew slightly (P<0.05) in the first 4 h and then dropped rapidly over the next 12 h compared with strain K279a (Fig. 5a, compared most closely with Fig. 1a). At the 24 h time point, UPSm1 was not recoverable; this was a marked departure from what had been observed for K279a. The second strain tested, UPSm4, obtained from a sputum sample, increased its numbers 10-fold (P<0.05) by 4 h post-inoculation, but then, unlike strain K279a, declined by 8 h (Fig. 5b, as compared with Fig. 1a). However, between 8 and 24 h, UPSm4 was more effective than K279a at maintaining its numbers 10-fold (Fig. 4a, as compared most closely with Fig. 1b). Late in the course of our study, another group reported infection of 7-week-old female DBA/2 mice with a different strain of S. maltophilia; however, as noted above, this study did not report bacterial replication (Di Bonaventura et al., 2010). Given our findings, the most likely reason that the other study did not observe replication was because it began with lung depositions that were already between 10^7 and 10^8 c.f.u. and then first monitored for changes in c.f.u. at 24 h post-inoculation. In the BALB/c mice, strain K279a did not increase its numbers but was able to fully maintain its c.f.u. for the first 8 h before dropping more than 10-fold at 12 h (Fig. 4b). In contrast to this result, a different blood culture isolate of S. maltophilia was very recently reported as being capable of replicating within 12 h following intranasal inoculation into 4–6-week-old male BALB/c mice (Zgair & Chhibber, 2010a). That bacterial replication was observed in that study and not ours could be due to differences in virulence between the bacterial strains used and/or differences in the age, sex or source (lineage) of the BALB/c mice. Additionally, the other study utilized a very large inoculating dose of 10^9 c.f.u., a dose that is 1000-fold higher than that used in our experiments. In the C57BL/6 mice, K279a appeared unable to replicate, showing a decline in c.f.u. recovery at 4, 8 and 12 h (Fig. 4c). An earlier study reported infection of neonatal (i.e. 7–10-day-old) C57BL/6 mice with different strains of S. maltophilia; but, as noted above, no evidence for bacterial replication was reported after determining lung c.f.u. at 16 h post-inoculation (Waters et al., 2007).

Taken together, our experiments, which represent the first side-by-side comparison of mouse strains for their susceptibility to S. maltophilia, indicate that the S. maltophilia bacterium replicates in some but not all strains of adult mice. Furthermore, the data indicate that there is a spectrum of susceptibility among commonly used inbred strains of mice, i.e. the A/J strain is the most permissive for S. maltophilia growth and persistence, followed by the DBA/2 strain, then the BALB/c mouse, and finally the non-permissive C57BL/6 strain. It is tempting to hypothesize that the reason that the A/J and DBA/2 mice are most permissive for S. maltophilia is that they both lack the complement factor C5, which is a mediator of neutrophil and macrophage recruitment, and C5 deficiency has been linked to susceptibility to other bacteria (Ahn et al., 2010; Ricklin et al., 2010). However, several points argue that the situation may not be that simple. First, the behaviour of strain K279a in the A/J mice was similar but not identical to that observed in the DBA/2 mice. Second, there are some bacteria for which the primary reason for A/J susceptibility is not linked to the absence of C5 (Qiu et al., 2009); e.g. in S. aureus, the key factors are encoded by the Tnfaip8 and Seh1l loci (Ahn et al., 2010; Qiu et al., 2009), and in L. pneumophila, the critical marker is Naip5/Bircle (Zamboni et al., 2006). Third, the susceptibility of DBA/2 mice to the replication of some bacteria, including P. aeruginosa, is also not linked to the C5 deficiency (Wilson et al., 2007). Based upon our data, genetic crosses between the permissive and non-permissive mice can map and then help define host factors that govern susceptibility to S. maltophilia, with the potential for discovering new attributes of host susceptibility that have implications for explaining human cases of S. maltophilia disease.

Fig. 3. Effect of prior infection with strain K279a on the susceptibility of A/J mice to S. maltophilia challenge. One group of mice (n=20) that had been infected with a sublethal dose of K279a 21 days earlier (○) and another group of mice (n=19) that had previously been inoculated with PBS only (■) were infected with K279a, and then the numbers of c.f.u. in the lungs were determined at 0, 4, 8 and 12 h post-inoculation. The values presented at each time point are the mean ± s.o obtained from five infected animals, with the exception of the 0 h point for the PBS control group (four animals). Mice that had previous exposure to K279a exhibited a significantly reduced bacterial load compared with the PBS control group at 8 h post-inoculation (Student’s t test, P<0.01).
numbers, e.g. at 24 h, it displayed ~100-fold more c.f.u. than did K279a. Taken together, these experiments indicated that S. maltophilia growth in the lungs is not specific to K279a. However, the data suggested that S. maltophilia strains can differ significantly in their ability to persist in the lungs of A/J mice, with UP\textsubscript{Sm}4 exhibiting strong persistence, K279a intermediate persistence and UP\textsubscript{Sm}1 weak persistence. Thus, we extended the c.f.u.

![Fig. 4. Growth of S. maltophilia strain K279a in the lungs of DBA/2, BALB/c and C57BL/6 mice. DBA/2 (a), BALB/c (b) and C57BL/6 (c) mice were intranasally inoculated with strain K279a (■), resulting in comparable levels of deposition (t=0), and then at 4, 8 and 12 h post-inoculation, the c.f.u. in lung homogenates were determined by plating. Data are the mean ± SD obtained from five infected animals and are representative of at least two independent experiments. Significant increases in c.f.u. relative to the value at t=0 were obtained at 4 h in (a) (Student's t test, P<0.05).](image)

![Fig. 5. Growth of different strains of S. maltophilia in the lungs of A/J mice. Animals were intranasally inoculated with strain UP\textsubscript{Sm}1 (a, ▲) or UP\textsubscript{Sm}4 (b, ○) resulting in comparable levels of deposition (t=0), and then at 4, 8, 12 and 24 h post-inoculation, the c.f.u. in total lung homogenates were determined by plating. Data are the mean ± SD obtained from five infected animals and are representative of at least two independent experiments. Significant increases in c.f.u. relative to the values at t=0 were obtained only at 4 h in (a) and (b) (Student's t test, P<0.05).](image)
determinations until ~40 h post-inoculation and added three more strains (i.e. UPSm2, obtained from a sputum sample of a CF patient, and UPSm3 and UPSm5, obtained from the respiratory sinus of two other patients) to the analysis. The prototype K279a persisted, showing 100–1000 c.f.u. in the lungs at 40 h post-inoculation (Fig. 6a). The other five could be placed roughly into three groups relative to K279a (Fig. 6b). UPSm1 and UPSm3 were unrecoverable after 40 h, indicative of strains with poor persistence. UPSm2 behaved comparably with K279a, yielding ~10^5 c.f.u. around the 20 h time point and then 100–1000 c.f.u. after 40 h. Finally, UPSm4 and UPSm5 were more effective at persistence than was K279a. UPSm4 was especially persistent, yielding ~10^5 c.f.u. after 40 h,

**Fig. 6.** Persistence of different strains of *S. maltophilia* in the lungs of A/J mice. Animals were intranasally inoculated with comparable numbers of strain K279a (a) or strain UPSm1 (▲), UPSm2 (△), UPSm3 (■), UPSm4 (○) or UPSm5 (●) (b), and then at indicated times post-inoculation, the c.f.u. in total lung homogenates were determined by plating. Data are the mean ± SD obtained from four infected animals and are representative of at least two independent experiments.

**Fig. 7.** Growth of *S. maltophilia* strains in bacteriological media. (a) Equal numbers of c.f.u. of strains K279a (■), UPSm1 (▲), UPSm2 (△), UPSm3 (■), UPSm4 (○) and UPSm5 (●) were inoculated into LB broth, and then the growth of the cultures was monitored spectrophotometrically. Data are the mean ± SD obtained from replicate samples and are representative of three independent experiments. The slightly reduced optical density readings for the UPSm2 cultures were only significant at the 205 min time point (Student’s *t* test, *P* < 0.05). Although the UPSm3 cultures were started at a slightly higher optical density in order to ensure equal c.f.u. at the outset, they subsequently displayed a growth pattern that was akin to that of the other strains. (b) As indicated, 10-fold dilutions of the five *S. maltophilia* strains were spotted onto LB agar and then incubated at 37, 30 and 25 °C. After 1 day of incubation, growth was recorded. The patterns of growth depicted here were observed on three independent occasions.
which was only about 10-fold less than its deposition. In contrast to these in vivo findings, the strains grew quite similarly when cultured at 37 °C in LB broth or on LB agar (Fig. 7). Incidentally, upon culturing at 30 and 25 °C, UPSm4 and to a lesser degree UPSm2 showed less growth than the other four strains (Fig. 7b). Taken together, these data document that strains of S. maltophilia grow and persist in the lungs to different degrees. That S. maltophilia strains should exhibit different levels of in vivo growth is compatible with past epidemiological analyses which describe high genetic diversity among clinical isolates (Schaumann et al., 2008; Valdezate et al., 2004). The molecular basis for differences in strain virulence may involve the presence or absence of large genomic islands that exist in S. maltophilia genomes but have not been functionally examined. For example, an island in K279a but not in an environmental isolate contains a putative type IV secretion system (Rocco et al., 2007; Figueirêdo et al., 2006; Nicoletti et al., 2011). Thus, future analysis of S. maltophilia virulence in the A/J mouse model should examine not only the sequenced K279a strain but also those strains that have an in vivo phenotype that is significantly different from that of the prototype.

Concluding thoughts

We have demonstrated that S. maltophilia is indeed capable of replicating in the murine lung. This observation lends support to the belief that S. maltophilia is infective and in some cases pathogenic for humans, especially as it relates to pulmonary infection. The different mouse models that we have presented here should prove very useful for future studies aimed at understanding both the bacterial and host factors that influence S. maltophilia infection and disease. Finally, the finding that S. maltophilia strains can differ dramatically in their ability to grow and persist in the lung means that efforts at strain-typing should be intensified in order to better define those strain types or clones that are most likely to trigger the more serious or chronic forms of human disease.

ACKNOWLEDGEMENTS

The authors thank Matthew Avison and Paul Edelstein for sending us bacterial strains. We also acknowledge past and present members of the Cianciotto lab for many helpful discussions, as well as Brendan Mulhern for assistance with figures. This work was supported in part by NIH grants R03 AI082541 and R21 AI076693 awarded to N. P. C.

REFERENCES


Edited by: H. Hilbi