

The Surfactant of *Legionella pneumophila* Is Secreted in a TolC-Dependent Manner and Is Antagonistic toward Other *Legionella* Species[†]

Catherine R. Stewart, Denise M. Burnside, and Nicholas P. Cianciotto*

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

Received 27 May 2011/Accepted 24 August 2011

When *Legionella pneumophila* grows on agar plates, it secretes a surfactant that promotes flagellum- and pilus-independent “sliding” motility. We isolated three mutants that were defective for surfactant. The first two had mutations in genes predicted to encode cytoplasmic enzymes involved in lipid metabolism. These genes mapped to two adjacent operons that we designated *bbcABCDEF* and *bbcGHIJK*. Backcrossing and complementation confirmed the importance of the *bbc* genes and suggested that the *Legionella* surfactant is lipid containing. The third mutant had an insertion in *tolC*. TolC is the outer membrane part of various trimolecular complexes involved in multidrug efflux and type I protein secretion. Complementation of the *tolC* mutant restored sliding motility. Mutants defective for an inner membrane partner of TolC also lacked a surfactant, confirming that TolC promotes surfactant secretion. *L. pneumophila* (*lspF*) mutants lacking type II protein secretion (T2S) are also impaired for a surfactant. When the *tolC* and *lspF* mutants were grown next to each other, the *lsp* mutant secreted surfactant, suggesting that TolC and T2S conjoin to mediate surfactant secretion, with one being the conduit for surfactant export and the other the exporter of a molecule that is required for induction or maturation of surfactant synthesis/secretion. Although the surfactant was not required for the extracellular growth, intracellular infection, and intrapulmonary survival of *L. pneumophila*, it exhibited antimicrobial activity toward seven other species of *Legionella* but not toward various non-*Legionella* species. These data suggest that the surfactant provides *L. pneumophila* with a selective advantage over other legionellae in the natural environment.

The aquatic bacterium *Legionella pneumophila* is the causative agent of Legionnaires’ disease, a potentially fatal pneumonia (69). The environmental life cycle of *L. pneumophila* includes growth, survival, and spread within the planktonic phase, protozoan hosts, and multiorganismal biofilms (58, 91). Human infection occurs after inhalation of *L. pneumophila*-containing droplets that are generated from a variety of devices, including cooling towers (74). Once the bacteria enter the lung, proliferation occurs inside the resident alveolar macrophages, with lung epithelial cells likely providing an additional replicative niche (64, 69). *L. pneumophila* secretes many factors, proteinaceous and nonproteinaceous, that permit the bacterium’s survival and proliferation in both the environment and the human host. Such factors include the many enzymes and effectors secreted via the type II and type IV protein secretion systems, the iron chelator legiobactin, and a pyromelanin pigment (3, 19, 23, 69).

Previously, we determined that *L. pneumophila* exhibits surface translocation when it is grown on buffered charcoal yeast extract (BCYE) plates containing 0.5% agar (90). The spreading legionellae appear in an amorphous, lobed pattern that is most manifest at 30°C. When bacteria were spotted onto the BCYE plates, the numbers and sizes of the lobes that formed

were variable, ranging from only one or a few main lobes to many large and small lobes. Furthermore, in some cases, the lobes emanate from one side of the inoculation point, whereas in other instances, they spread out in all directions. This translocation is displayed by some, but not all, *Legionella* species. Because *L. pneumophila* mutants that lack flagella and/or type IV pili spread on low-percentage agar, this surface translocation is not swarming or twitching motility (90). Importantly, a translucent film appears atop the agar, moving ahead of the spreading legionellae. This film consisted of a surfactant, based on its ability to disperse water droplets and promote surface translocation of nonspreading *Escherichia coli* (90). *L. pneumophila* type II protein secretion (T2S) mutants are defective for surface translocation and surfactant. When these mutants are spotted onto the film produced by the wild type, they are able to spread, indicating that T2S, directly or indirectly, promotes the elaboration of a surfactant (90). Thus, *L. pneumophila* secretes a surfactant which promotes a form of surface translocation that is best described as “sliding motility.”

Biosurfactants (i.e., surfactants made by microbes) are produced by many types of bacteria, including *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (8, 85). As a group, they display diversity in structure, possessing lipid, sugar, and/or peptide moieties (8). Although biosurfactants have been intensely studied for their role in motility as well as industrial applications (8, 50), their role in the growth, ecology, and virulence of pathogens has, with the exception of the *P. aeruginosa* rhamnolipid, been minimally addressed (1). The results reported here suggest that the surfactant made by *L. pneumophila* has lipid characteristics and is secreted by a pro-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Northwestern University Medical School, 320 East Superior St., Chicago, IL 60611-3010. Phone: (312) 503-0385. Fax: (312) 503-1339. E-mail: n-cianciotto@northwestern.edu.

† Supplemental material for this article may be found at <http://jbb.asm.org/>.

[‡] Published ahead of print on 2 September 2011.

TABLE 1. Wild-type and mutant strains used in this study

Species and strain(s) ^a	Description	Reference or source
<i>L. pneumophila</i>		
130b (ATCC BAA-74)	Clinical isolate	90
NU275	<i>lspF</i> mutant	83
NU388, NU393	lpw_24151 (<i>bbcB</i>) mutants	This study
NU389, NU394	lpw_24111 (<i>bbcI</i>) mutants	This study
NU390, NU395	lpw_07761 (<i>tolC</i>) mutants	This study
NU391, NU392	lpw_23601 mutants	This study
NU396, NU397	lpw_24191 (<i>bbcF</i>) mutants	This study
NU400, NU401	lpw_27401 mutants	This study
NU402, NU403	lpw_07981 mutants	This study
<i>lpnE::km</i> strain	lpw_24081 (<i>lpnE</i>) mutant	70
<i>L. anisa</i> ATCC 35292	Environmental isolate	35
<i>L. feeleii</i> ATCC 35072	Environmental isolate	42
<i>L. hackeliae</i> ATCC 35250	Clinical isolate	13
<i>L. jordanis</i> ATCC 33263	Environmental isolate	21
<i>L. moravica</i> ATCC 43877	Environmental isolate	94
<i>L. micdadei</i> ATCC 33218	Clinical isolate	39
<i>L. wadsworthii</i> ATCC 33877	Clinical isolate	28
<i>Bacillus subtilis</i> KY42	Environmental isolate	31
<i>Escherichia coli</i> DH5 α	Laboratory strain	Invitrogen
<i>Klebsiella pneumoniae</i> DMDS 92-08-28a	Environmental isolate	67
<i>Listeria monocytogenes</i> EGDe	Clinical isolate	84
<i>Micrococcus luteus</i>	Environmental isolate	63
<i>Pseudomonas aeruginosa</i> ATCC 27700	Environmental isolate	67

^a All *L. pneumophila* strains listed under 130b are derivatives of strain 130b. ATCC, American Type Culture Collection.

cess that involves TolC, an outer membrane protein that is best known for its role in antibiotic efflux. Although surfactant was not critical for the extra- or intracellular growth of *L. pneumophila*, it displayed a unique antimicrobial activity against other species of *Legionella*.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *L. pneumophila* 130b, also known as AA100 or Wadsworth, served as our wild-type strain. Additional wild-type bacteria, as well as mutants of 130b that were also examined are listed in Table 1. Legionellae were routinely grown at 37°C in buffered yeast extract (BYE) broth or on BCYE agar containing 1.5% agar (90). When appropriate, bacteria were supplemented with 2.5 μ g of gentamicin/ml, 25 μ g of kanamycin/ml, or 4.5 μ g of chloramphenicol/ml. In some experiments, *L. pneumophila* was also grown in a deferrated chemically defined medium (CDM) (20). *E. coli* strain DH5 α (Invitrogen, Carlsbad, CA), as a host for recombinant plasmids, was routinely grown in Luria-Bertani medium. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Assays for surface translocation and biosurfactant activity. Surface translocation and surfactant production were monitored as described before (90). Briefly, *Legionella* strains were grown, with shaking, in BYE broth at 37°C to early stationary phase, and then 10 μ l of culture was spotted onto fresh (i.e., <24-h-old) BCYE plates containing 0.5% agar. In some BCYE plates, we added 5 μ g/ml surfactin (Sigma catalog no. S3523), a surfactant produced by *B. subtilis* (53). The inoculated plates were incubated at 30°C, and growth was observed for the next 14 days. Surface translocation was evident as lobed, wavelike areas of growth emanating from the point of inoculation, and the surfactant was seen as a film advancing ahead of the spreading bacteria. The presence of the surfactant was confirmed by the collapse of a water droplet when it was spotted onto the film-covered area of the plate.

Extracellular growth and effects of a biosurfactant. To assess the extracellular growth capacity of *L. pneumophila*, strains were inoculated into BYE broth and then incubated in air, with shaking. Growth was assessed by measuring the optical density at 660 nm (OD₆₆₀) of cultures, using a DU720 UV-visible light

spectrophotometer (Beckman Coulter, Fullerton, CA). To judge the efficiency of plating on solid medium, legionellae were precultured for 3 days on BCYE agar and suspended in phosphate-buffered saline (PBS) at 10⁹ CFU per ml, and then 10- μ l aliquots taken from 10-fold serial dilutions in PBS were spotted onto the medium. Growth was recorded after 3 to 5 days of incubation at various temperatures. To ascertain the effect of the *L. pneumophila* surfactant on the extracellular growth of other bacteria (Table 1), the heterologous bacteria were spotted, as described above, onto standard BCYE containing 1.5% agar, BCYE containing 0.5% agar, and the surfactant film produced by *L. pneumophila* previously grown on the low-percentage agar medium. After 1 to 3 days at 37°C in an air incubator, the growth of the bacteria in the presence or absence of the surfactant was monitored and recorded.

DNA and protein sequence analysis. DNA was isolated from *L. pneumophila* as described before (24). Sequencing was done at the Northwestern Biotech Laboratory, with primers from Integrated DNA Technologies (Coralville, IA). Sequences were analyzed using Lasergene (DNASTAR, Madison, WI). Protein alignments were done using the Clustal method, and predictions for the cellular locations of proteins were done using versions 3.0 of both PSORTb and SignalP (32, 71). BLAST homology searches were done through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>) and *Legionella* databases at <http://genolist.pasteur.fr/LegioList/>, <http://www.ncbi.nlm.nih.gov/genomeprj/48801>, and <http://www.ebi.ac.uk/ena/data/view/FR687201>.

RT-PCR analysis of *L. pneumophila* gene expression. Reverse transcription (RT)-PCR was done as described previously (4, 20). The RNA was isolated from late log and early stationary BYE cultures grown at 30°C using the RNA Stat-60 reagent (IsoTex, Friendswood, TX). Controls in which reverse transcriptase was omitted from the reaction mixtures were included to rule out contributions from contaminating DNA in the DNase-treated samples. The primers used are described in Table 2. Primers 24151rtF and 24151rtR were used to assess transcription of lpw_24151. To assess cotranscription in the lpw_24151-containing operon, four intergenic regions, denoted as regions 1 to 4 in Fig. 3A, were amplified with primers 24141rt and 24151rt2R for region 1, primers 24151rt2F and 24161rtR for region 2, primers 24161rt and 24171rt for region 3, and primers 24191F and 24181rt for region 4. Primers 24111rtF and 24111rtR were used to assess transcription of lpw_24111. To assess cotranscription in the lpw_24111-containing operon, four intergenic regions, denoted as regions 1 to 4 in Fig. 3B, were amplified with primers 24091rt and 24101rt for region 1, primers 24101dsrt and 24111rt2 for region 2, primers 24111rt and 24121rt2 for region 3, and primers 24121rt and 24131rtF for region 4. Primers tolCF3 and TolC-5'-StuI assessed transcription of lpw_07761. To judge cotranscription within the lpw_07761-containing operon, two intergenic regions, denoted as regions 1 and 2 in Fig. 4A, were amplified with primers TolC-3'-F2 and TolC-R2-XbaI for region 1 and primers TolC-3'-F2-XbaI and TolC-5'-R2 for region 2.

Construction and screening of an *L. pneumophila* mutant library. Strain 130b was electroporated with mini-Tn10-containing pCDP05, and transposon-containing transformants were selected on BCYE agar with kanamycin (76). Individual mutants were inoculated into the wells of a 96-well, nontreated, polystyrene microtiter plate containing 200 μ l of BYE and then grown at 37°C with shaking. Following 14 to 16 h of culturing, 5- μ l aliquots from the wells were spotted onto BCYE plates containing 0.5% agar and incubated at 30°C. Over the next 3 to 5 days, the inocula were examined for the presence of surface translocation and surfactant. For mutants displaying impaired translocation and surfactant, the location of the mini-Tn10 insertion was ascertained by inverse PCR and subsequent sequence determination as previously described (19, 60). To confirm that the mutant phenotypes were due to transposon insertions, we backcrossed the Tn10-linked mutations into the chromosome of wild-type strain 130b. DNA from the transposon-containing mutants was treated with SacI, and then the digested DNA was mixed with competent 130b, giving a final DNA concentration of 4 μ g per ml. DNA transformation proceeded as previously described (90), with the mixture incubating for 72 h at 30°C with shaking (i.e., 100 rpm). Transformants were selected by plating bacteria on kanamycin-containing BCYE agar.

Additional mutant constructions and gene complementation. To obtain mutants specifically lacking lpw_23601, a 7-kb region containing the gene was amplified from 130b DNA using primers 23601F and 23601R (see Table 2 for these and all other primers). The resultant fragment was then cloned into pGEM-T Easy (Promega, Madison, WI) to give pG23601. Plasmid pG23601 was then digested with NruI, producing a 2-kb deletion in the lpw_23601 gene, and ligated to a kanamycin resistance (Km^r) cassette derived from pMB2190 (83), giving pG23601K1. Mutated lpw_23601 was introduced into strain 130b by transformation (90). Verification of the mutant genotype was carried out by PCR, using primers 23601F and 23601R. A similar allelic exchange procedure was used to introduce mutations into the lpw_24191 and lpw_27401 genes of strain 130b, with primers 24191F and 24191Rcomp being used to construct the lpw_24191

TABLE 2. Primers used in this study

Primer name	Sequence (5'→3') ^a
24151rtF	CCCTGGTTGATGTCGTAAGG
24151rtR	TGTGATCCGCAATCATCAGT
24141rt	GATGCTGATACCATAGGC
24151rtR	TGTGATCCGCAATCATCAGT
24151rtF	GTCCTGATTCTCTTAAAGCAATG
24161rtR	GGTGAAGAGAATAAGTGCTGTCTGG
24161rt	CAAACGCCTACTACCCAGGA
24171dsrt	GAAACCCGTCACGTCAATGTT
24191F	GCGTTCACCTCGGGTCACC
24181dsrt	CCAGTTGAAAATCCAATCACGG
24111rtF	GGCAGCCCAGCATTCTACTA
24111rtR	AAAGACCTCATCAGGCATGG
24091rt	GGTAGCAGGCTATTCATTGAC
24101rt	GGAGAGTCCTTGCTTCATGG
24101dsrt	TTGGTATCGGTTGGCTGAG
24111rt2	GCATGGGCAAACCAAC
24111rt	TGCCAACAACTCTTCTGAT
2412rt2	TGACCCACGCAGTTTAGC
24121rt	TTGGAAAGTCAAATCGGTTCC
24131rtF	TCATTGGGCTTGATTGATGA
tolCF3	GTGTTTCAACCCATGTGTTTG
TolC-5'-R-StuI	AGGCCTGTAAGAGCGGCACGAGC
TolC-3'-F2	GATTAACCTCATGGTTAGCAACT
TolC-R2-XbaI	AAAAAATCTAGACTTTTCCGCAGAC
TolC-3'-F2-XbaI	AAAAAATCTAGACGCAATCCCTGGTA
TolC-5'-R2	ATGGTATCGTTTCTCCAGAGC
23601F	CCCGGGTCTGAGACAAATGAATGAAAG AATGC
23601R	TCTAGATTTCGCTTCTATAGTATGGC
27401F	TTATGGTCAAATCACGAACGC
27401R	AGTTGTTTCGCTCCCTGAATA
07981F	CAGGCTCAGGCGAAAGTAG
07981-5'-R-HpaI	GTTAACAAATCCTGCTATGAGTTCAGC
07981-3'-F-HpaI	GTTAACGACTCGTTATCTCCACAGGTT
07981R	TGGCAACGGCACAAATCACC
24191Fcomp	GGTACCCGACCCAGTCGATACCCCTGTT
24191Rcomp	TCTAGACGCAAGCCTGATTGTTACC
24111Fcomp	GGTACCAAGGAGGATAGACTCGGTT TGTT
24111Rcomp	TCTAGACCTCATACTCGCTATCATC ATTG
07981F2comp	GGCTTGTTGTTCTTTACTACC
07981R3comp	GTCGCAGGTATCCACAAAGT
tolCF	CGTCTGCCTTGAGTGTTTC
tolCR	TCACATTGGATTGACTGCGT

^a Restriction sites are underlined.

mutant and primers 27401F and 27401R being used to make the lpw_27401 mutant. In order to mutate lpw_07981, the 5' and 3' ends of the gene were separately amplified from 130b DNA by using the primer pairs 07981F/07981-5'-R-HpaI and 07981-3'-F-HpaI/07981R, respectively. Each of the generated fragments was ligated into pGEM-T Easy, and then the two resulting plasmids were digested with HpaI and SacI. Finally, a trimolecular ligation was done by placing the Km^r-containing cassette between the beginning of lpw_07981 and the end of the gene. The plasmid thus obtained (i.e., pGEM-lpw_07981-Kan) carried a 2.7-kb deletion in the central lpw_07981 coding region. Introduction and confirmation of the mutation in strain 130b was achieved as noted above for the other mutants. For each of the targeted genes, two independent mutants were obtained by performing separate transformations of the mutagenizing plasmid. Genetic complementation of each of the mutants was achieved by PCR amplifying the corresponding intact gene, ligating the resulting fragment into pGEM-T Easy, and then following a unique restriction digestion, moving the gene into pMMB2002 as described previously (20). The primer pairs used for complementation were 24191Fcomp and 24191Rcomp to create pMlpw24191 for analysis of the lpw_24191 mutant, 24111Fcomp and 24111Rcomp to make pMlpw_24111 for analysis of the lpw_24111 mutant, and 07981F2comp and 07981R3comp to make pM07981 for analysis of the lpw_07981 mutant. For the lpw_07761 mutant, a 3-kb PCR fragment of *tolC* generated using primers tolCF and tolCR was

digested with HindIII-XmnI, and the resulting fragment was cloned into vector pMMB2002 to generate pMtolC.

Infection assays. To test the ability of strains to grow intracellularly, *Acanthamoeba castellanii* and *Hartmannella vermiformis* amoebae, U937 macrophages, and A549 and WI-26 lung epithelial cells were infected as described previously (20, 64). The production of cytokines by infected U937 cells was also ascertained as described before (64). To assess the ability of *L. pneumophila* to grow within the lung, strains were inoculated into the tracheas of 6- to 8-week-old A/J mice (Jackson Laboratory, Bar Harbor, ME), and then at various times postinoculation, the numbers of CFU in the lungs were determined by plating bacteria on BCYE agar (20).

Assays for PHB content, secreted enzymes, and sensitivity to antibiotics. In order to measure the level of poly-3-hydroxybutyrate (PHB) in *L. pneumophila*, strains were grown at 37°C in broth and then stained with the lipophilic dye Nile red as previously described (47). Following various periods of incubation in deferrated CDM broth, bacteria were collected by centrifugation, resuspended to an OD₆₆₀ of 0.3, and then fixed with 1% formaldehyde. After being washed and resuspended in PBS, the cells were finally stained with Nile red (at 25 μM) for 60 min. Fluorescence was recorded at a λ_{excitation} of 545 nm and λ_{emission} of 600 nm and blanked against a sample of unstained bacteria. The presence of secreted proteins in *L. pneumophila* culture supernatants was confirmed by assaying for lipase and phosphatase activities (5, 6). Antibiotic and detergent sensitivity assays were performed as described previously (79, 81). Briefly, strains were grown at 37°C in BYE broth to early stationary phase and then diluted in fresh BYE to 5 × 10⁵ CFU per ml. In triplicate wells of a microtiter polystyrene tray, 50 μl of the bacterial suspension was added to an equal volume of BYE containing various concentrations of SDS or erythromycin. Following incubation at 37°C for 3 days, the lowest concentration of detergent or antibiotic resulting in no visible growth was deemed to be the MIC for that compound for that strain.

RESULTS

Isolation of *L. pneumophila* mutants defective for surfactant production. As a first approach to identifying *L. pneumophila* genes that promote surfactant production, we examined the genome database of strain 130b (87) for a gene(s) that is predicted to encode a protein with sequence similarity to a bacterial enzyme known to be involved in the synthesis of a surfactant. The open reading frame (ORF) lpw_23601 was predicted to encode a product sharing 30% amino acid (aa) identity (E value = 2 × 10⁻⁶¹) with serrawettin W1 synthetase; serrawettin is a surfactant produced by *S. marcescens* (59). Using allelic exchange, we isolated two mutants lacking lpw_23601 but found that both produced normal levels of a surfactant and surface translocation (see Fig. S1 in the supplemental material). As a next approach, we screened a population of Tn10-mutagenized 130b for strains lacking the ability to elaborate a surface film and/or undergo surface translocation when spotted on BCYE plates containing 0.5% agar. Out of the 1,536 mutants screened, three (NU388, NU389, and NU390) reproducibly displayed the desired phenotype. Each mutant lacked both film production and surface translocation (Fig. 1), confirming that surfactant spreading and surface spreading are linked phenotypes in *Legionella*. A similar result was obtained when we reexamined our T2S (*lspF*) mutant of strain 130b (Fig. 1). However, NU388, NU389, and NU390 did not appear to be T2S mutants, because they exhibited a yellow coloration when grown on 0.5% agar BCYE plates whereas *lsp* mutants have a bluish-gray appearance (82). Also, they were not defective for secreted enzyme activities that are dependent upon T2S (data not shown). In theory, the inability of NU388, NU389, and NU390 to undergo surface translocation could be due to the loss of a surfactant or a cell-associated defect such as loss of a motility organelle. Differentiating between these possibilities, each of the new mutants recovered the ability to

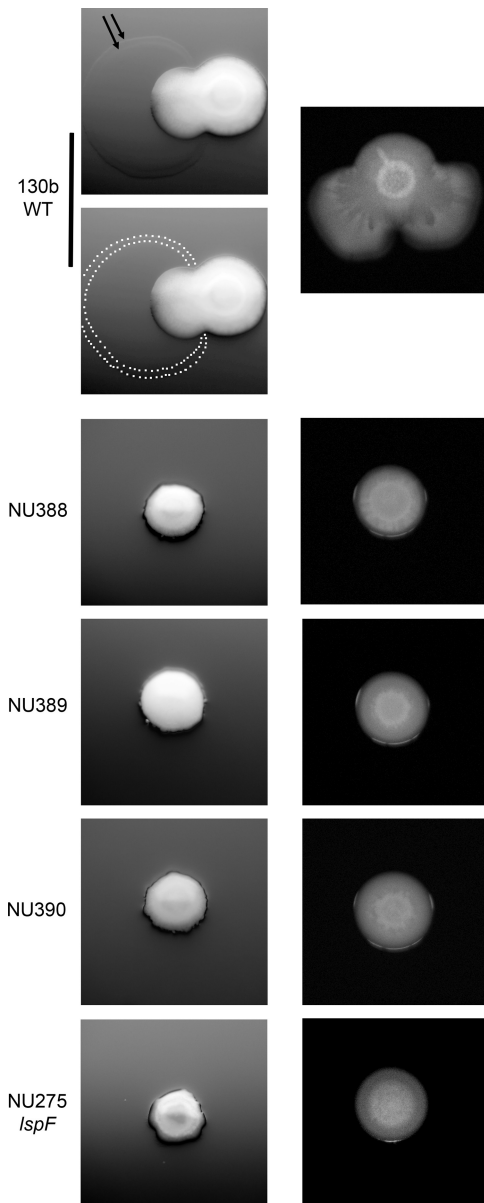


FIG. 1. *L. pneumophila* mutants that lack surfactant and sliding motility. Wild-type (WT) strain 130b, mini-Tn10-containing mutants NU388, NU389, and NU390, and type II secretion (*lspF*) mutant NU275 were grown on BCYE plates containing 0.5% agar at 30°C. Imaged after 4 days, the left panel depicts, in the case of the WT but not the mutants, the early stage of surface translocation and a surfactant film front displaying two delineating rings. To aid in visualization of the surfactant film, two images are presented for the WT, with the upper one showing the delineating rings marked by two black arrows and the lower one having white dots mark the boundary of the film. Imaged after 10 days, the right panel shows the progression of surface translocation by the WT and its continued absence for the mutants. The images presented are representative of those observed for at least 12 experiments.

spread when spotted onto the film made by parental 130b (Fig. 2). Mutant bacteria spread in the direction away from the central area of wild-type growth, suggesting that they were moving into areas with sufficient surfactant production or away from areas of nutrient depletion. Together, these data suggest

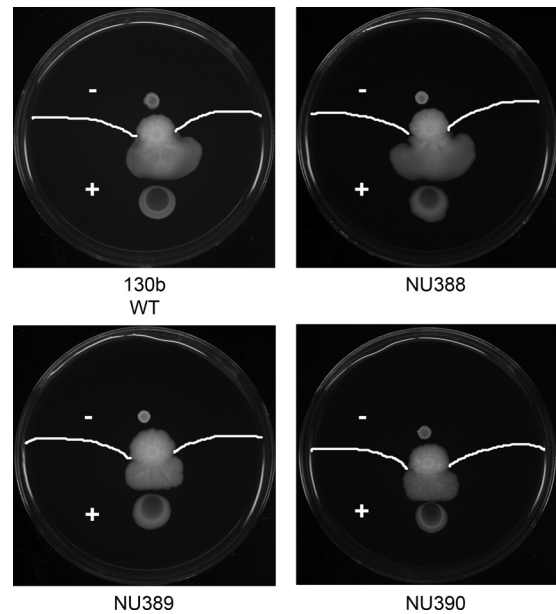


FIG. 2. Surface translocation by *L. pneumophila* mutants when spotted onto film produced by wild-type legionellae. WT 130b was inoculated onto the center of a BCYE plate containing 0.5% agar at 30°C. After 10 days, when a layer of surface film was clearly evident beyond the spreading legionellae, a 10- μ l aliquot of the WT, mutant NU388, mutant NU389, and mutant NU390 was spotted onto the agar surface either in the surfactant-covered area (“+”) or at a point outside of it but still equidistant from the center of WT growth (“-” area). After 3 days of further incubation, images were taken in order to visualize any differences in spreading. The white lines show the film front. The images are representative of three independent experiments.

that NU388, NU389, and NU390 are defective for the synthesis and/or secretion of a surfactant.

Identification of *L. pneumophila* genes required for biosurfactant biosynthesis. To discern the genetic basis for the observed losses of surfactant, we used inverse PCR and sequence analysis to identify the location of the Tn10 insertions. Mutant NU388 had its insertion at nucleotide (nt) 757 in the 1,746-bp gene designated *lpw_24151* in the 130b genome database (87). The *lpw_24151* gene was predicted to be part of a six-gene operon (Fig. 3A). The operon structure was confirmed by RT-PCR (data not shown), identifying transcripts spanning each intergenic region (Fig. 3A). Transcription of *lpw_24151* was detected in the wild type grown in BYE broth at both 30°C and 37°C (data not shown). Using allelic exchange, we isolated a new mutant (NU393) specifically defective for *lpw_24151*. This mutant, like NU388, was impaired for both the production of film and surface translocation (Fig. 3A), confirming that NU388’s loss of a surfactant was due to mutation of *lpw_24151* and not an unlinked spontaneous mutation. When an insertion mutation was introduced into *lpw_24191*, the last gene in the *lpw_24151* operon (Fig. 3A), the resulting mutants (NU396 and NU397) displayed the simultaneous loss of a surfactant and surface translocation (Fig. 3A). The introduction of *lpw_24191* on a plasmid (i.e., pMlpw_24191) into NU396 reversed the mutant phenotype (Fig. 3A). Thus, these data indicate that *lpw_24191* and most likely the entire *lpw_24151*-containing operon are required for the production of a

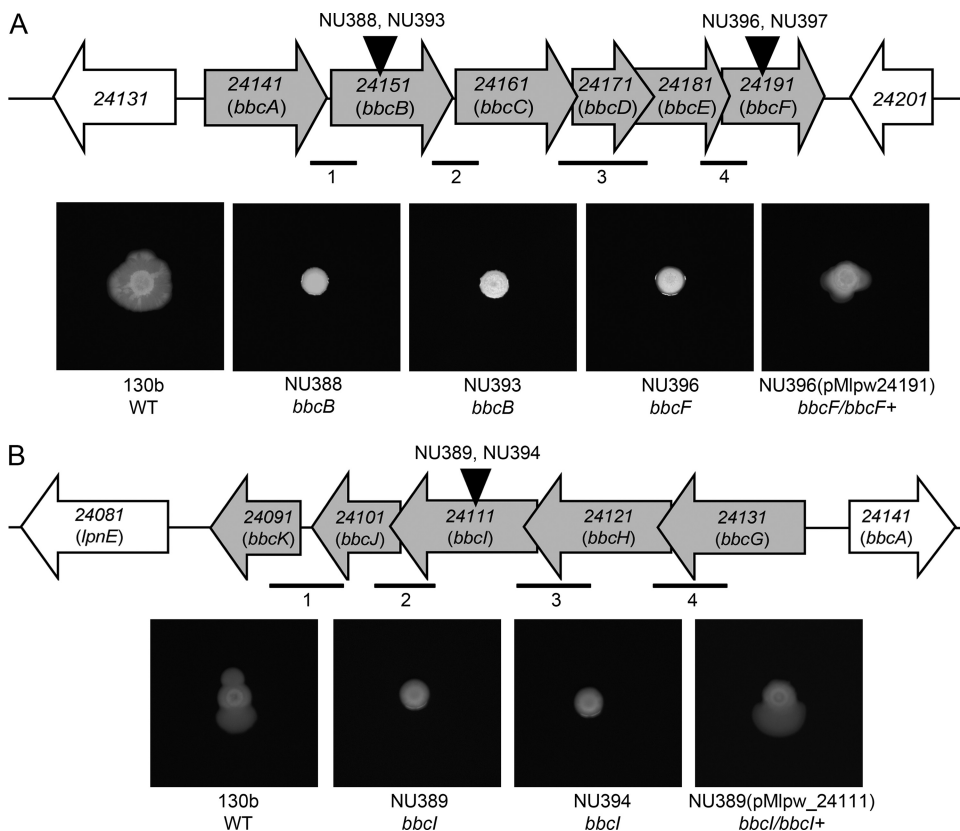


FIG. 3. *L. pneumophila* *bbcABCDEF* and *bbcGHIJK* operons and their impact on surfactant and surface translocation. (A, top) Depiction of the region of the strain 130b chromosome containing genes *lpw*_24141 through *lpw*_24191, i.e., the *bbcABCDEF* operon. The horizontal gray arrows denote the relative size and orientation of the *bbc* genes, whereas the horizontal white arrows denote the two flanking genes. The vertical black arrowheads mark the approximate locations of the insertion mutations in mutants NU388, NU393, NU396, and NU397. The thinner horizontal lines below the gene map signify the approximate size and location of the four intergenic transcripts identified by RT-PCR analysis. (A, bottom) Surface translocation and surfactant phenotypes of *bbcB* and *bbcF* mutants. WT 130b, *bbcB* surfactant mutants NU388 and NU393, *bbcF* surfactant mutant NU396, and complemented mutant NU396(pMlpw24191) were grown on BCYE plates containing 0.5% agar at 30°C for 7 days. The *bbcF* mutant NU397 also consistently lacked surface translocation and surfactant (data not shown). (B, top) Depiction of the region of the strain 130b chromosome containing genes *lpw*_24131 through *lpw*_24091, i.e., the *bbcGHIJK* operon. The black arrowhead marks the location of the insertion in mutants NU389 and NU394, and the thinner horizontal lines signify the four intergenic transcripts identified by RT-PCR analysis. (B, bottom) Surface translocation and surfactant phenotypes of *L. pneumophila* *bbcI* mutants. WT 130b, *bbcI* surfactant mutants NU389 and NU394, and complemented mutant NU389(pMlpw_24111) were grown on BCYE plates containing 0.5% agar at 30°C for 7 days. The images of bacterial growth presented in panels A and B are representative of those obtained from at least three independent experiments.

surfactant by strain 130b. Utilizing the *L. pneumophila* databases (18, 22, 26, 34), we determined that the operon containing *lpw*_24141 through *lpw*_24191 (*lpw*_24141-*lpw*_24191) is largely conserved in the five other sequenced strains of *L. pneumophila* (Table 3). Bioinformatics analysis indicated that all of the proteins encoded within the *lpw*_24141-*lpw*_24191 operon are cytoplasmic proteins. We next used BLAST to begin to gain insight into the activities or functions associated with the proteins encoded by the *lpw*_24141-*lpw*_24191 operon. Although the protein encoded by *lpw*_24171 did not share sequence similarity to any known or hypothetical non-*Legionella* protein in the database, each of the five other predicted proteins shared significant similarity with cytoplasmic enzymes involved in lipid metabolism (Table 4) (36, 49, 57). *Lpw*_24191, for example, had a predicted ketoacyl-acyl carrier protein synthase III domain, which, in proteins from other bacteria, is known to initiate elongation in fatty acid biosynthesis (57). Based upon our mutational and bioinformatics

analyses, we designated the *lpw*_24141-containing operon *bbcABCDEF* for *Legionella* biosurfactant biosynthesis cluster genes A through F (Fig. 3A). We posited that the surfactant made by *L. pneumophila* has a lipid component and that the enzymes encoded by *bbcABCDEF* help mediate the production of that lipid component.

Mutant NU389 contained its *Tn10* insertion at nt 723 in the 1,530-bp gene denoted *lpw*_24111. The *lpw*_24111 ORF was predicted to be part of an operon, and this arrangement was confirmed by RT-PCR analysis (data not shown), which identified transcripts spanning four intergenic regions (Fig. 3B). Transcripts from *lpw*_24111 were detected in 130b bacteria grown in BYE broth at both 30°C and 37°C (data not shown). When we backcrossed the mutation in *lpw*_24111, the new mutant (NU394) lacked the ability to both produce a film and spread over the agar (Fig. 3B). These data indicated that the loss of the surfactant that we had seen for NU389 was due to mutation in *lpw*_24111 and not a spontaneous second-site mu-

TABLE 3. Surfactant biosynthetic operons in six sequenced *L. pneumophila* strains

Strain 130b ORF (gene)	Gene annotation using indicated database:				
	Alcoy ^b	Corby	Lens	Paris	Philadelphia
lpw_24141 (<i>bbcA</i>)	lpa_03202	lpc_1695	lpl2153	lpp2180	lpg2228
lpw_24151 (<i>bbcB</i>)	lpa_03203	lpc_1696	lpl2154	lpp2181	lpg2229
lpw_24161 (<i>bbcC</i>)	lpa_03204	lpc_1697	lpl2155	lpp2182	lpg2230
lpw_24171 (<i>bbcD</i>)	lpa_03205	lpc_1698	lpl2156	lpp2183	— ^c
lpw_24181 (<i>bbcE</i>)	lpa_03207	lpc_1699	lpl2157	lpp2184	lpg2231
lpw_24191 (<i>bbcF</i>)	lpa_03209	lpc_1700	lpl2158	lpp2185	lpg2232
lpw_24131 (<i>bbcG</i>)	lpa_03201	lpc_1694	lpl2152	lpp2178	lpg2227
lpw_24121 (<i>bbcH</i>)	lpa_03200	lpc_1693	lpl2151	lpp2177	lpg2226
lpw_24111 (<i>bbcI</i>)	lpa_03198	lpc_1692	lpl2150	lpp2176	lpg2225
lpw_24101 (<i>bbcJ</i>) ^a	lpa_03196	lpc_1691	lpl2149	lpp2175	lpg2223
lpw_24091 (<i>bbcK</i>) ^a	lpa_03196	lpc_1691	lpl2149	lpp2175	lpg2223

^a The genome databases are slightly divergent at this junction, such that *bbcJ* and *bbcK* are annotated as two open reading frames in strain 130b but as a single open reading frame in the other strains.

^b In the Alcoy database, there are no genes annotated as lpa_03206, lpa_03208, and lpa_03199.

^c —, the Philadelphia-1 annotation does not include this gene.

tation. Genetic complementation of NU389 with a plasmid copy of lpw_24111 (Fig. 3B) confirmed that lpw_24111 promotes the production of a surfactant. Examination of the genome database revealed that the first three genes in the lpw_24111-containing operon (i.e., lpw_24131, lpw_24121, and lpw_24111) are conserved within the five other sequenced strains of *L. pneumophila* (Table 3). Bioinformatics analysis indicated that most of the proteins encoded within the lpw_24091-lpw_24131 operon are cytoplasmic proteins, with the exception being Lpw_24121, an inner membrane protein. BLAST analysis and examination of the conserved domain database indicated that Lpw_24091 and Lpw_24101 encoded by the last two genes in the 130b operon did not share sequence similarity to any known or hypothetical non-*Legionella* protein in the database. However, Lpw_24111 and the two remaining proteins from the 130b locus had similarity with enzymes involved in lipid metabolism (Table 4) (25, 36). These findings support our hypothesis that the surfactant of *L. pneumophila* has a lipid component. Interestingly, this second operon identified by our screen mapped immediately upstream but transcribed in the direction opposite from *bbcABCDE* (compare Fig. 3A and B). Thus, we designated the lpw_24111-containing operon *bbcGHIJK* (Fig. 3B). Although all of the predicted activities associated with the *bbc* loci relate to lipid

metabolism, those from *bbcABCDE* were distinct from those of *bbcGHIJK*.

Incidentally, the gene (lpw_24081) downstream of the *bbcGHIJK* operon is *lpnE* (Fig. 3B), which encodes a protein that is important for bacterial entry into host cells (70). An *lpnE* mutant had no alteration in surfactant or surface translocation (data not shown).

Outer membrane protein TolC promotes the secretion of *L. pneumophila* biosurfactant. The third mutant (NU390) isolated in our screen had its *Tn10* insertion mutation in lpw_07761, which encodes the outer membrane protein TolC. TolC has been very well studied in a variety of Gram-negative bacteria and is best known for its key role in antibiotic efflux (54, 96). Previous mutational analysis of *L. pneumophila* strain Lens determined that TolC also promotes antibiotic and detergent efflux by *Legionella* (30), and we confirmed this when we compared our *tolC* mutant NU390 to parental 130b for sensitivity to erythromycin and SDS (data not shown). In strain 130b, *tolC* (i.e., lpw_07761) was predicted to be the second gene in a three-gene operon (Fig. 4A), with the upstream gene lpw_07751 predicted to encode a protein with some similarity to nucleotide synthesis enzymes and the downstream gene lpw_07771 predicted to encode protein-L-isoaspartate-*O*-methyltransferase. This operon structure was confirmed by

TABLE 4. Conserved domains in surfactant biosynthetic proteins of *L. pneumophila*

Strain 130b ORF	Gene	Conserved domain in the predicted protein ^a	E value
lpw_24141	<i>bbcA</i>	Ketoacyl-acyl carrier protein synthase III	2×10^{-39}
lpw_24151	<i>bbcB</i>	Acyl-CoA synthetase	5×10^{-120}
lpw_24161	<i>bbcC</i>	Acyl-CoA/AMP-acid ligase II	9×10^{-54}
lpw_24171	<i>bbcD</i>	None	
lpw_24181	<i>bbcE</i>	3-Ketoacyl-acyl carrier protein reductase	4×10^{-30}
lpw_24191	<i>bbcF</i>	Ketoacyl-acyl carrier protein synthase III	8×10^{-71}
lpw_24131	<i>bbcG</i>	Carboxyltransferase of acetyl-CoA carboxylase	5×10^{-96}
lpw_24121	<i>bbcH</i>	Acyl-CoA dehydrogenase	1×10^{-52}
lpw_24111	<i>bbcI</i>	GH3 domain	7×10^{-90}
lpw_24101	<i>bbcJ</i>	None	
lpw_24091	<i>bbcK</i>	None	

^a Protein sequences from the 130b genome (<http://www.ebi.ac.uk/ena/data/view/FR687201>) were analyzed using the conserved domain database at the NCBI. CoA, coenzyme A.

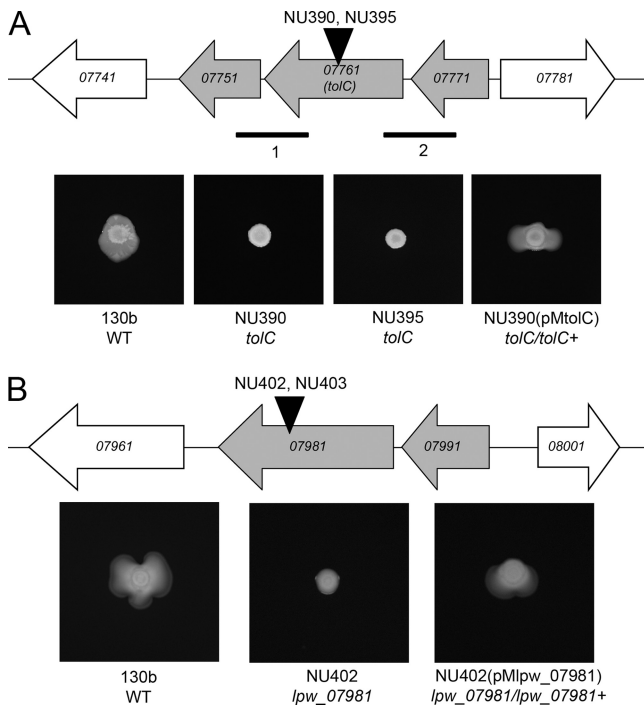


FIG. 4. *L. pneumophila tolC* and *lpw_07981* and their impact on surfactant and surface translocation. (A, top) Depiction of the region of the 130b chromosome containing genes *lpw_07771*, *lpw_07761*, and *lpw_07751*; i.e., the *tolC* operon. The horizontal gray arrows denote the size and orientation of the *tolC* operon, and the white arrows denote the two flanking genes. The vertical black arrowheads mark the locations of the insertion mutations in mutants NU390 and NU395. The thinner horizontal lines below the gene map signify the size and location of the two intergenic transcripts identified by RT-PCR. (A, bottom) Surface translocation and surfactant phenotypes of *tolC* mutants. WT 130b, *tolC* mutants NU390 and NU395, and complemented mutant NU390(pM*tolC*) were grown on BCYE plates containing 0.5% agar and 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) at 30°C for 7 days. (B, top) Depiction of the region of the 130b chromosome containing genes *lpw_07991* and *lpw_07981*. The black arrowhead marks the locations of the insertions in *lpw_07981* mutants NU402 and NU403. In the 130b database, the *lpw_07981* ORF is also (erroneously) denoted *lpw_07971*. (B, bottom) Surface translocation and surfactant phenotypes of *lpw_07981* mutants. WT 130b, *lpw_07981* mutant NU402, and complemented mutant NU402(pM*lpw_07981*) were grown on BCYE plates containing 0.5% agar at 30°C for 7 days. The *lpw_07981* mutant NU403 also consistently lacked surface translocation and surfactant (data not shown). The images presented in panels A and B are representative of those obtained from at least three independent experiments.

using RT-PCR (data not shown) to amplify the junction between *lpw_07761* and *lpw_07771* and the region between *lpw_07761* and *lpw_07751* (Fig. 4A). TolC transcript was detected in cultures at both 37°C and 30°C (data not shown). When we backcrossed the mutation in *tolC*, the newly derived mutant (NU395) lacked the ability to both produce a film and spread over agar (Fig. 4A), implying that the loss of the surfactant that we had seen for NU390 was due to mutation in *tolC* and not a spontaneous second-site mutation. Complementation of NU390 with a plasmid copy of *tolC* (Fig. 4A) confirmed that TolC is required for the elaboration of a surfactant by *L. pneumophila*. Given the well-known outer membrane location of TolC, we hypothesized that our *tolC* mutants were

defective for surfactant secretion and not impaired for biosynthesis.

In other systems studied, TolC forms a trimolecular complex with multiple pairs of inner membrane and membrane fusion (adaptor) proteins in order to mediate efflux of different types of substrates (10, 72). The inner membrane anchor protein and its associated adaptor protein are often referred to as the “translocase.” Therefore, in order to gain further support for the role of TolC-mediated efflux in surfactant secretion, we scanned the *L. pneumophila* database for putative TolC partners and then mutated the corresponding genes in strain 130b. The operon containing *lpw_07981* and *lpw_07991* (Fig. 4B) encoded a protein (Lpw_07981) with sequence relatedness to the *E. coli* O157:H7 inner membrane protein AcrB (i.e., 32% aa identity and 52% aa similarity, with an E value of 2×10^{-141}) and a protein (Lpw_07991) showing sequence relatedness to the *E. coli* membrane fusion (adaptor) protein AcrA (i.e., 22% aa identity and 38% aa similarity, with an E value of 5×10^{-11}) (10). Two strains (NU402 and NU403) containing a mutation in *lpw_07981* were made. As would be predicted, both mutants showed increased sensitivity to erythromycin and SDS, similar to the *tolC* mutant (data not shown). More significantly, NU402 and NU403 were impaired for both surfactant and surface translocation (Fig. 4B). Complementation of NU402 with a plasmid copy of *lpw_07981* (Fig. 4B) confirmed that the targeted AcrB-like protein is required for elaboration of the surfactant by *L. pneumophila*. In contrast to these results, mutants NU400 and NU401, defective for *lpw_27401*, a gene predicted to encode an adaptor protein similar to *E. coli* OqxA (37% aa identity and 53% aa similarity, with an E value of 3×10^{-51}) (37), exhibited a surfactant phenotype that was similar to that of the wild type (see Fig. S2 in the supplemental material). Based upon the combination of the results obtained from analyzing a TolC mutant (and its complement) and an AcrB-like mutant (and its complement), we hypothesized that a TolC-containing membrane complex is required for secretion of the *L. pneumophila* surfactant. To our knowledge, these data represent the first genetic proof for the role of TolC and its partners in surfactant secretion.

In considering the different ways in which TolC might promote surfactant secretion, it was important to revisit a key result from our previous study, namely, that an intact T2S system is also required for elaboration of the *L. pneumophila* surfactant (90). Therefore, we posited that the TolC and T2S systems work together to mediate secretion, with one directly exporting the surfactant and the other exporting a signaling molecule and/or activating enzyme. To test this hypothesis, we spotted an *lspF* mutant and a *tolC* mutant next to each other on the low-percentage-agar BCYE plates and then monitored bacterial growth, surface translocation, and surfactant film production over time (Fig. 5A). After 4 to 5 days of growth, the *lspF* mutant showed surface translocation and surfactant film emerged from the mutant’s spreading lobe. By 7 days, the expanding film resulted in spreading from the *tolC* mutant spots. A similar result was obtained when we spotted the *lspF* mutant next to a mutant lacking the presumptive AcrB-like, inner membrane partner of TolC (Fig. 5B). Taken together, these results indicate that the *lspF* mutant is able to secrete a surfactant when exposed to a diffusible factor (that is still) made by mutants lacking a TolC complex. These data also

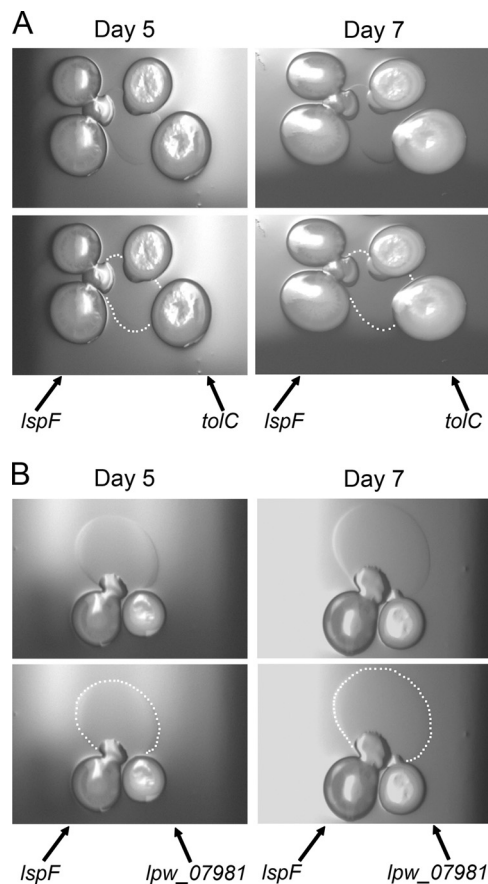


FIG. 5. Surfactant secretion by an *L. pneumophila* *lspF* mutant when grown in the presence of an *L. pneumophila* *tolC* or *lpw_07981* mutant. (A) As indicated by the arrows, two inocula of the *lspF* mutant NU275 and two inocula of the *tolC* mutant NU390 were spotted next to each other on a BCYE plate containing 0.5% agar. After incubation at 30°C for 5 days or 7 days, bacterial growth and surface translocation were imaged (top row). To help with visualization of the surfactant that was produced by the sliding *lspF* mutant, white dots have been added to mark the boundary of the spreading film (bottom row). The surfactant film was also evident when single spots of the two mutants were placed next to each other (data not shown). (B) As indicated, *lspF* mutant NU275 and *lpw_07981* mutant NU402 were spotted next to each other on low-agar BCYE plates, and then after incubation at 30°C for 5 or 7 days, bacterial growth, surface translocation, and surfactant production were imaged. As in panel A, the bottom images depict the boundary of the film produced by the *lspF* mutant with white dots. The results depicted in panels A and B are representative of those obtained from three independent experiments.

imply that the lack of surfactant secretion that we had observed for the T2S and TolC mutants was most likely due to the specific loss of mediators of secretion and not due solely to indirect effects on global gene expression and/or growth rate when *lsp*, *tolC*, or *lpw_07981* is mutated. Because it was the *lspF* mutant and not the TolC mutant that was able to now produce a surfactant, it would appear that a TolC complex is the conduit for surfactant secretion and that the T2S apparatus mediates the export of a signaling or activating factor.

Effect of a surfactant on *L. pneumophila* growth. Utilizing our new mutants, we next sought to determine the importance of surfactant for *L. pneumophila* growth. The *bbcB* mutant

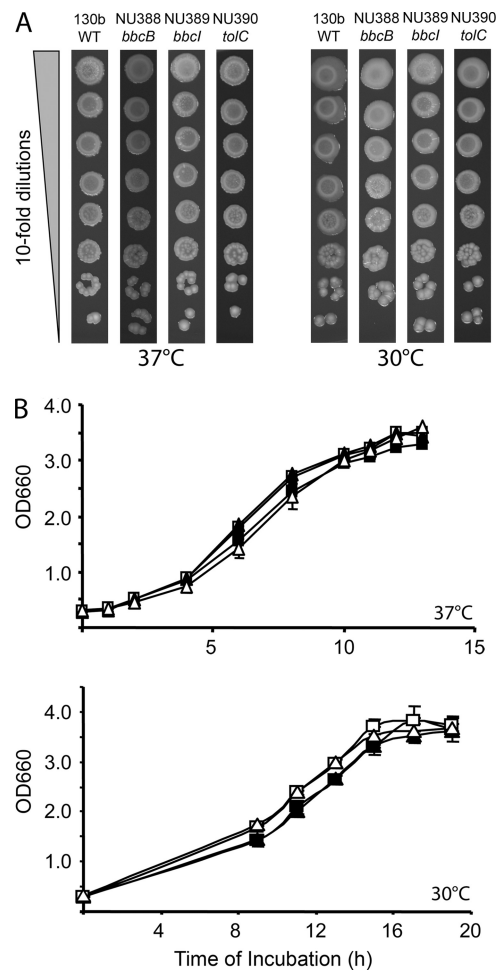


FIG. 6. Extracellular growth of the *L. pneumophila* wild type and *bbcB*, *bbcI*, and *tolC* mutants. (A) Growth of legionellae on BCYE agar. We spotted dilutions of WT 130b, *bbcB* mutant NU388, *bbcI* mutant NU389, and *tolC* mutant NU390 onto standard BCYE agar and incubated the plates at various temperatures. Bacterial growth was then imaged at 3 days for the 37°C plates and 4 days for the 30°C plates. (B) Growth of legionellae in BYE broth. WT 130b (■), *bbcB* mutant NU388 (□), *bbcI* mutant NU389 (▲), and *tolC* mutant NU390 (△) were inoculated into BYE broth and then incubated at 37°C or 30°C. At various times postinoculation, the extent of growth was monitored spectrophotometrically. The data points represent the means and standard deviations for triplicate cultures. The results presented in both panels A and B are representative of three independent experiments.

NU388, *bbcI* mutant NU389, and *tolC* mutant NU390 had an efficiency of plating on standard BCYE agar that was similar to that of wild-type 130b, whether the plates were incubated at 30°C or 37°C (Fig. 6A). In a similar vein, NU388, NU389, and NU390 grew like the wild type did in standard BYE broth (Fig. 6B). Previous studies had established that the *lspF* T2S mutant also grows normally on standard BCYE agar and in BYE broth at 30°C and 37°C (90). These data indicate that these mutants do not have a generalized growth defect and that surfactant is not required for optimal extracellular growth by *L. pneumophila* at least under standard lab conditions.

In some cases, the enzymes involved in surfactant synthesis are also required for the synthesis of other metabolites; e.g., *P.*

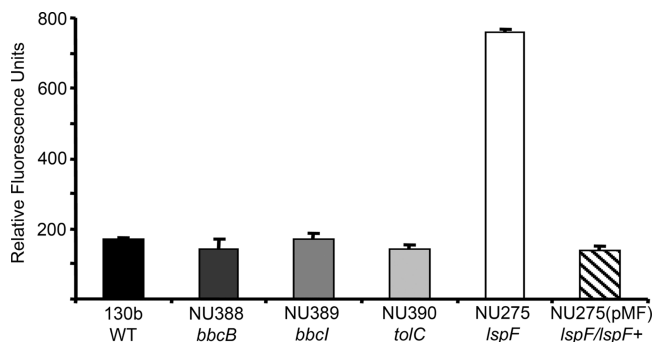


FIG. 7. PHB in the *L. pneumophila* wild type and *bbcB*, *bbcl*, *tolC*, and *lspF* mutants. WT 130b, *bbcB* mutant NU388, *bbcl* mutant NU389, *tolC* mutant NU390, *lspF* mutant NU275, and complemented NU275(pMF) were grown in BYE to early log phase. The cells were then subcultured into CDM and grown at 37°C. After staining with Nile red, fluorescence was measured using a spectrophotometer blanked with unstained bacteria. Data are the means and standard deviations for three replicates obtained from duplicate cultures. The levels of PHB exhibited by the NU275 mutant were significantly increased compared to those for the other strains ($P < 0.05$, Student's *t* test). The results shown here are representative of at least four independent experiments.

aeruginosa RhlG is needed for synthesis of rhamnolipids and poly- β -hydroxyalkanoate lipid storage molecules (16). *L. pneumophila* produces poly-3-hydroxybutyrate (PHB) storage molecules when grown in bacteriological medium, but the basis of its synthesis is only partly known (7, 47). Given this and the fact that BbcE belongs to the same FabG-like family as RhlG, we considered the possibility that our surfactant mutants might lack PHB. However, the *bbc* and *tolC* mutants accumulated PHB levels similar to those of the wild type (Fig. 7). On the other hand, the T2S mutant NU275, but not its complemented derivative (83), had higher levels of PHB (Fig. 7), indicating that in *L. pneumophila* a T2S-dependent factor unrelated to the surfactant is critical for maintaining normal levels of PHB. To our knowledge, these data are the first link between T2S and PHB. Since T2S is not known to directly influence factors in the cytoplasm, which is where enzymes involved in PHB synthesis and utilization would reside (7), a T2S-dependent enzyme may be indirectly influencing PHB metabolism.

When the *bbcB* and *bbcl* mutants were tested for their relative ability to infect and replicate in *A. castellanii* and *H. vermiformis*, no defect was observed at a coculture incubation temperature of 35°C (Fig. 8A and B) or 30°C (data not shown). The surfactant biosynthesis mutants also grew in the way that the wild type did when tested in the U937 macrophage cell line, the A549 type II lung epithelial cell line, and the WI-26 type I lung epithelial cell line (Fig. 8C to E). Recently, we defined the cytokine output produced when U937 cells are infected with *L. pneumophila* 130b (64). Since some biosurfactants can influence host cytokine responses (33, 86), we examined the levels of interleukin-6 (IL-6) in supernatants from U937 cell cultures infected with the *bbc* mutants but did not find an altered cytokine profile (data not shown). Lastly, the *bbcl* mutant NU389 was tested for replication in the lungs of A/J mice and found to grow and persist in a manner similar to that of the wild type (Fig. 8F). Together, these data indicate

that the surfactant linked to the *bbc* loci is not required for optimal intracellular infection or pathogenicity.

The *L. pneumophila* surfactant impacts the growth of other legionellae. Because some biosurfactants possess antimicrobial activity (8), we determined whether the surfactant produced by *L. pneumophila* 130b influenced the growth of heterologous bacteria. Wild-type 130b and surfactant mutant NU389 had no effect on the efficiency of plating (i.e., numbers of CFU) of Gram-negative *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* as well as Gram-positive *B. subtilis* and *Listeria monocytogenes* (Fig. 9), indicating that the *L. pneumophila* surfactant does not affect the growth of these microbes. As might be expected, the *L. pneumophila* surfactant did promote spreading of some of these bacteria; e.g., *B. subtilis* (Fig. 9) and surfactants produced by other bacteria promoted spreading of *L. pneumophila* (see Fig. S4 in the supplemental material). In contrast to these results, *L. pneumophila* greatly stimulated the growth of Gram-positive *Micrococcus luteus* (Fig. 9). However, this effect was not dependent on the surfactant, because the *bbcl* mutant promoted growth just as the wild type did.

We had previously determined that surfactant production levels vary among the different *Legionella* species (90). Most notably, *Legionella micdadei* lacks film production and surface translocation when spotted onto BCYE agar containing a low percentage of agar (90). Therefore, we next examined the impact of *L. pneumophila* and its surfactant on the growth of nonsliding *L. micdadei*. In marked contrast to the observations made with nonlegionellae, the growth of *L. micdadei* was deleteriously affected by the presence of *L. pneumophila* (Fig. 10). Compared to its behavior when plated alone on standard BCYE agar or BCYE containing 0.5% agar, the growth of *L. micdadei* was reduced ca. 10^6 -fold when spotted on the film produced by nearby strain 130b (Fig. 10). However, when *L. micdadei* was spotted next to the surfactant-lacking *bbcl* mutant NU2388, there was no reduction in CFU (Fig. 10), indicating that this impaired growth was due, directly or indirectly, to the surfactant. In support of this conclusion, a similar result was obtained when we spotted *L. micdadei* next to *bbcB* mutant NU388, *tolC* mutant NU390, and *lspF* mutant NU275 (Fig. 10 and data not shown). Further confirmation was obtained when the reduction in *L. micdadei* growth was observed after the strain was spotted adjacent to a complemented *bbcl* mutant (Fig. 10). Given these results, we next assessed the effect of the *L. pneumophila* surfactant on the growth of six additional species of *Legionella*. As we had observed for *L. micdadei*, *L. anisa*, *L. feeleii*, *L. hackeliae*, *L. jordanis*, *L. moravica*, and *L. wadsworthii* were all impaired by the presence of wild-type 130b but not the *bbcl* surfactant mutant (Fig. 11). Taken together, these data indicate that the surfactant made by *L. pneumophila* has an antagonistic or antibacterial activity which may be specific against species of *Legionella*. An antagonism such as this, surfactant or otherwise, has not been reported before.

DISCUSSION

Surfactants are amphipathic molecules that reduce surface and interfacial tension (66, 95). Hence, the various bacteria and fungi that make surfactants are usually capable of spreading across an agar surface (90). In our previous survey of

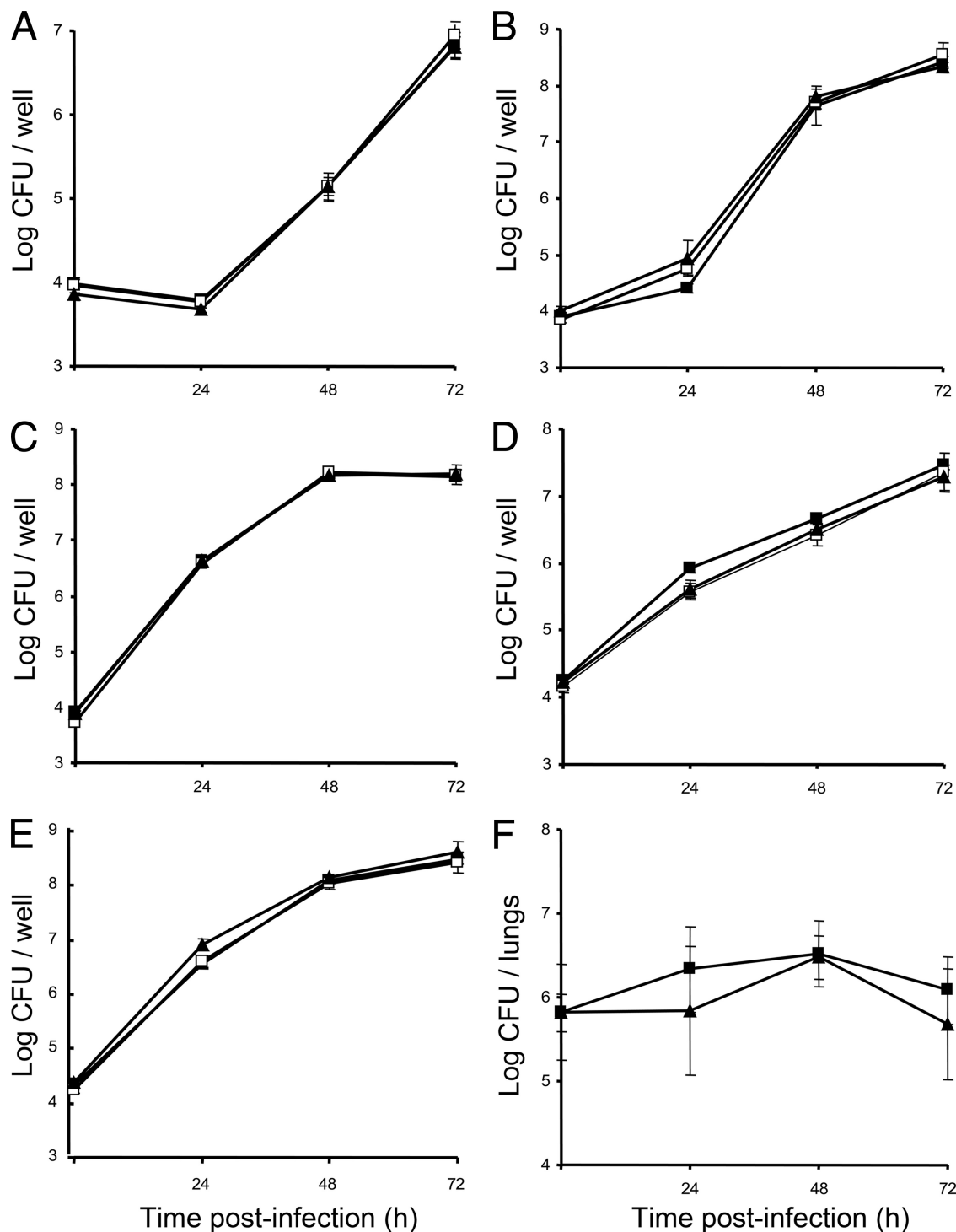


FIG. 8. Intracellular and intrapulmonary growth of the *L. pneumophila* wild type and *bbcB* and *bbcI* mutants. (A to E) *H. vermiformis* (A), *A. castellanii* (B), U937 macrophages (C), A549 type II epithelial cells (D), and WI-26 type I epithelial cells (E) were infected with WT 130b (■), *bbcB* mutant NU388 (□), and *bbcI* mutant NU389 (▲), and then at the indicated times, the numbers of CFU in the infected monolayers were determined by plating. Data are the means and standard deviations for four infected wells. Each panel is representative of three independent experiments. (F) WT 130b (■) and *bbcI* mutant NU389 (▲) were inoculated into the lungs of A/J mice, and then after 24, 48, and 72 h, the numbers of CFU in total lung homogenates were determined by plating. Data are the means and standard deviations for five infected animals. While there might appear to be no growth and reduced recovery of the mutant at 24 h, this result was not statistically significant and was not obtained in the repeat experiment (data not shown).

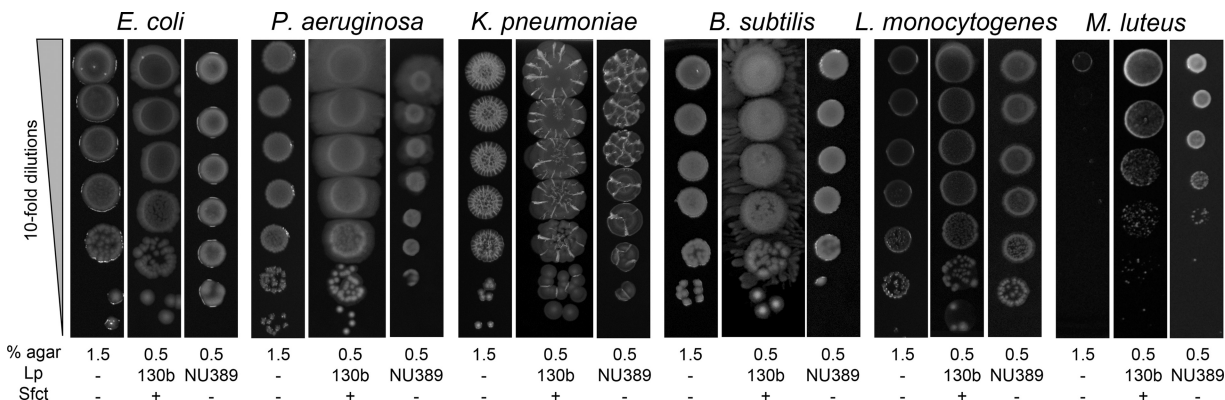


FIG. 9. Effect of the *L. pneumophila* surfactant on heterologous bacteria. As indicated, we spotted 10-fold dilutions of *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. subtilis*, *L. monocytogenes*, and *M. luteus* onto BCYE agar plates that were free of *L. pneumophila* (Lp -), contained a nearby row of WT *L. pneumophila* that had grown and made a surfactant film (Lp 130b), or contained a row of a *bbcI* mutant that had grown but not produced a surfactant (Lp NU389). After incubation at 37°C for another day, the growth of the heterologous bacteria in the absence (Sfct -) or presence (Sfct +) of a surfactant was imaged. The results depicted here were observed in at least three independent experiments. On BCYE agar not containing *L. pneumophila*, the efficiencies of plating for these bacteria were the same whether the medium contained 0.5% agar or 1.5% agar, although plating on low-percentage-agar BCYE did result in greater spreading for those bacteria, such as *P. aeruginosa*, that are known to make a surfactant and/or undergo surface translocation (see Fig. S3 in the supplemental material).

wild-type *Legionella* that included 10 strains of *L. pneumophila* and 12 non-*pneumophila* strains, the expression of the surfactant correlated with surface translocation (90). That all *L. pneumophila* surfactant mutants, including the five (*bbcB*, *bbcF*, *bbcI*, *tolC*, and *lpw_07981*) currently isolated and the four (*lspDE*, *lspF*, *lspG*, and *pilD*) from our past study (90), lack both a surfactant and surface translocation indicates that the

surfactant made by *L. pneumophila* is promoting translocation. We have still not uncovered a role for flagella, pili, or any other motility organelle in the *L. pneumophila* surface translocation described here. Thus, we continue to use the term sliding as a way of denoting the surface translocation exhibited by *L. pneumophila*; sliding is a passive form of motility produced by the expansive forces in a growing culture in combination with the reduced friction between a cell and a substrate that is mediated by a surfactant (40, 48, 50). Other bacteria that exhibit sliding include species of *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Mycobacterium*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Vibrio* (2, 29, 38, 40, 44, 68, 78).

Surfactants are represented by a wide variety of molecular structures, including fatty acids, neutral lipids, phospholipids, glycolipids, glycopeptidolipids, polysaccharides, lipopeptides, and lipoproteins (2, 9, 11, 66, 73, 95). Based upon the genes within the *bbcABCDEF* and *bbcGHIJK* operons, it is probable that the *L. pneumophila* surfactant contains a lipid structure and is being synthesized, at least in part, by a polyketide synthase (PKS) (92). More specifically, our data are most compatible with the involvement of a type II PKS (41, 52). Indeed, the *bbcABCDEF* locus is predicted to encode monofunctional enzymes and provide three of the four enzymes that typically constitute a minimal type II PKS. *BbcA* and *BbcF* would represent two ketosynthases, whereas the gene directly downstream of *bbcF* (*lpw_24201* in Fig. 3) would be the acyl carrier protein. The fourth typical enzyme, an acyltransferase, might be encoded by one of the more novel genes in the locus (e.g., *bbcD*) or encoded elsewhere in the chromosome, perhaps as part of a fatty acid biosynthetic pathway (52). The identification of the *bbcI* gene permits one further prediction, that the surfactant is a lipopeptide or lipoprotein. As a GH3-like enzyme (89), *BbcI* is predicted to conjugate an amino acid, from a peptide or protein, to an acyl substrate. In the future, the structural determination of a purified surfactant, when combined with the genetic information obtained here, should re-

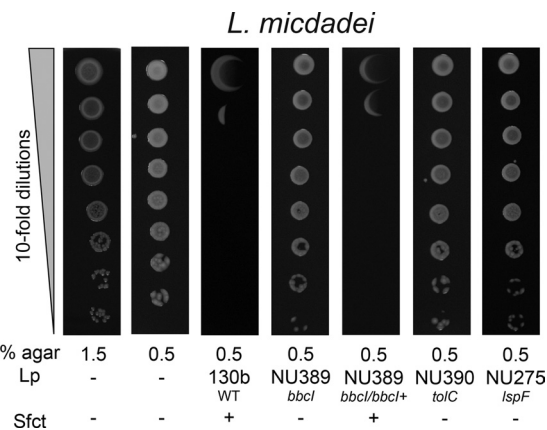


FIG. 10. Effect of the *L. pneumophila* surfactant on the growth of *L. micdadei*. For the two leftmost panels, we spotted dilutions of *L. micdadei* onto BCYE plates containing either 1.5% agar or 0.5% agar but no nearby *L. pneumophila* (Lp -). For the next five panels, we spotted *L. micdadei* onto 0.5% agar BCYE containing a nearby row of WT *L. pneumophila* that had grown and made a surfactant film (Lp 130b; third panel), a row of a *bbcI* mutant that had grown but not produced a surfactant (Lp NU389; fourth panel), a row of the complemented *bbcI* mutant that had restored surfactant production [Lp NU389(pMlpw_24111); fifth panel], a row of a *tolC* mutant that had grown but not made a surfactant (Lp NU390; sixth panel), or a row of an *lspF* mutant that had grown but lacked a surfactant (Lp NU275; last panel). After incubation at 37°C for another 3 days, growth of *L. micdadei* in the absence (Sfct -) or presence (Sfct +) of a surfactant was imaged. The results depicted here were observed in at least three independent experiments.

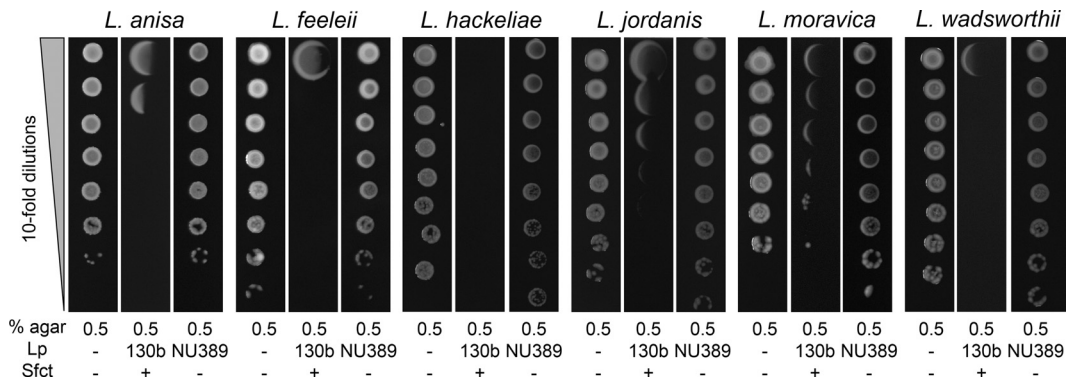


FIG. 11. Effect of the *L. pneumophila* surfactant on the growth of *L. anisa*, *L. feeleii*, *L. hackeliae*, *L. jordanis*, *L. moravica*, and *L. wadsworthii*. For the leftmost panel, we spotted dilutions of the indicated strains onto BCYE plates containing 0.5% agar but no nearby *L. pneumophila* (Lp –). For the next two panels, we spotted the bacteria onto 0.5% agar BCYE containing either a nearby row of WT *L. pneumophila* that had grown and made a surfactant film (Lp 130b; second panel) or a row of a *bbcI* mutant that had grown but not produced a surfactant (Lp NU389; third panel). After incubation at 37°C for another 3 days, growth of the legionellae in the absence (Sfct –) or presence (Sfct +) of a surfactant was imaged. The results depicted here were observed in at least three independent experiments.

veal much of the mechanism of synthesis for this new *Legionella* surfactant.

Among the other *Legionella* species that we had previously found to produce a surfactant and undergo sliding (90), *L. longbeachae* is the only one for which a published genome database is available (17, 55). *L. longbeachae* has homologs of *bbcABCDEF* and *bbcGHI*, i.e., llo2601 to llo2609 in strain NSW150 and llb_2731 to llb_2739 in D-4968. These data suggest that the surfactant produced by *L. longbeachae* is the same as the one made by *L. pneumophila*. The ability of different species within the same genus to produce identical or similar surfactants has been observed before; e.g., several species of *Pseudomonas* produce rhamnolipids (1). Complete genome databases are not (yet) available for any other *Legionella* species, with the exception of the obligate intracellular parasite *L. drancourtii* (65). Therefore, we cannot immediately say whether those species that do not produce a surfactant lack *bbc* genes.

The data presented here are both the first documentation of a link between surfactant secretion and TolC and the first indication that a TolC complex may act as the conduit for surfactant secretion. However, several past studies which examined putative TolC partners in other bacteria have suggested that such a connection could exist. For example, an AcrB-like protein known as SwrC is needed for secretion of surfactin by *B. subtilis* (51), and the loss of a putative TolC partner results in the reduced production of putisolvin by *Pseudomonas putida* and arthrofactin by *Pseudomonas* sp. strain MIS38 (27, 61). That TolC may act as the conduit for the export of a surfactant is not unreasonable for two more reasons; first, TolC complexes are known to mediate the export of a wide variety of substrates, ranging from small antibiotics to proteins (10), and second, surfactants are usually small, amphipathic molecules that should be compatible with the TolC outer membrane channel. In pondering the mechanism by which T2S conjoins with TolC to promote surfactant secretion by *L. pneumophila*, several scenarios are possible. In the first case, T2S promotes the secretion of a “regulatory” molecule that provides a necessary signal to the cell for when to make and/or secrete the surfactant. In the second scenario, T2S

mediates the secretion of a factor (e.g., an enzyme) that is needed for the maturation or activation of the surfactant after it exits the cell envelope. In the third case, the T2S system secretes a factor that is necessary for the ability of TolC to export the surfactant. That secreted factors can influence the export of a surfactant is supported by observations in the literature; e.g., quorum sensing has been linked to regulation of surfactant production in *B. subtilis*, *P. aeruginosa*, and *Serratia liquefaciens* (62, 75, 77). Since a connection between TolC and T2S has not been reported previously, further study of the *L. pneumophila* surfactant should yield novel insight into the mechanism and regulation of Gram-negative secretion. By continuing to screen our mutant library for strains that lack a surfactant and sliding, we should be able to uncover additional factors, such as a hypothetical signal or modifying enzyme, that provide the link between TolC and T2S as it relates to surfactant secretion.

Although we did not find a required role for the surfactant in the extracellular growth, intracellular infection, or intrapulmonary survival of *L. pneumophila*, we did determine that the substance is antagonistic toward other *Legionella* species. This inhibitory effect most likely represents a direct antimicrobial activity, since there are numerous past studies ascribing antimicrobial properties to biosurfactants, including those that are lipoproteins (77). Additionally, though fewer, there are other surfactants that are known to act within a genus; e.g., surfactin is a lipoprotein that is produced by *B. subtilis* and has activity against *Bacillus cereus* (45). It is also formally possible that the *L. pneumophila* surfactant impaired the growth of the other legionellae by obstructing nutrient acquisition or somehow negatively affecting the microenvironment. Whatever its precise mode(s) of action, the surfactant may help strains of *L. pneumophila* gain a selective advantage over other *Legionella* species that it encounters in natural habitats. Besides providing an antimicrobial activity, the surfactant could foster the environmental survival of *L. pneumophila* in several other ways. For example, *L. pneumophila* is known to exist in multiorganismal biofilms (91), and based upon work in other microbial systems, surfactants help bacteria to modulate their biofilm architectures as well as to promote their ability to detach from

and/or disperse biofilms (12, 46, 56). Moreover, surfactants are known to mediate solubilization, uptake, and utilization of hydrophobic compounds, solubilization of quorum-sensing molecules, solubilization of outer membrane vesicles, and binding of heavy metals and prevention of their toxicity (2, 14, 15, 43, 80, 88, 93). Thus, another direction for future research will focus on more fully defining the role of the surfactant in *L. pneumophila* ecology, particularly given that environmental legionellae are directly linked to human disease.

ACKNOWLEDGMENTS

We thank past and present members of the Cianciotto lab for their helpful comments and suggestions. We also thank Nancy Freitag for sending us strains of *Micrococcus* and *Bacillus* and Barry Fields for strains of *Pseudomonas* and *Klebsiella*. We gratefully acknowledge Emily Yip for designing *tolC* primers and performing some of the antibiotic testing and Kessler McCoy-Simandle for help with the cytokine analysis and animal infections.

This study was funded by NIH grant AI043987 awarded to N.P.C.

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