ACS Infectious Diseases

Article

pubs.acs.org/journal/aidcbc

A Secreted Ankyrin-Repeat Protein from Clinical *Stenotrophomonas maltophilia* Isolates Disrupts Actin Cytoskeletal Structure

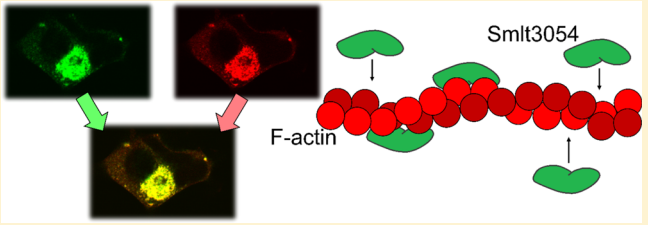
Logan C. MacDonald,[†] Sean O'Keefe,[†] Mei-Fan Parnes,[†] Hanlon MacDonald,[†,§] Lindsey Stretz,[†] Suzanne J. Templer,[‡] Emily L. Wong,[‡,⊥] and Bryan W. Berger*[,†]

[†]Program in Bioengineering and Department of Chemical and Biomolecular Engineering, Lehigh University, Bethlehem, Pennsylvania 18015, United States

[‡]Division of Infectious Disease, Lehigh Valley Health Network, 1250 South Cedar Crest Boulevard, Suite 200, Allentown, Pennsylvania 18103, United States

Supporting Information

ABSTRACT: *Stenotrophomonas maltophilia* is an emerging, multidrug-resistant pathogen of increasing importance for the immunocompromised, including cystic fibrosis patients. Despite its significance as an emerging pathogen, relatively little is known regarding the specific factors and mechanisms that contribute to its pathogenicity. We identify and characterize a putative ankyrin-repeat protein (Smlt3054) unique to clinical *S. maltophilia* isolates that binds F-actin in vitro and co-localizes with actin in transfected HEK293a cells. Smlt3054 is endogenously expressed and secreted from clinical *S. maltophilia* isolates, but not an environmental isolate (R551-3). The in vitro binding of Smlt3054 to F-actin resulted in a thickening of the filaments as observed by TEM. Ectopic expression of Smlt3054−GFP exhibits strong co-localization with F-actin, with distinct, retrograde F-actin waves specifically associated with Smlt3054 in individual cells as well as formation of dense, internal inclusions at the expense of retrograde F-actin waves. Collectively, our results point to an interaction between Smlt3054 and F-actin. Furthermore, as a potentially secreted protein unique to clinical *S. maltophilia* isolates, Smlt3054 may serve as a starting point for understanding the mechanisms by which *S. maltophilia* has become an emergent pathogen.



KEYWORDS: *Stenotrophomonas maltophilia, ankyrin-repeat protein, actin-binding protein*

Stenotrophomonas maltophilia is a nonfermenting, Gram-negative, multidrug-resistant bacilli that has gained significant attention as an emerging, opportunistic pathogen in immunocompromised patients.[1,2] In particular, *S. maltophilia* is among the most prevalent bacteria responsible for ventilator-associated pneumonia, with longer treatment times and associated treatment costs required for *S. maltophilia* versus other prevalent pathogens.[3] *S. maltophilia* is also increasingly found as part of chronic, multispecies bacterial infections in cystic fibrosis (CF) patients, with approximately 9% of CF patients in recent studies having co-infection of *Pseudomonas aeruginosa* and *S. maltophilia*.[4] As a result, the attributed crude mortality of *S. maltophilia* infection was recently estimated to be as high as 37%, and *S. maltophilia* is among the 15 most prevalent pathogens isolated from pediatric patients in North America in the 2004 SENTRY Antimicrobial Surveillance Program.[2]

Although the significance of *S. maltophilia* as a nosocomial pathogen is clear, relatively few specific virulence factors that act directly on host cells have been identified. *S. maltophilia* clinical isolates test positive for extracellular protease and lipase activity, and recent studies have indicated that loss of extracellular protease activity associated with a type II secretion system (T2SS) reduces virulence in lung epithelial cell (A549) cultures.[5−7] Several studies have focused on the role of biofilm formation in *S. maltophilia* invasion and virulence, particularly in clinical isolates from CF patients. Of particular note, biofilm formation from both CF and non-CF isolates was associated with increased adhesion to epithelial respiratory cells, and clinical *S. maltophilia* isolates were observed to be capable of internalizing in both A549 and IB3-1 bronchial cells with rates ranging from 0.4 to 4.9%.[8,9] Internalization provides one mechanism by which bacteria evade host detection, and in the case of *S. maltophilia*, the presence of internalized bacteria was consistent with its increased ability to persist during chronic lung infection.[10] Interestingly, whereas adhesion to host cells was observed to correlate with biofilm formation, the rate of invasion was not correlated with biofilm formation. Thus, the specific factors responsible for *S. maltophilia* invasion and persistence, particularly in chronic lung infection, remain an active area of research.

We describe the identification and characterization of a putative ankyrin-repeat containing protein unique to clinical isolates of *S. maltophilia* (Smlt3054, NCBI accession no.

Received: September 7, 2015
Published: October 20, 2015

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

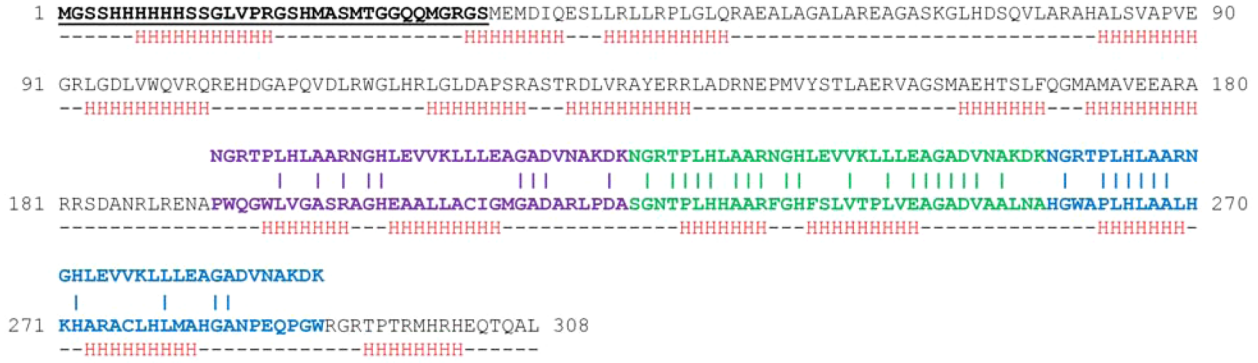


Figure 1. Primary amino acid sequence of recombinant His_6−Smlt3054. The primary amino acid sequence of His_6−Smlt3054 contains 308 amino acids, including the His_6-tag added by pET28a(+) (highlighted in bold and underlined) used for IMAC purification and three predicted ANKs (highlighted in purple, green, and blue). The secondary structure of recombinant His_6−Smlt3054 is 44.5% α-helical, as shown by the residues designated with a red "H".[19] The alignment of each ANK with the consensus sequence for ankyrin-repeats is also shown. Whereas overall sequence identity of each ANK is <58%, the most frequently conserved amino acids in the 33-residue motif are conserved for each ANK in Smlt3054.[37]

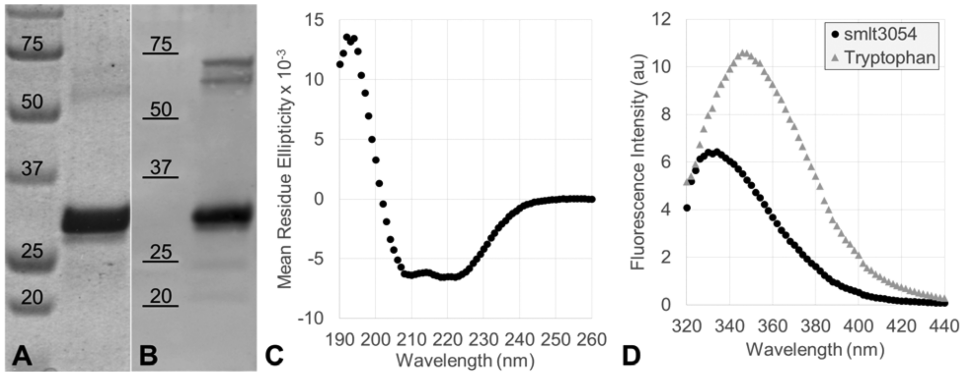


Figure 2. Expression and purification of recombinant Smlt3054. (A) SDS-PAGE gel of IMAC-purified, His_6-tagged Smlt3054 expressed recombinantly from *E. coli* BL21 (DE3) cells. The molecular weight of Smlt3054 based on primary sequence plus the N-terminal His_6-tag is 33.4 kDa. (B) Western blot using anti-His6 monoclonal antibody confirming the presence of His6-tagged Smlt3054 in purified protein sample. (C) Circular dichroism spectra of purified Smlt3054 revealing an α-helical secondary structure, consistent with the predicted 44.5% α-helical structure for Smlt3054 from Jpred4.[19] (D) Tryptophan emission spectra for purified Smlt3054 blue-shifted relative to free tryptophan, consistent with burial of tryptophan residues present in Smlt3054.

SMLT_RS14540) that is secreted from clinical isolates of *S. maltophilia*, binds F-actin in vitro, and affects cellular F-actin localization. Several bacterial pathogens, including *Salmonella*, *Shigella*, *Rickettsia*, and *Listeria*, are known to secrete effectors that disrupt F-actin structure and bundling in order to invade host cells during infection, with a steadily growing list of virulence factors that either act directly on actin or disrupt actin-dependent signaling pathways.[11,12] Furthermore, numerous bacterial pathogens secrete ankyrin-repeat containing proteins into their host to hijack various host functions.[13] We find that Smlt3054 is endogenously expressed and secreted from *S. maltophilia* stains associated with infection and leads to disruption of cellular F-actin structure and localization when overexpressed in HEK293 cells. By tracking the fate of F-actin in HEK293 cells overexpressing Smlt3054, we observe specific Smlt3054 binding to retrograde F-actin waves appearing at the leading edge of cells, which are regions of high membrane and F-actin turnover involved in cell motility. Purified Smlt3054 binds F-actin in vitro and TEM images reveal thickened, distorted F-actin fibers formed in the presence of added Smlt3054. Collectively, our results indicate Smlt3054 is a secreted protein from *S. maltophilia* clinical isolates that acts on F-actin in a manner consistent with a role in host cell cytoskeleton disruption, which may enhance invasion.

RESULTS

Expression and Purification of Smlt3054 Resulted in a Soluble, Folded Protein. We originally identified Smlt3054 through a comparative analysis of published genomes from *S. maltophilia* clinical isolates (K279a, Ab55555) versus environmental isolates (R551-3, SKA14), where it was identified as a hypothetical protein present exclusively in a subset of clinical isolates.[1,14] The Smlt3054 gene (NCBI GeneID 6391502) was isolated from *S. maltophilia* K279a genomic DNA and subcloned into pET28a(+). The primary amino acid sequence of the resulting recombinant protein is presented in Figure 1, including the N-terminal His_6-tag added by pET28a(+) for IMAC purification (shown in bold and underlined) and three predicted ankyrin-repeat domains (ANKs) located near the C-terminal of Smlt3054 (highlighted in purple, green, and blue). The 33-residue ankyrin-repeat is one of the most common amino acid motifs observed in nature and frequently appears in tandem repeats which cooperatively fold into structures that undergo protein−protein interactions, including interactions between host and microbial proteins.[13] In addition to interacting with other target proteins, ankyrin-repeat containing proteins are also known to interact with themselves to form homomultimers.[15−17] Recombinant Smlt3054 was expressed and purified from *Escherichia coli* (Figure 2A,B). The predicted

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

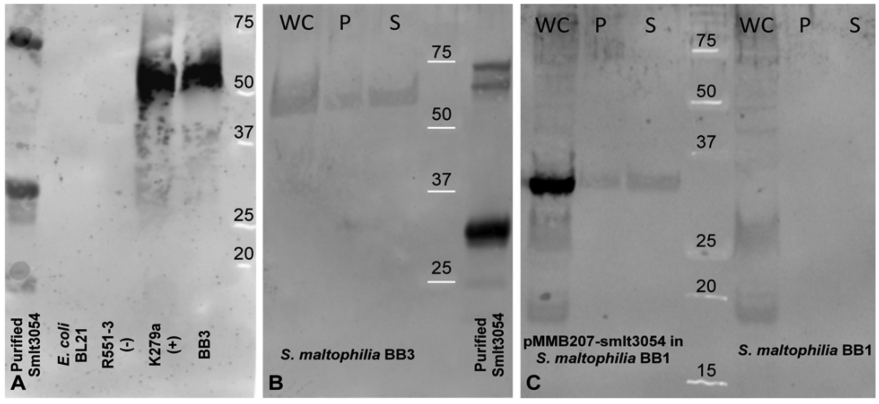


Figure 3. Smlt3054 is secreted from clinical *S. maltophilia* isolates. (A) Western blot using a rabbit polyclonal antibody raised against purified Smlt3054 indicating expression of Smlt3054 in *S. maltophilia* clinical isolates K279a and BB3 but not environmental isolate R551-3. (B) Western blot using anti-Smlt3054 antibody indicating the presence of Smlt3054 in the periplasmic space and culture supernatant of BB3 cultures. (WC, whole cell extract; P, periplasmic space; S, culture supernatant.) (C) Western blot for HA-tagged Smlt3054 overexpressed in clinical strain BB1 indicating Smlt3054 is expressed and present in culture supernatants. Note that the genotype for strain BB1 includes Smlt3054.

molecular weight of His_6-tagged Smlt3054 is 33.4 kDa, consistent with the most prominent band in Figure 2A,B.[18] However, higher molecular weight species were detected by the anti-His_6 antibody as well (Figure 2B), at approximately twice the molecular weight of monomeric His_6−Smlt3054. Thus, our results imply that Smlt3054 forms SDS-PAGE resistant multimers, consistent with other ankyrin-repeat containing proteins.[15−17]

Prediction of secondary structure using JPred4[19] indicates that Smlt3054 is 44.5% α-helical (Figure 1) and is therefore consistent with the α-helical signal measured by circular dichroism (Figure 2C). Likewise, tryptophan fluorescence emission spectra show a blue-shift as compared to free tryptophan (Figure 1D), indicative of partial burial of tryptophan side chains. Thus, we conclude that Smlt3054 is expressed and purified in a soluble form, with secondary and tertiary structures consistent with those of a folded protein.

Smlt3054 Is Endogenously Expressed and Secreted in *S. maltophilia* Clinical Isolates. Given that *S. maltophilia* is noteworthy in terms of its genetic diversity,[20] we were interested in determining whether the Smlt3054 gene was prevalent in clinical isolates and how well conserved the primary amino acid sequence was between the various strains. Of the 20 clinical strains tested, 10 screened positive for the Smlt3054 gene. Sequencing of the Smlt3054 PCR products from each positive clinical strain revealed seven unique primary amino acid sequences, one of which was identical to Smlt3054 from *S. maltophilia* K279a and six that shared between 93 and 95% sequence identity with Smlt3054 from *S. maltophilia* K279a (Figure S1). Each of the six divergent sequences contained between 11 and 17 point mutations distributed throughout the entirety of the protein (Figure S1). Our results provide further evidence that *S. maltophilia* exhibits an exceptional degree of genetic diversity even in strains isolated from the same hospital.[20]

We were also interested in whether the Smlt3054 protein was endogenously expressed from the clinical isolates. Using purified Smlt3054 (Figure 2), a polyclonal antibody against Smlt3054 was raised and tested against eight clinical *S. maltophilia* strains that screened positive for the gene. An anti-Smlt3054 Western blot revealed expression of Smlt3054 for two of eight strains under standard growth conditions as well as the reference strain K279a that contains Smlt3054, whereas no expressed protein was detected for the environmental isolate R551-3, which lacks Smlt3054. A representative Western blot using anti-Smlt3054 for one of the clinical isolates (BB3) is provided in Figure 3A, illustrating positive endogenous expression in strains K279a and BB3, both of which contain the gene product for Smlt3054, but not in the environmental strain R551-3. Furthermore, Smlt3054 was detected by anti-Smlt3054 Western blotting in the perisplasm and culture supernatant of BB3 cultures (Figure 3B). Additionally, Smlt3054 was subcloned with a C-terminal HA tag into plasmid pMMB207, which contains a tac expression promoter and has been used previously for protein overexpression in *S. maltophilia* as well as in demonstrating secretion of actin-binding proteins from *Vibrio parahemolyticus*.[21,22] When transformed into the clinical strain BB1, we find that Smlt3054 overexpressed from plasmid pMMB207 is present in the periplasm as well as the culture supernatant as detected by Western blotting with an anti-HA antibody, whereas no expression is detected in the absence of pMMB207−Smlt3054 (Figure 3C). Thus, we conclude that Smlt3054 is endogenously expressed and secreted from *S. maltophilia* clinical isolates grown under standard conditions.

It should be noted that on each anti-Smlt3054 Western blot (Figure 3A,B) endogenously expressed Smlt3054 is detected at a molecular weight of approximately twice that of the predicted molecular weight of Smlt3054 (29.8 kDa). As stated previously, purified recombinant Smlt3054 samples contained higher order oligomers as observed by anti-His_6 (Figure 2B) and anti-Smlt3054 (Figure 3A,B) Western blots, and numerous ankyrin-repeat containing proteins have been shown to form multimers.[15−17] Thus, our results are consistent with Smlt3054 existing as a dimer as well as higher order oligomer when expressed endogenously as well as overexpressed recombinantly.

Smlt3054 Is Associated with Actin in Mammalian Cells. To determine a possible role for Smlt3054 as a host effector, we overexpressed Smlt3054 with a C-terminal GFP fusion in transfected HEK293 cells and compared its intracellular distribution to that of actin, which was overexpressed with an N-terminal tdTomato fusion, via multichannel confocal microscopy. Similar approaches have been used previously in determining intracellular localization and function for secreted ankyrin-repeat effectors from *Coxiella* (AnkJ) as

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

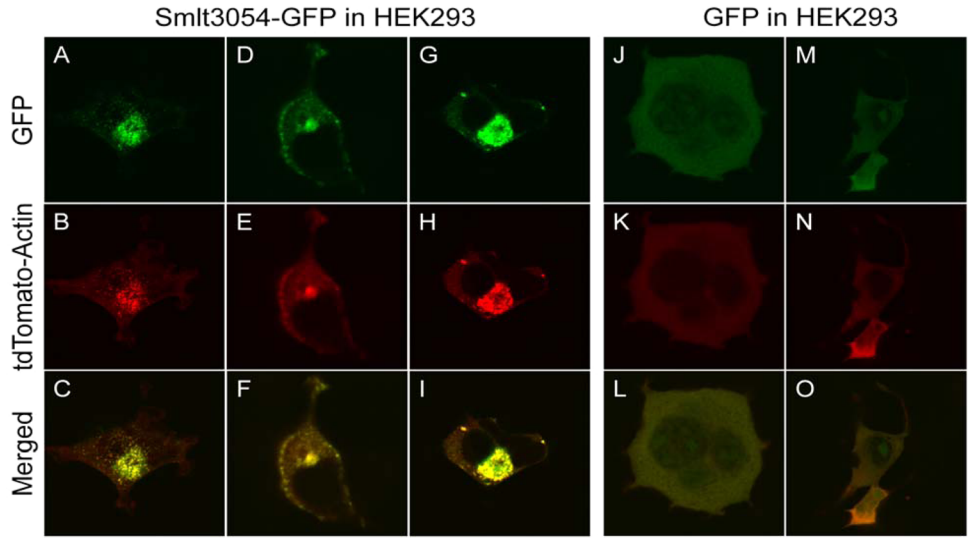


Figure 4. Ectopically expressed Smlt3054−GFP co-localizes with tdTomato−actin in transfected HEK293 cells. The left nine panels (A−I) correspond to coexpression of pEGFP2−Smlt3054 and ptdTomato−Actin-7, whereas the right six panels (J−O) correspond to coexpression of pEGFP2 and ptdTomato−Actin-7. The first row panels (A, D, G, J, M) are the green (Smlt3054−GFP or GFP) channel alone, the second row panels (B, E, H, K, N) are the red (tdTomato−actin) channel alone, and the third row panels (C, F, I, L, O) are merged images. Note the lack of co-localization between GFP and tdTomato−actin in the panels J−O as indicated by the lack of overlap (yellow) in panels L and O, whereas the panels A−I exhibit strong co-localization between Smlt3054−eGFP and tdTomato−actin as indicated by the overlap (yellow) in panels C, F, and I.

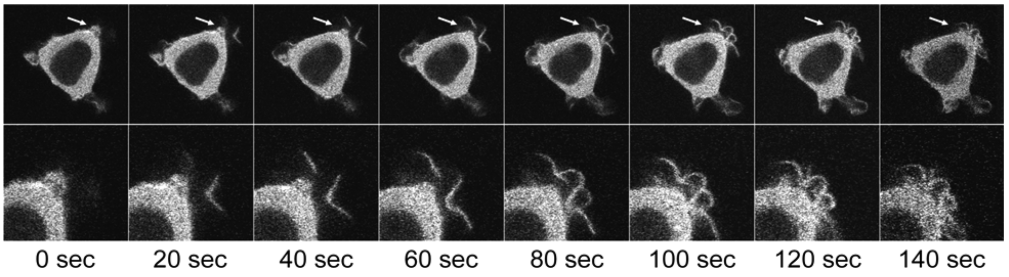


Figure 5. Ectopically expressed Smlt3054−GFP associates with retrograde F-actin waves. Each panel indicates individual images from time-lapse confocal microscopy images of HEK293 cells overexpressing Smlt3054−GFP taken 20 s apart for 4 min. The white signal indicates expression of Smlt3054−GFP. Smlt3054 is associated with transient waves at the edge of individual cells as indicated by the white arrows. Videos S1 and S2 assembled from time-lapse confocal microscopy images are also provided to illustrate the retrograde F-actin waves observed in individual cells overexpressing Smlt3054−GFP.

well as for actin-specific effectors from *Salmonella* (SopB, SopE).[23,24] We observe essentially complete overlap (yellow) between the Smlt3054 (green) and actin (red) channels (Figure 4, left), with a nonuniform distribution of actin−Smlt3054 overlap throughout the cell; in particular, internal dense regions of locally higher actin−Smlt3054 concentration and co-localization are observed throughout the transfected cells. This same pattern is not observed in cells co-expressing GFP alone with tdTomato−actin, with regions of high actin (red) concentration observed within cells distinct from GFP, and no significant overlap between the two proteins (Figure 4, right). Thus, the nonuniform, co-localized regions of actin−Smlt3054 are consistent with a specific actin-Smlt3054 interaction.

Using time-lapse confocal microscopy, we tracked the fate of Smlt3054−GFP expressed within individual cells. Snapshots from the time-lapse images (Figure 5) and videos based on these images (Video S1) reveal the presence of Smlt3054−GFP (white) at prominent, retrograde F-actin "waves" propagating from the leading edges of individual cells (Figure 5 and Video S1). Dense, intracellular inclusions consistent with the co-localization images (Figure 4) are also observed in individual cells overexpressing Smlt3054−GFP, and considerably diminished retrograde F-actin waves are observed in cells containing internal inclusions (Video S2). Retrograde F-actin waves are known to occur in the leading edges of lamellapodia and fillipodia during cell movement, and F-actin-driven cell motility is associated with a wide range of cell types and biological processes.[25] The distinctness of the observed retrograde F-actin waves (Figure 5), as well as the accumulation of dense, internal inclusions at the expense of these waves (Video S2), provides further evidence in support of a specific Smlt3054−actin interaction.

Smlt3054 Binds F-Actin in Vitro. To demonstrate a specific interaction between F-actin and Smlt3054, we used a centrifugal assay based on the density difference between G-actin, F-actin, and Smlt3054. In this assay, purified Smlt3054 (Figure 2) is co-incubated with F-actin, and mixtures are ultracentrifuged to sediment F-actin and associated proteins from solution; proteins that do not associate with F-actin remain in solution. As expected, F-actin filaments alone sediment when centrifuged (Figure 6A, lane 1), whereas Smlt3054 alone does not (Figure 6A, lane 2). However, when Smlt3054 is pre-incubated with F-actin, Smlt3054 is observed

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

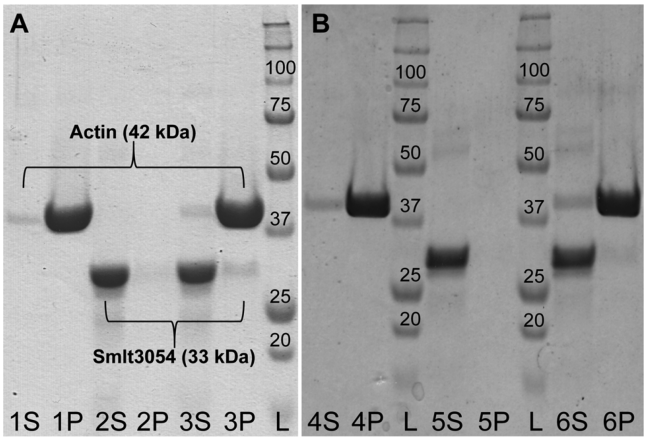


Figure 6. Purified Smlt3054 co-sediments with F-actin in vitro. Association of purified Smlt3054 with F-actin (A) and the effect of Smlt3054 on actin polymerization (B) in vitro was tested using a centrifugal assay as described under Materials and Methods. (P, pellet formed after centrifugation; S, supernatant after centrifugation.) Purified Smlt3054 does not sediment at high centrifugal speeds (lanes 2 and 5), remaining in the soluble fraction (lanes 2S and 5S), whereas purified F-actin does sediment at high centrifugal speeds (lanes 1 and 4), accumulating in the insoluble fraction (lanes 1P and 4P). (A) F-actin binding experiment. When F-actin is pre-incubated with Smlt3054 (lane 3), Smlt3054 is observed in the insoluble fraction (lane 3P), indicative of association with F-actin. (B) Polymerization test. G-actin was incubated with Smlt3054 for 30 min before inducing polymerization for 30 min. Any remaining G-actin will stay in the supernatant, whereas polymerized F-actin will sediment upon ultracentrifugation. Although Smlt3054 does co-sediment with F-actin (lane 6P), there is no significant change in the amount of actin that sediments when pre-incubated with Smlt3054 (lane 6) and the amount of actin that sediments in the absence of Smlt3054 (lane 4), indicating that Smlt3054 does not alter the rate of actin polymerization.

to co-sediment with F-actin (Figure 6A, lane 3). Additionally, we repeated this experiment with G-actin to test if Smlt3054 influenced the polymerization of G-actin into F-actin (Figure 6B). Briefly, Smlt3054 is pre-incubated with G-actin in a low-salt buffer, and then actin polymerization is induced by increasing the salt concentration and allowed to proceed for precisely 30 min at room temperature. Mixtures are then ultracentrifuged, and the resulting supernatant and pellet are analyzed for protein content via SDS-PAGE. The ultracentrifugation causes F-actin to sediment; however, G-actin will remain in solution. Therefore, if Smlt3054 either sequesters G-actin, preventing it from polymerizing into F-actin, or promotes the polymerization of G-actin into F-actin, the relative amount of actin protein in the supernatant and pellet lanes containing both actin and Smlt3054 will be significantly different from the actin alone lanes. As shown in Figure 6B, the majority of the actin-alone sample sediments (lane 4) and Smlt3054-alone does not (lane 5). Whereas we did observe Smlt3054 co-sedimentation with the F-actin (lane 6P), there is no significant difference between the amount of F-actin that sediments when pre-incubated with Smlt3054 (lane 6) and the amount of F-actin that sediments in the absence of Smlt3054 (lane 4). Thus, our results indicate that Smlt3054 interacts directly with F-actin in vitro and does not significantly influence actin polymerization, providing evidence in favor of a specific, direct Smlt3054 interaction with F-actin filaments.

To investigate further what specific effects bound Smlt3054 may have on F-actin, purified F-actin samples incubated with Smlt3054 were imaged via TEM. Preparation of F-actin and Smlt3054−F-actin was performed using previously described techniques for TEM imaging of F-actin.[26] As expected, F-actin samples exhibit long, straight filaments consistent with previous results for TEM imaging of F-actin.[26] However, in the presence of Smlt3054 distinct changes in F-actin filament structure are observed. First, individual filaments are thicker in the presence of Smlt3054 (Figure 7C,D) versus F-actin filaments alone

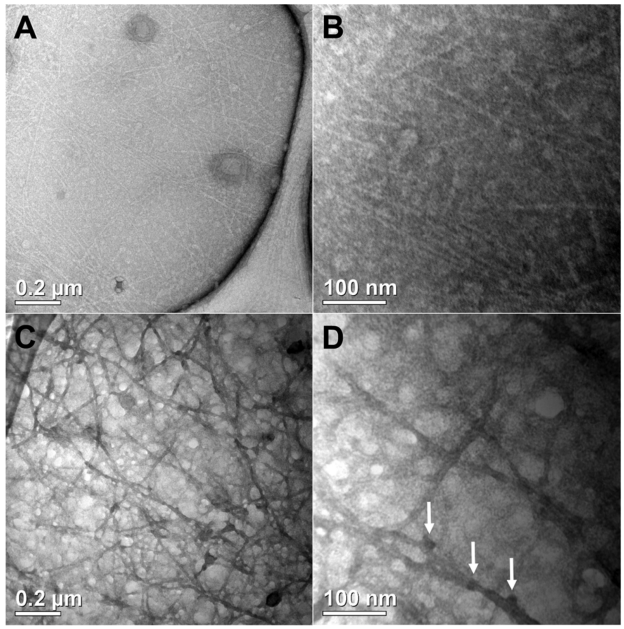


Figure 7. Smlt3054 causes thickening of F-actin filaments. F-actin filaments were negatively stained and imaged using TEM as described previously, comparing F-actin alone (panels A and B) to F-actin treated with Smlt3054 (panels C and D).[26] Regions of higher electron density and thicker F-actin filaments are observed for Smlt3054-treated F-actin filaments (panels C and D) versus F-actin alone (panels A and B). Rounded protrusions with higher electron density are also observed on individual, thickened filaments (highlighted by arrows in panel D).

(Figure 7A,B), with darkened regions of increased electron density observable throughout the Smlt3054-treated samples. Second, at 100 nm scale, regions of increased local electron density are observed on individual filaments in the presence of Smlt3054 (Figure 7D), with rounded protrusions observable along individual filaments (highlighted by arrows in Figure 7D). Collectively, these results support the observations in cells and in vitro indicating Smlt3054 binds F-actin and provide further insight into the specific effects of Smlt3054 on F-actin bundle structure.

DISCUSSION

Bacterial pathogens have evolved a wide range of effector types and mechanisms to deliver them to host cells to manipulate actin cytoskeletal structure. In the case of Smlt3054, we provide evidence in favor of it being a specific actin-binding protein in transfected cells (Figures 4 and 5) as well as in vitro (Figures 6 and 7). Bacterial actin-binding proteins have been identified in a wide range of pathogens, where they contribute directly to

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

bacterial internalization, cell movement, and intracellular replication.[11] Among the most well-studied of these is SipA from *Salmonella typhimurium*, which decreases the critical concentration of actin required for F-actin polymerization, stabilizes F-actin filaments by preventing depolymerization, and enhances F-actin bundling, thereby promoting extension of membrane ruffles and filipodia to facilitate invasion.[12,27,28] For Smlt3054, we do not observe a reduction in the concentration of G-actin required for F-actin polymerization (Figure 6B), but when overexpressed in transfected mammalian cells, we observe Smlt3054 co-localization with overexpressed actin (Figure 4) and Smlt3054 bound specifically to retrograde F-actin waves associated with lamellapodia and fillipodia (Figure 5). We also observe significant, morphological changes in F-actin filaments in the presence of Smlt3054: F-actin filaments are thicker and contain rounded, dense protrusions from individual filaments (Figure 7). In the case of SipA, addition of SipA to F-actin filaments resulted in an increase in electron density around the fibers when observed by TEM. This increase was interpreted as SipA binding laterally to individual F-actin filaments, and the resulting TEM images were used to build a 3D predictive model of the SipA/F-actin structure.[12,28] In the case of Smlt3054, we observe a similar increase in electron density around individual F-actin filaments, implying that Smlt3054 also binds laterally to F-actin, essentially "coating" the filament. However, unlike SipA, we also observed nodules of electron density protruding from individual filaments in Smlt3054-treated F-actin filaments (Figure 7C,D, white arrows). A recent study of T2SS in *S. maltophilia* K279a indicates significant changes in A549 actin cytoskeletal structure for cells treated with K279a culture supernatants, and these changes were dependent on expression of specific proteins associated with assembly of the identified T2SS.[5] Another recent study demonstrated two serine proteases (StmPr1 and StmPr2) secreted by *S. maltophilia* via a T2SS contributed either directly or indirectly to the actin rearrangement associated with the co-culturing of *S. maltophilia* K279a with A549 cells, which ultimately resulted in cell rounding, detachment, and death.[29] In the current study, secretion of Smlt3054 from clinical *S. maltophilia* isolates (Figure 3) occurred under the same growth conditions as those used in identification of type II secreted effectors from strain K279a. In the case of *S. typhimurium*, several other effectors are secreted to independently affect microtubule structure and other aspects of cytoskeletal structure, and in the case of *S. maltophilia*, it will be interesting to identify what, if any, other effectors work in concert with Smlt3054 to modulate host cytoskeletal structure.[24]

Although Smlt3054 is a hypothetical protein, we demonstrate that it is endogenously expressed and secreted from *S. maltophilia* clinical isolates containing the gene product for Smlt3054 versus reference strains lacking the gene product for Smlt3054 (Figure 3) and exhibits a well-defined secondary and tertiary structure (Figure 2), providing further evidence in favor of its being a specific *S. maltophilia* actin-binding effector protein. Using the sequence-specific domain prediction tool PFAM, Smlt3054 is predicted to contain three C-terminal ANKs (Figure 1).[30] Interestingly, bacterial ANKs have previously been identified in other intracellular bacterial pathogens as host effectors, including *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Legionella pneumophila*, and *Coxiella burnetii*, where in each case the ANK is secreted via a type II secretion system (T2SS) to modulate host cell behavior, facilitate bacterial internalization, and promote intracellular replication during infection.[13] For *E. chaffeensis*, the effector AnkA has been shown to interfere with dissociation of the host IκB/NF-κB complex necessary to activate host cytokine production and pro-inflammatory response; specifically, it is though the ANKs present in AnkA act as mimics of IκB, thereby sequestering NF-κB and preventing its transcriptional activation.[31] For *L. pneumophila*, AnkX has been shown to interfere with microtubule-dependent transport of vesicles from the ER to the Golgi, whereas AnkB contains an additional F-box domain that interacts with host ubiquitination machinery; loss of AnkB severely reduced intracellular replication of *L. pneumophila* in a mouse model of infection.[32,33] Thus, our results add to the growing list of specific, virulence-associated functions attributable to ANKs as secreted effectors from bacterial pathogens; to date, no bacterial ANKs have been reported whose function is to disrupt host actin cytoskeletal structure (Figures 4 and 5) and directly bind F-actin (Figures 6 and 7) as is observed for Smlt3054.[13] Given that previous studies of *S. maltophilia* virulence have indicated actin rearrangement in A549 cells occurs in a T2SS-dependent manner, it is interesting to speculate whether Smlt3054 is secreted via a T2SS or instead via a type I or type IV system as has been described for other secreted bacterial ankyrin-repeat effectors.[5,13,34]

In summary, we provide evidence that the hypothetical protein Smlt3054 is expressed and secreted in clinical *S. maltophilia* isolates and acts directly on F-actin filaments to disrupt cytoskeletal structure and bind retrograde F-actin waves. Our work provides the first study of a specific, secreted protein from *S. maltophilia* having function linked to actin cytoskeletal disruption and suggests a role as a possible effector involved in facilitating *S. maltophilia* internalization during infection. Clinical *S. maltophilia* isolates from cystic fibrosis patients with chronic, drug-resistant infections were capable of internalization in cell culture studies using bronchial epithelial cells, and internalization was thought to contribute to both drug resistance and persistence. Thus, our work serves as a useful starting point for understanding mechanisms that may contribute to the multidrug resistance and persistence of this important, emerging bacterial pathogen.[2]

MATERIALS AND METHODS

Bacterial Strains and Genomic DNA Preparation. *Stenotrophomonas maltophilia* K279a (TAX ID 522373)[14] was kindly provided by Robert Ryan (University College Cork). Clinical isolates of *S. maltophilia* were collected from patients as part of an IRB-approved study with the Lehigh Valley Health Network in Allentown, PA, USA. All bacterial strains were cultured in 5 mL of LB broth at 37 °C for 16 h before resuspension in 1 mL of LB broth containing 10% glycerol for long-term storage at −80 °C. Genomic DNA was prepared from each isolate via standard molecular biology techniques.[35]

Cell Lines. HEK293 (ATCC CRL-1573) cells were cultivated in Dulbecco's modified Eagle medium supplemented with 4.5 g/L glucose and sodium pyruvate (Corning), 4 mM L-glutamine (Lonza), 10% fetal bovine serum (BioWest), and 1× antibiotic antimycotic solution (100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B) (Hyclone). Medium lacking phenol red was used for live cell imaging experiments.

Genomic DNA Screening and Plasmid Preparation. Gene-specific oligonucleotide primers were designed on the basis of the nucleotide sequences of Smlt3054 (NCBI GeneID

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

6391502) with the necessary restriction enzymes added for cloning. Genomic DNA prepared from *S. maltophilia* K279a and the 20 clinical isolates of *S. maltophilia* were used as PCR templates. Smlt3054 was cloned into pMMB207 (ATCC 37809) as *Eco*RI/*Hin*dIII insert with a C-terminal HA epitope tag (YPYDVPDYA) for expression in *S. maltophilia*, pGFP^2-N3 (BioSignal Packard) as a *Nhe*I/*Sac*I insert with no stop codon for ectopic expression in HEK293, and pET28a(+) as a *Bam*HI/*Xho*I insert with a stop codon for overexpression in *E. coli* BL21(DE3) cells.

Expression of Smlt3054 in *S. maltophilia* and Cell Fractionation. Either pMMB207−Smlt3054 or empty pMMB207 was electroporated into electrically competent *S. maltophilia* BB1 cells and plated on LB agar plates containing 10 μg/mL chloramphenicol. All media were supplemented with 10 μg/mL chloramphenicol. Individual colonies were grown in 5 mL of LB broth for 16 h at 37 °C and 200 rpm. The cultures were then diluted to an A_{600} of 0.8 in 5 mL of brain−heart infusion broth and grown at 37 °C and 200 rpm for 1 h. After 1 h, protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside. Two 1 mL samples of cultures were harvested 16 h post-induction by centrifugation at 17000*g* for 30 min at 4 °C. The medium supernatant was collected and filtered through a 0.2 μm syringe filter. One of the resulting pellets was resuspended in 200 μL of 4 M urea and designated the whole cell lysate sample. The periplasmic fraction of the second pellet was extracted as described previously.[36] The whole cell lysate, periplasmic fraction, and medium supernatant were analyzed for the presence of HA-tagged Smlt3054 via Western blotting as described below. For endogenous expression of Smlt3054, cells were processed as described above, and the medium supernatant was analyzed for the presence of Smlt3054 via Western blotting as described below.

Ectopic Expression in Mammalian Cells. HEK293 cells were suspended in ice-cold HEPES-buffered saline at a cell density of approximately 106 cells/mL, and 200 μL of the cell suspension was mixed with vector DNA (15 μg per construct) in a total volume of 350 μL. The mixture was transferred to prechilled 2 mm electroporation cuvettes (USA Scientific) and electroporated via a Bio-Rad Gene Pulse XCell system using the preset HEK293 parameters. Immediately after electroporation, cells were transferred to 5 mL of medium and placed in two 35 mm dishes containing gelatin-coated glass coverslips. After 24−36 h, the cells were fixed with 3.7% (w/v) paraformaldehyde in PBS. Coverslips were then washed twice with PBS and once with ddH_2O before mounting onto microscope slides with fluoromount (Sigma-Aldrich).

Epifluorescence and Confocal Laser Scanning Microscopy. Slides containing HEK293 cells were imaged via epifluorescence microscopy (Zeiss) at 20× and 40× magnification and via confocal laser scanning microscopy (Zeiss) at 63× magnification with an excitation wavelength of 488 nm for imaging GFP-fused proteins and 543 nm for imaging tdTomato-fused proteins. For live cell imaging, HEK293 cells transfected with pGFP^2−N3−Smlt3054 were cultivated on gelatin-coated 30 mm round glass coverslips and mounted into a POCmini chamber heaters (PeCon GmbH, Erbach, Germany) containing phenol-red free medium and maintained at 37 °C via stage and objective heaters (PeCon GmbH). Images were collected on the Zeiss LSM confocal microscope every 20 s for 4 min.

Expression and Purification of Recombinant Smlt3054 in *E. coli*. The pET28a(+)−Smlt3054 vector was electroporated into *E. coli* BL21(DE3) cells and plated on LB agar plates containing 50 μg/mL kanamycin. For expression, all media were supplemented with 50 μg/mL kanamycin. An individual colony was grown in 5 mL of LB broth for 16 h at 37 °C and 200 rpm. Then 2 mL of saturated culture was added to 200 mL of LB broth and incubated for an additional 16 h at 37 °C and 200 rpm. The culture was then diluted to an A_{600} of 0.8 in 800 mL of LB broth and grown for 1 h at 18 °C and 200 rpm. After 1 h, protein expression was induced by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside, and the culture was incubated at 18 °C and 200 rpm for 20 h. Expression was confirmed via SDS-PAGE and Western blotting as described below.

For purification, cells were harvested at 8000*g* for 15 min at 4 °C, washed once in 20 mL of ice-cold PBS, resuspended in 20 mL of PBS, and sonicated at 15 W, 50% duty, for 30 min total processing time. The insoluble fraction containing recombinant His_6-tagged Smlt3054 was isolated by centrifugation at 17000*g* for 30 min at 4 °C and washed once in 20 mL of PBS to remove any contaminating soluble proteins. Smlt3054 was extracted by incubating the resulting pellet in buffer A (1× PBS, 250 mM NaCl, 0.4% (w/v) sarkosyl) for 1 h at room temperature, followed by centrifugation at 17000*g* for 30 min at 4 °C. The solution was passed over a column containing 15 mL of Ni^{2+}-bound Chelating Sepharose Fast Flow resin (GE Healthcare) pre-equilibrated in buffer A at a flow rate of 2 mL/min via a BioLogic LP chromatography system (Bio-Rad) with a fraction collector. The column was washed for 80 min with buffer A before a gradient from 0 to 30% buffer B (1× PBS, 250 mM NaCl, 0.4% (w/v) sarkosyl, 500 mM imidazole) was applied over the course of 120 min to remove weakly bound contaminating proteins. Smlt3054 was eluted from the column by running 100% buffer B for 30 min. Fractions were analyzed via SDS-PAGE, and samples containing purified Smlt3054 were pooled together and dialyzed against 4 L of 20 mM sodium phosphate buffer, pH 7.5, plus 0.05% sarkosyl for 48 h with two buffer exchanges.

SDS-PAGE and Immunoblotting. Protein samples (40 μL) were mixed with 10 μL of 5× Lammeli sample buffer and heated for 10 min at 90 °C before loading 25 μL onto 4% stacking, 12% separating acrylamide gels with MES running buffer. Precision Plus All Blue Standard (Bio-Rad) was used as a molecular weight standard. Samples were run at 150 V and transferred to a nitrocellulose membrane (Amersham Biosciences Hybond ECL) for 90 min at 200 mA at 4 °C. Transfer was confirmed by staining with 0.1% Ponceau S. The membrane was then blocked overnight at 4 °C with 5% fat-free milk in TBST and incubated for 1 h at room temperature with primary antibody. For expression of Smlt3054 in *S. maltophilia* via pMMB207−Smlt3054, the primary antibody was mouse monoclonal anti-HA.11 tag (BioLegend) diluted 1000-fold in 5% BSA in TBST. For endogenous expression of Smlt3054 from clinical *S. maltophilia* isolates, a rabbit polyclonal antibody was developed against purified Smlt3054 (Lampire) and used at 1:5000 dilution. For expression of Smlt3054 in *E. coli* via pET28a(+)−Smlt3054, the primary antibody was mouse monoclonal anti-His_6 tag (Cell Signaling) diluted 5000-fold in 5% fat-free milk in TBST. Membranes were then washed five times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Cell Signaling) diluted

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

1000-fold in 5% BSA in TBST for pMMB207−Smlt3054 expression and diluted 5000-fold in 5% fat-free milk in TBST for pET28a(+)−Smlt3054 expression. Membranes were washed five times with TBST and twice with TBS and developed with ECL plus Western blotting detection reagents (RPN2132, GE Healthcare), a chemiluminescent horseradish peroxidase substrate.

Circular Dichroism and Tryptophan Fluorescence Measurements. Smlt3054 protein samples (300 μL) at 320 μg/mL (9.6 μM) in 2 mM sodium phosphate buffer, pH 7.5, plus sarkosyl ranging in concentration from 0.01 to 1% (w/v) were added to 1 mm path length quartz cuvettes (Starna), and ellipticity was measured from 190 to 260 nm in a J-815 circular dichroism spectrometer (JASCO). The scan speed was 200 nm/min with three accumulations per sample. The same Smlt3054 protein samples (100 μL) were added to a 96-well half-area UV transparent plate (USA Scientific) along with equimolar tryptophan solutions in equivalent buffers. Fluorescence emission spectra was collected from 310 to 450 nm with an excitation wavelength of 295 nm via an Infinite M200 PRO plate reader (Tecan).

Actin-Binding Protein Centrifugal Assay. For F-actin experiments, F-actin was prepared from rabbit skeletal muscle actin, and an F-actin binding assay (cytoskeleton) was performed as described previously.[27] Briefly, 10 μL of Smlt3054 (85 μM) in 20 mM sodium phosphate buffer, pH 7.5, plus 0.2% sarkosyl was mixed with 40 μL of F-actin (21 μM) in Actin Polymerization Buffer (5 mM Tris, pH 8, 0.2 mM $CaCl_2$, 50 mM KCl, 2 mM $MgSO_4$, 1 mM ATP) for final Smlt3054 and F-actin concentrations of 17 μM and sarkosyl concentration of 0.4% (w/v). Control samples were also prepared with F-actin only and Smlt3054 only at equivalent concentrations and buffers, as well as F-actin plus α-actinin and F-actin plus BSA as positive and negative controls for F-actin binding, respectively (Figure S2). After incubation at room temperature for 30 min, samples were fractionated via ultracentrifugation at 150000*g* for 1.5 h in an Airfuge (Beckman) to sediment actin fibers. The resulting pellet and supernatant of each condition were analyzed via SDS-PAGE.

For actin polymerization experiments, 10 μL of Smlt3054 (85 μM) in 20 mM sodium phosphate buffer, pH 7.5, plus 0.2% sarkosyl was mixed with 40 μL of G-actin (21 μM) in General Actin Buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$) for a final Smlt3054 and G-actin concentration of 17 μM and sarkoysl concentration of 0.4% (w/v). After incubation at room temperature for 30 min, 2.5 μL of 10× Actin Polymerization Buffer was added to induce the polymerization of G-actin into F-actin. Actin polymerization was allowed to proceed for precisely 30 min before samples were fractionated via ultracentrifugation at 150000*g* for 1.5 h in an Airfuge (Beckman) to sediment actin fibers. The resulting pellet and supernatant of each condition was analyzed via SDS-PAGE.

Transmission Electron Microscopy (TEM). Solutions containing F-actin alone and F-actin plus purified Smlt3054 were prepared as described above with the exception of a final sarkosyl concentration of 0.01% (w/v). Samples were incubated for 30 min at room temperature before 10 μL of each was added to 300 mesh gold TEM disks containing an ultrathin carbon film on a holey carbon support film (Ted Pella). After incubation for 30 s at room temperature, excess liquid was blotted off, and samples were negatively stained with 1% uranyl acetate for 1 min. Excess liquid was blotted off again, and samples were air-dried for 30 min. Disks were imaged on a JEM-2000FX scanning electron microscope (JEOL) at 100000× and 300000× magnifications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00103.

Figure S1, sequence alignment of Smlt3054 from *S. maltophilia* clinical isolates; Figure S2, control experiment for actin-binding protein centrifugal assay (PDF)

Video S1, time-lapse confocal microscopy image of HEK293 cells overexpressing Smlt3054−GFP binding retrograde F-actin waves (AVI)

Video S2, time-lapse confocal microscopy image of HEK293 cells overexpressing Smlt3054−GFP with reduced retrograde F-actin wave binding and formation of intracellular inclusions (AVI)

■ AUTHOR INFORMATION

Corresponding Author

*(B.W.B.) Mail: B320 Iacocca Hall, 111 Research Drive, Bethlehem, PA 18015, USA. Phone: (610) 758-6837. E-mail: bwb209@lehigh.edu.

Present Address

⊥(E.L.W.) Delaware County Memorial Hospital, Infectious Disease, Drexel Hill, PA 19026, USA.

Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

§In memory of Hanlon MacDonald, who we lost on August 22, 2013.

■ ACKNOWLEDGMENTS

We thank Matthias Falk (Lehigh) for providing the tdTomato−actin fusion construct, Robert Ryan (University College Cork) for providing *S. maltophilia* strain K279a, and Robert Keyse (Lehigh) for assistance with TEM. This work was supported by Lehigh University, including funds provided through a Faculty Innovation Grant (FIG), as well as by the Lehigh Valley Health Network, including funds provided through the Infectious Disease Endowment. Undergraduate research scholarships from the Lehigh University Grants for Experiential Learning in Health (GEHL) program and from the P. C. Rossin College of Engineering and Applied Science are gratefully acknowledged.

■ ABBREVIATIONS

CF, cystic fibrosis; T2SS, type II secretion system; ANK, ankyrin repeat domain

■ REFERENCES

(1) Rocco, F., De Gregorio, E., Colonna, B., and Di Nocera, P. P. (2009) *Stenotrophomonas maltophilia* genomes: a start-up comparison. *Int. J. Med. Microbiol. 299*, 535−546.

(2) Brooke, J. S. (2012) *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin. Microbiol. Rev. 25*, 2−41.

(3) Looney, W. J., Narita, M., and Muhlemann, K. (2009) *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. *Lancet Infect. Dis. 9*, 312−323.

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70

(4) LiPuma, J. J. (2003) Burkholderia and emerging pathogens in cystic fibrosis. *Semin. Respiratory Crit. Care Med. 24*, 681−692.
(5) Karaba, S. M., White, R. C., and Cianciotto, N. P. (2013) *Stenotrophomonas maltophilia* encodes a type II protein secretion system that promotes detrimental effects on lung epithelial cells. *Infect. Immunity 81*, 3210−3219.
(6) Travassos, L. H., Pinheiro, M. N., Coelho, F. S., Sampaio, J. L., Merquior, V. L., and Marques, E. A. (2004) Phenotypic properties, drug susceptibility and genetic relatedness of *Stenotrophomonas maltophilia* clinical strains from seven hospitals in Rio de Janeiro, Brazil. *J. Appl. Microbiol. 96*, 1143−1150.
(7) Windhorst, S., Frank, E., Georgieva, D. N., Genov, N., Buck, F., Borowski, P., and Weber, W. (2002) The major extracellular protease of the nosocomial pathogen *Stenotrophomonas maltophilia*: characterization of the protein and molecular cloning of the gene. *J. Biol. Chem. 277*, 11042−11049.
(8) Pompilio, A., Crocetta, V., Confalone, P., Nicoletti, M., Petrucca, A., Guarnieri, S., Fiscarelli, E., Savini, V., Piccolomini, R., and Di Bonaventura, G. (2010) Adhesion to and biofilm formation on IB3-1 bronchial cells by *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients. *BMC Microbiol. 10*, 102.
(9) Di Bonaventura, G., Prosseda, G., Del Chierico, F., Cannavacciuolo, S., Cipriani, P., Petrucca, A., Superti, F., Ammendolia, M. G., Concato, C., Fiscarelli, E., Casalino, M., Piccolomini, R., Nicoletti, M., and Colonna, B. (2007) Molecular characterization of virulence determinants of *Stenotrophomonas maltophilia* strains isolated from patients affected by cystic fibrosis. *Int. J. Immunopathol. Pharmacol. 20*, 529−537.
(10) De Vidipo, L. A., De Marques, E. A., Puchelle, E., and Plotkowski, M. C. (2001) *Stenotrophomonas maltophilia* interaction with human epithelial respiratory cells in vitro. *Microbiol. Immunol. 45*, 563−569.
(11) Goldberg, M. B. (2001) Actin-based motility of intracellular microbial pathogens. *Microbiol. Mol. Biol. Rev.: MMBR 65*, 595−626.
(12) Lilic, M., Galkin, V. E., Orlova, A., VanLoock, M. S., Egelman, E. H., and Stebbins, C. E. (2003) *Salmonella* SipA polymerizes actin by stapling filaments with nonglobular protein arms. *Science (Washington, DC, U. S.) 301*, 1918−1921.
(13) Al-Khodor, S., Price, C. T., Kalia, A., and Abu Kwaik, Y. (2010) Functional diversity of ankyrin repeats in microbial proteins. *Trends Microbiol. 18*, 132−139.
(14) Crossman, L. C., Gould, V. C., Dow, J. M., Vernikos, G. S., Okazaki, A., Sebaihia, M., Saunders, D., Arrowsmith, C., Carver, T., Peters, N., Adlem, E., Kerhornou, A., Lord, A., Murphy, L., Seeger, K., Squares, R., Rutter, S., Quail, M. A., Rajandream, M. A., Harris, D., Churcher, C., Bentley, S. D., Parkhill, J., Thomson, N. R., and Avison, M. B. (2008) The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Genome Biol. 9*, R74.
(15) Witt, S. H., Labeit, D., Granzier, H., Labeit, S., and Witt, C. C. (2005) Dimerization of the cardiac ankyrin protein CARP: implications for MARP titin-based signaling. *J. Muscle Res. Cell Motil. 26*, 401−408.
(16) Boice, J. A., and Fairman, R. (1996) Structural characterization of the tumor suppressor p16, an ankyrin-like repeat protein. *Protein Sci. 5*, 1776−1784.
(17) Thomas, J. C., Matak-Vinkovic, D., Van Molle, I., and Ciulli, A. (2013) Multimeric complexes among ankyrin-repeat and SOCS-box protein 9 (ASB9), ElonginBC, and Cullin 5: insights into the structure and assembly of ECS-type Cullin-RING E3 ubiquitin ligases. *Biochemistry 52*, 5236−5246.
(18) Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D., and Hochstrasser, D. F. (1999) Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol. (Clifton, N.J.) 112*, 531−552.
(19) Drozdetskiy, A., Cole, C., Procter, J., and Barton, G. J. (2015) JPred4: a protein secondary structure prediction server. *Nucleic Acids Res. 43*, W389−W394.
(20) Valdezate, S., Vindel, A., Martin-Davila, P., Del Saz, B. S., Baquero, F., and Canton, R. (2004) High genetic diversity among *Stenotrophomonas maltophilia* strains despite their originating at a single hospital. *J. Clin. Microbiol. 42*, 693−699.
(21) de Lorenzo, V., Eltis, L., Kessler, B., and Timmis, K. N. (1993) Analysis of *Pseudomonas* gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes. *Gene 123*, 17−24.
(22) Zhou, X., Konkel, M. E., and Call, D. R. (2010) Vp1659 is a *Vibrio parahaemolyticus* type III secretion system 1 protein that contributes to translocation of effector proteins needed to induce cytolysis, autophagy, and disruption of actin structure in HeLa cells. *J. Bacteriol. 192*, 3491−3502.
(23) Voth, D. E., Howe, D., Beare, P. A., Vogel, J. P., Unsworth, N., Samuel, J. E., and Heinzen, R. A. (2009) The *Coxiella burnetii* ankyrin repeat domain-containing protein family is heterogeneous, with C-terminal truncations that influence Dot/Icm-mediated secretion. *J. Bacteriol. 191*, 4232−4242.
(24) Galan, J. E., and Zhou, D. (2000) Striking a balance: modulation of the actin cytoskeleton by *Salmonella*. *Proc. Natl. Acad. Sci. U. S. A. 97*, 8754−8761.
(25) Pollard, T. D., Blanchoin, L., and Mullins, R. D. (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct. 29*, 545−576.
(26) Svitkina, T. (2009) Imaging cytoskeleton components by electron microscopy. *Methods Mol. Biol. (N. Y., NY, U. S.) 586*, 187−206.
(27) Zhou, D., Mooseker, M. S., and Galan, J. E. (1999) An invasion-associated *Salmonella* protein modulates the actin-bundling activity of plastin. *Proc. Natl. Acad. Sci. U. S. A. 96*, 10176−10181.
(28) Galkin, V. E., Orlova, A., VanLoock, M. S., Zhou, D., Galan, J. E., and Egelman, E. H. (2002) The bacterial protein SipA polymerizes G-actin and mimics muscle nebulin. *Nat. Struct. Biol. 9*, 518−521.
(29) DuMont, A. L., Karaba, S. M., and Cianciotto, N. P. (2015) Type II secretion-dependent degradative and cytotoxic activities mediated by *Stenotrophomonas maltophilia* serine proteases StmPr1 and StmPr2. *Infect. Immun. 83*, 3825−3837.
(30) Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L., Tate, J., and Punta, M. (2014) Pfam: the protein families database. *Nucleic Acids Res. 42*, D222−230.
(31) Huang, H., Lin, M., Wang, X., Kikuchi, T., Mottaz, H., Norbeck, A., and Rikihisa, Y. (2008) Proteomic analysis of and immune responses to *Ehrlichia chaffeensis* lipoproteins. *Infect. Immunity 76*, 3405−3414.
(32) Pan, X., Luhrmann, A., Satoh, A., Laskowski-Arce, M. A., and Roy, C. R. (2008) Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science (Washington, DC, U. S.) 320*, 1651−1654.
(33) Price, C. T., Al-Khodor, S., Al-Quadan, T., Santic, M., Habyarimana, F., Kalia, A., and Kwaik, Y. A. (2009) Molecular mimicry by an F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. *PLoS Pathog. 5*, e1000704.
(34) Min, C. K., Kwon, Y. J., Ha, N. Y., Cho, B. A., Kim, J. M., Kwon, E. K., Kim, Y. S., Choi, M. S., Kim, I. S., and Cho, N. H. (2014) Multiple *Orientia tsutsugamushi* ankyrin repeat proteins interact with SCF1 ubiquitin ligase complex and eukaryotic elongation factor 1 alpha. *PLoS One 9*, e105652.
(35) Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.
(36) MacDonald, L. C., and Berger, B. W. (2014) A polysaccharide lyase from *Stenotrophomonas maltophilia* with a unique, pH-regulated substrate specificity. *J. Biol. Chem. 289*, 312−325.
(37) Mosavi, L. K., Minor, D. L., Jr., and Peng, Z. Y. (2002) Consensus-derived structural determinants of the ankyrin repeat motif. *Proc. Natl. Acad. Sci. U. S. A. 99*, 16029−16034.

DOI: 10.1021/acsinfecdis.5b00103
ACS Infect. Dis. 2016, 2, 62−70