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Revisiting the Gram-Negative Lipoprotein Paradigm

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ABSTRACT

The processing of lipoproteins (Lpps) in Gram-negative bacteria is generally considered an essential pathway. Mature lipoproteins in these bacteria are triacylated, with the final fatty acid addition performed by Lnt, an apolipoprotein N-acyltransferase. The mature lipoproteins are then sorted by the Lol system, with most Lpps inserted into the outer membrane (OM). We demonstrate here that the *lnt* gene is not essential to the Gram-negative pathogen *Francisella tularensis* subsp. *tularensis* strain Schu or to the live vaccine strain LVS. An LVS Δ*lnt* mutant has a small-colony phenotype on sucrose medium and increased susceptibility to globomycin and rifampin. We provide data indicating that the OM lipoprotein Tul4A (LpnA) is diacylated but that it, and its paralog Tul4B (LpnB), still sort to the OM in the Δ*lnt* mutant. We present a model in which the Lol sorting pathway of *Francisella* has a modified ABC transporter system that is capable of recognizing and sorting both triacylated and diacylated lipoproteins, and we show that this modified system is present in many other Gram-negative bacteria. We examined this model using *Neisseria gonorrhoeae*, which has the same Lol architecture as that of *Francisella*, and found that the *lnt* gene is not essential in this organism. This work suggests that Gram-negative bacteria fall into two groups, one in which full lipoprotein processing is essential and one in which the final acylation step is not essential, potentially due to the ability of the Lol sorting pathway in these bacteria to sort immature apolipoproteins to the OM.

IMPORTANCE

This paper describes the novel finding that the final stage in lipoprotein processing (normally considered an essential process) is not required by *Francisella tularensis* or *Neisseria gonorrhoeae*. The paper provides a potential reason for this and shows that it may be widespread in other Gram-negative bacteria.

Bacterial lipoproteins (Lpps) are a class of secreted, membrane-bound proteins with diverse functions, such as stabilizing the outer membrane (OM), energy production, establishing host invasion, intracellular survival, and influencing the immune response of the host (1–8). Lipoproteins are a microbial molecular pattern sensed via Toll-like receptor 2 (TLR2) in conjunction with either TLR1 or TLR6 (1, 2). This recognition can stimulate the activation of signaling pathways, resulting in the production of proinflammatory cytokines and antimicrobial effector molecules (3).

The pathways for lipoprotein processing and sorting have been developed from extensive studies in *Escherichia coli* (4). Processing (Fig. 1A) begins with the translation of a preprolipoprotein that has an N-terminal secretion signal consisting of three regions: an n-region, an h-region, and a c-region. The n-region contains positively charged amino acids, the h-region is made up of hydrophobic amino acids, and the c-region contains the conserved lipobox ([LVI][ASTVI][AGS] ↓ C), with an invariant cysteine at position +1. The preprolipoprotein is usually exported from the cytosol by the general secretory pathway (although exceptions exist) and modified first by preprolipoprotein diacylglyceryl transferase (Lgt), which transfers a diacylglyceride to the cysteine sulfhydryl of the preprolipoprotein, forming a prolipoprotein. The signal peptide of the prolipoprotein is then cleaved by prolipoprotein signal peptidase (Lsp), resulting in a diacylated apolipoprotein with a new N-terminal cysteine that is then acylated by apolipoprotein N-acyltransferase (Lnt), creating a mature triacylated lipoprotein.

The mature protein either remains in the inner membrane or is transported to the outer membrane via the Lol sorting machinery (Fig. 1B), which, in *E. coli* consists of an ABC transporter (LolCDE), periplasmic chaperone protein (LolA), and an OM lipoprotein (LolB) (5, 6). A homodimer of LolD constitutes the ATPase component, and LolC and LolE are the membrane-spanning units (5). ATP hydrolysis drives the passage of the lipoprotein from LolCDE to the chaperone LolA in the periplasm (7). The LolA-lipoprotein complex crosses the periplasmic space to the OM receptor LolB, which is itself a lipoprotein (8). LolB releases the lipoprotein from LolA and inserts it into the OM. It is thought that the OM is the default destination for lipoproteins unless they contain a species-specific "Lol avoidance" signal, in which case they are retained in the inner membrane (IM). This signal is an aspartic acid at position +2 in *E. coli*, and a lysine and a serine at positions +3 and +4 in *Pseudomonas* spp. (9–11). Most OM lipoproteins are thought to face into the periplasm; however, it is apparent that a few lipoproteins are exposed on the outer surface

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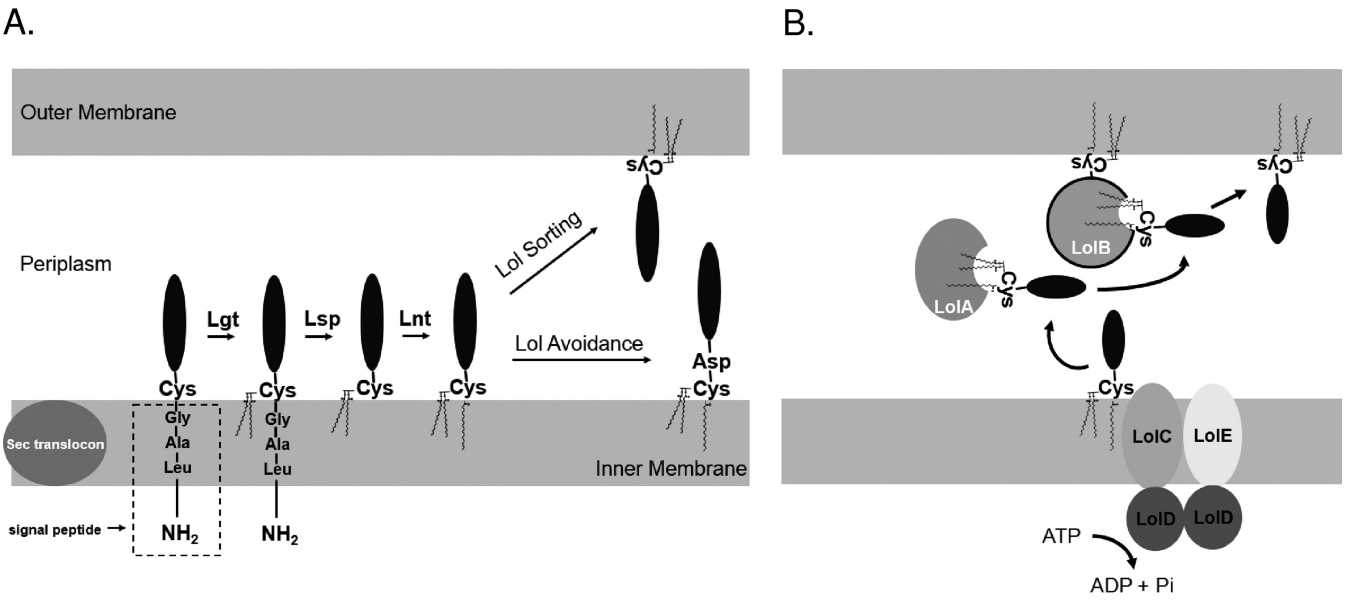

FIG 1 General lipoprotein processing (A) and sorting pathway (B) in Gram-negative bacteria (4). All steps in the processing pathway are considered essential to Gram-negative bacteria. In *E. coli*, the Asp at position +2 is an Lol avoidance signal that prevents sorting of lipoproteins to the OM. Most lipoproteins are sorted by the Lol system to the OM by utilizing the LolCDE ABC transporter, the periplasmic protein LolA, and the OM lipoprotein LolB, all of which are essential proteins.

of the OM and face the extracellular milieu (12, 13). An exception to this is found with the spirochete *Borrelia burgdorferi*, in which the majority of OM lipoproteins are surface exposed (14).

Gram-positive bacteria were previously considered to have a lipoprotein processing pathway that is similar to that of Gram-negative bacteria, except that Gram-positive bacteria lack the Lnt enzyme and, thus, only produce diacylated lipoproteins (4). However, this paradigm has recently shifted with the discoveries that mycobacteria and staphylococci possess apolipoprotein *N*-acyltransferase activity and can produce triacylated lipoproteins (15–19). A recurring theme in studies of lipoprotein physiology is that processing is not essential in Gram-positive bacteria but that processing and sorting are essential in enteric bacteria and are generally thought to be essential in other Gram-negative bacteria (4).

In this study, we report the novel finding that the lipoprotein processing gene *lnt*, encoding apolipoprotein N-acyltransferase, is not essential in the Gram-negative bacteria *F. tularensis* subsp. *tularensis* strain Schu, the causative agent of tularemia, and the *F. tularensis* subsp. *holarctica* live vaccine strain LVS. Furthermore, we found that the loss of Lnt has little effect on cellular physiology under laboratory conditions. We developed a model that posits that a difference between the organization of the *F. tularensis* and *E. coli* Lol sorting systems may be responsible for the difference in lipoprotein processing essentiality between these two organisms. We tested this hypothesis by successfully deleting *lnt* in *Neisseria gonorrhoeae*, which has the same Lol sorting system organization as *F. tularensis*. Our results suggest that lipoprotein maturation and sorting in Gram-negative bacteria may be more flexible than previously appreciated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strain DH10B was used as the cloning strain for all plasmid constructions and was grown in Luria-Bertani (LB) broth (BD Biosciences) or on LB agar, which is LB broth and 1.5% (wt/vol) Bacto agar (BD Biosciences). *F. tularensis* strains were grown in modified Mueller-Hinton (MMH) broth, which is Mueller-Hinton broth (BD Biosciences) supplemented with 0.5% (wt/vol) NaCl, 1.0% (wt/vol) glucose, 0.025% (wt/vol) ferric pyrophosphate, and 250 μg/ml of L-cysteine, or on MMH agar, which is MMH broth containing 1% (wt/vol) tryptone, 2.5% (vol/vol) defibrinated sheep's blood, and 1.5% (wt/vol) Bacto agar. For MMH broth, L-cysteine was added just prior to inoculation using L-cysteine (free base) stocks prepared at 250 mg/ml and stored frozen at −20°C, while for MMH agar, L-cysteine (free base) was added directly to the medium prior to autoclaving. *Neisseria gonorrhoeae* strains were grown on Difco GC medium base (BD Biosciences) agar with 1% (vol/vol) Kellogg's supplement (GCK). The β-galactosidase substrate 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal; Fermentas) was used at 20 μg/ml in MMH agar. The antibiotic

TABLE 1 Bacterial strains used in this study

Strain	Description	Source or reference
E. coli DH10B	F⁻ *mcrA* Δ(*mcrBC-hsdRMS-mrr*) [ϕ80d*lacZ*ΔM15] Δ*lacX74* *deoR recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK* λ⁻ *rpsL nupG*	Invitrogen
F. tularensis strains		
LVS	*F. tularensis* subsp. *holarctica* live vaccine strain	J. Benach
Schu	*F. tularensis* subsp. *tularensis*	M. Shriefer
PM2006	LVS Δ*lnt*	This work
PM2012	Schu Δ*lnt*	This work
N. gonorrhoeae strains		
F62	Pro⁻	V. Clark
PM2475	F62 Δ*lnt*::*aphA1*	This work

TABLE 2 Plasmids used in this study

Plasmid	Description	Source or reference
pMP716	Km^r, *E. coli-F. tularensis* shuttle vector, higher copy no.	35
pMP780	Km^r, *sacB* suicide vector derived from pMP590 with P_{dnak}-*sacB*	58
pMP789	Km^r, pMP780 suicide vector bearing Δ*lnt* allele	This work
pMP790	Km^r, *blaB* integration vector based on pMP741 with P_{rpsL}-*lacZ*	58
pMP812	Km^r, 192 bp smaller, improved *sacB* suicide vector derived from pMP780	58
pMP822	Hyg^r, *E. coli-F. tularensis* shuttle vector stable with P_{blaB}-*mcs* (multiple-cloning site)	35
pMP823	Hyg^r, *E. coli-F. tularensis* shuttle vector unstable with P_{blaB}-*mcs*	35
pMP874	Hyg^r, pMP822 stable P_{blaB}-*lnt*$^+$	This work
pMP882	Km^r, pMP716 higher copy no., P_{blaB}-*lsp*$^+$	This work
pMP872	Km^r, *E. coli* vector encoding full-length LpnA with C-terminal c-Myc tag	This work
pMP902	Km^r, pMP812 suicide vector bearing Δ*lolCD* allele	This work
pMP942	Km^r, pMP812 suicide vector bearing Δ*bamD* allele	This work
pMP985	Hyg^r, pMP823 *E. coli-F. tularensis* shuttle vector bearing P_{rpsL}-*lacZ* from pMP790	This work
pMP992	Hyg^r, pMP985 unstable P_{blaB}-*lolCD*$^+$	This work
pMP1003	Hyg^r, pMP985 unstable P_{blaB}-*bamD*$^+$	This work
pMP1181	Km^r, *E. coli-F. tularensis* shuttle vector, higher copy no., P_{rpsL}-Δ$lpnA_{102}$ ("short" c-Myc-tagged reporter)	

kanamycin was used at 50 μg/ml (*E. coli* and *N. gonorrhoeae*) or 5 μg/ml (*Francisella*), while hygromycin was used at 200 μg/ml (*E. coli* and *Francisella*). All cultures were grown at 37°C. *N. gonorrhoeae* cultures were grown in the presence of 5% CO_2.

Plasmids and DNA methods. DNA manipulations for plasmid construction were performed as previously described (20). DNA fragments were isolated using agarose gel electrophoresis and QIAquick spin columns (Qiagen Inc.). Oligonucleotides were synthesized by Invitrogen Life Technologies. All restriction endonucleases and DNA-modifying or polymerase enzymes were from New England BioLabs or Fermentas. PCRs were performed with Iproof high-fidelity DNA polymerase (Bio-Rad) according to the manufacturer's recommendations. All plasmids used in this study (Table 2) were from the laboratory collection and were prepared from *E. coli* using Qiagen columns (Qiagen Inc.). Preparation of genomic DNA from *F. tularensis* was done as previously reported (20). *N. gonorrhoeae* genomic DNA was prepared by using a Qiaprep miniprep kit (Qiagen Inc.) as recommended by the manufacturer, but after addition of buffer P2 the solution was mixed by using a high-speed vortexer prior to addition of buffer P3. Electroporation of *Francisella* bacteria was performed as previously described (20). Detailed descriptions of the plasmids constructed for this work, other plasmids, and bacterial strains can be obtained from the corresponding author.

Mutant construction. Deletion of the *lnt* gene in *Francisella tularensis* subsp. *tularensis* strains Schu and LVS was done using *sacB* counterselection (20) and the suicide plasmid pMP789, which contains an in-frame deletion of the *lnt* open reading frame along with approximately 1 kb of DNA flanking each side of the deletion. The *lnt* deletion mutant of *N. gonorrhoeae* was constructed by replacing the *lnt* coding region, between the start and stop codon, with an *aphA1* resistance cassette. Two DNA fragments flanking *lnt* were amplified from *N. gonorrhoeae* F62 genomic DNA using PCR: the 5′ fragment included 1 kb of DNA upstream from the *lnt* start site and an XhoI restriction site on the 3′ end. The second fragment had a HindIII restriction site on the 5′ end and included 1 kb of DNA downstream of the *lnt* stop codon. The two fragments were ligated with a complementarily digested *aphA1* resistance cassette, and the resulting construct was used to transform *N. gonorrhoeae* F62 by a previously reported method (21). A second, independent mutant was made for verification purposes by utilizing this PCR product for allelic exchange using another culture of F62. All mutants were verified by PCR.

Construction of the Tul4A (LpnA) reporter. The "short" Tul4A reporter lipoprotein gene (Δ$lpnA_{102}$) was constructed by inverse PCR, using the plasmid pMP872, which carries the gene for a full-length version of LpnA with a C-terminal polyhistidine tag and a c-Myc epitope. pMP872 was used as a template in an inverse PCR, and this resulted in an internal, in-frame deletion with the coding region of *lpnA*, beyond the lipobox, which reduced the size of the translated product from 176 amino acids to 121 amino acids, prior to processing by Lsp. After processing, this "short" LpnA reporter is predicted to be only 102 amino acids. The shortened Δ$lpnA_{102}$ allele was then cloned into a derivative of the high-copy-number *E. coli-Francisella* plasmid pMP716 bearing the *Francisella rpsL* promoter, resulting in pMP1181.

Susceptibility assays. For susceptibility testing of *F. tularensis*, 3-ml cultures of either wild-type (WT) LVS or the Δ*lnt* mutant were grown to an optical density at 600 nm (OD_{600}) of ~1.0; for *N. gonorrhoeae*, cells were grown overnight on GCK agar, scraped, and resuspended in GCK broth and normalized to an OD_{600} of ~1.0. The bacteria were then spread with a sterile cotton swab on measured agar plates to obtain a lawn. Either prepared Sensi-Discs (BD Biosciences) or compound-soaked sterile discs (BD Biosciences), as indicated below, were placed on the plates. After 2 days of incubation for *Francisella* or 1 day for *Neisseria*, the inhibition zones were measured to the nearest millimeter. These determinations were performed in triplicate for each strain and drug, and results are presented as averages ± the standard deviations. Data were analyzed using an unpaired *t* test with GraphPad software (La Jolla, CA).

Intracellular survival assays. The human monocyte cell line THP-1 (a generous gift from M. Wellington, University of Rochester) grown in RPMI with 10% fetal bovine serum at 37°C in 5% CO_2 was treated with 100 ng/ml phorbol myristate acetate (PMA; Invivogen) for 3 days prior to survival assays, to allow for differentiation, and then seeded in 12-well plates at a population of 1×10^6 cells per well. Prior to infection, the wells were washed with 1× phosphate-buffered saline (PBS), and fresh medium was added. To each well, a suspension of bacteria at a multiplicity of infection (MOI) of 100 was added, and bacterial uptake was allowed to occur for 4 h. Afterwards, the wells were washed twice to remove any free bacteria, and then fresh medium was added that contained 25 μg/ml gentamicin. After 6 or 24 h, the wells were washed twice and the THP-1 cells were lysed by the addition of 0.5 ml distilled water and agitation by pipetting. After addition of 0.5 ml 2× PBS, 10 μl of each lysate, serially diluted in PBS, was then spotted onto MMH agar for determinations of viable counts. These assays were performed in triplicate, and results are reported as averages ± standard deviations. Data were analyzed for each time point by using an unpaired *t* test with GraphPad software (La Jolla, CA).

Cell fractionation and immunoblotting. LVS wild-type and Δ*lnt* strains were fractionated by sucrose gradient ultracentrifugation as previously described (22). Gradient fractions were separated by SDS-PAGE and subjected to immunoblotting using rabbit antibodies against the IM protein SecY, the OM protein FopA, and two OM lipoproteins, LpnA (Tul4A) and LpnB (Tul4B), as previously described (22).

LVS strains bearing the "short" Δ$lpnA_{102}$ reporter gene were grown to saturation in MMH broth. One milliliter of cell suspension was pelleted and resuspended in 100 μl of Laemmli loading buffer with 5% (vol/vol) β-mercaptoethanol (23) and boiled for 5 min. Twenty microliters of each sample was loaded per well and then separated on a 12% SDS-PAGE denaturing gel and immunoblotted using a polyvinylidene difluoride membrane. The blot was probed with mouse anti-cMyc antibody at a

1:5,000 dilution (Invitrogen). Detection was done using rabbit anti-mouse antibody conjugated to horseradish peroxidase at a dilution of 1:5,000, followed by incubation with the Novex enhanced chemiluminescence substrate (Life Technologies).

RESULTS

The *lnt* gene is not essential to *Francisella*. We inspected a transposon saturation mutagenesis library (24) of *Francisella novicida*, which was designed to identify essential genes, for hits for all the genes encoding the lipoprotein processing and sorting enzymes, and we found that there were no mutants in the library with inserts in the processing genes *lgt* and *lsp* or in any of the *lol* genes, suggesting that these genes are essential. However, the *F. novicida* library contained two mutants with different insertions in the *lnt* gene, suggesting that the gene is not essential to this strain. To test the essentiality of the gene in *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, we performed a standard two-step, *sacB*-mediated allelic exchange with the suicide plasmid pMP789 (20), bearing an unmarked, in-frame deletion of *lnt*, in strains Schu and LVS. Kanamycin-resistant (Km^r), sucrose-sensitive primary recombinants resulting from a single-crossover event were readily obtained, and sucrose-resistant secondary recombinants were subsequently obtained at the expected frequency of $\sim 10^{-3}$. For both strains, we observed that ~50% of the sucrose-resistant recombinants had a small-colony phenotype, and this morphotype was shown to be mutant by PCR (see Fig. S1A in the supplemental material). The small-colony phenotype was only seen on medium containing sucrose. This phenotype was not due to acid production from sucrose fermentation, since the mutant does not have an acid-sensitive phenotype (data not shown). The Δ*lnt* mutants grow similarly to the wild type in liquid media and solid media (data not shown). The LVS Δ*lnt* mutant is strain PM2006, while the corresponding Schu mutant is PM2012.

Tul4A (LpnA) is not fully mature in the Δ*lnt* mutant. The ability to delete *lnt* was surprising, suggesting that sorting of immature diacylated lipoproteins to the OM can occur in *Francisella*. Alternatively, the *lnt* mutant may be viable, because lipoproteins were still triacylated, the result of a second, unidentified apolipoprotein's N-acyltransferase activity that is capable of suppressing the Δ*lnt* defect. To test the latter possibility, we constructed a lipoprotein reporter to allow us to infer the acylation state of the protein in the wild type or the Δ*lnt* mutant. This approach was based upon a recent study that used a tagged reporter lipoprotein that was sufficiently small such that the diacylated and triacylated species could be distinguished from each other after separation by SDS-PAGE followed by Western blotting (17). To do the same kind of analysis with *Francisell*a, we chose to use the well-studied OM lipoprotein Tul4A (LpnA) (25, 26), which is synthesized as a preprolipoprotein of 155 amino acids and processed by a prolipoprotein signal peptidase to 136 amino acids. We constructed a tagged version of LpnA with a C-terminal polyhistidine tag and c-Myc epitope, totaling 176 amino acids in length and processed to 157 amino acids in the mature form. Unfortunately, this construct was too large for our purposes, and so we engineered a "short" derivative with an internal deletion that removed 55 amino acids, resulting in the Δ$lpnA_{102}$ allele, which encoded a tagged preprolipoprotein of 121 amino acids that would ultimately be cleaved to a 102-amino-acid lipoprotein. This allele was cloned into the high-copy-number vector pMP716, under the control of the *Francisella rpsL* promoter, and introduced into wild-type LVS and the LVS Δ*lnt* mutant. Extracts from two independent transformants for each strain were separated by SDS-PAGE and immunoblotted with anti-cMyc antibody. As shown in Fig. 2, the mobility of the reporter protein was faster in the Δ*lnt* transformants (lanes 4 and 6) than in the wild-type transformants (lanes 3 and 5). The smaller size of the reporter in the Δ*lnt* mutant was consistent with a change in acylation state at the N terminus, since the reporter is tagged at the C terminus. A C-terminal cleavage event would result in loss of detection by Western blotting. Given that such increased mobility has been shown by mass spectrometry to be the result of incomplete acylation (17), it appears that LpnA is not triacylated in the Δ*lnt* mutant but is diacylated, as expected.

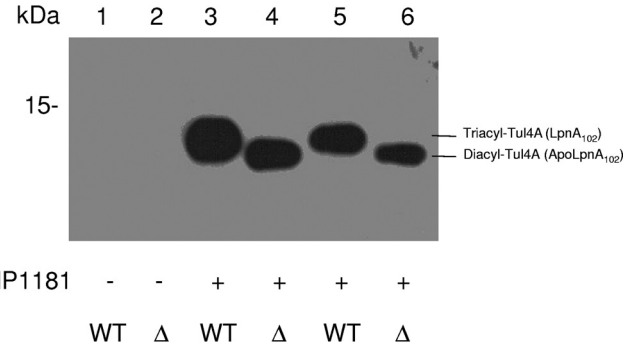

FIG 2 Tul4A ($LpnA_{102}$) acylation Western blotting results for the wild-type LVS (lanes 1, 3, and 5) and the Δ*lnt* strain (Δ; lanes 2, 4, and 6). Lanes 1 and 2 contain lysates from negative controls lacking the $lpnA_{102}$ reporter plasmid, while lanes 3, 4, 5, and 6 contain lysates from strains bearing the plasmid pMP1181 which harbors the $lnpA_{102}$ gene. Lanes 3 and 5 represent two LVS transformants, and lanes 4 and 6 represent two Δ*lnt* transformants. The blot was probed with mouse anti-c-Myc antibody.

Most of the *Francisella* lipoproteins are OM proteins. Since we were able to rule out suppression of the Δ*lnt* defect, we next examined the possibility that *Francisella* does not require the sorting of lipoproteins to the OM. The major lipoprotein in *E. coli* is Braun's lipoprotein (encoded by *lpp*), which has roles in stabilizing the cell wall and OM (27). While *F. tularensis* lacks an *lpp* homolog, there are homologs of several other *E. coli* OM lipoproteins (e.g., Pal, LolB, Blc, and BamD) (28–32) encoded in the genome (33). We extended our analysis of the *F. tularensis* Schu S4 genome annotation and found 69 putative lipoproteins (see Table S1 in the supplemental material). Because the default localization of Lpps in *E. coli* is the OM unless there is an "Lol avoidance" signal at +2 or beyond in the mature Lpp, we reasoned it would be possible to infer the localization of these lipoproteins, even if the actual Lol avoidance sequence for *Francisella* were unknown, by comparing all the Lpps to those known to be located in the OM. The *Francisella* OM lipoproteins Blc, LolB, Pal, and BamD, which are shared with other Gram-negative bacteria, have valine, alanine, serine, and glycine at position +2, while the *Francisella*-specific OM lipoproteins Tul4A (LpnA) and Tul4B (LpnB) both have a serine at position +2 and the IglE proteins have an isoleucine at +2; thus, any potential Lol avoidance signal in *Francisella* should not have a valine, alanine, serine, glycine, or isoleucine at the +2 position. The only inner membrane lipoprotein in *E. coli* that is present in *Francisella* is the subunit II of ubiquinol oxidase (CyoA), but the *E. coli* protein does not have an Lol avoidance signal and is localized to the IM by two transmembrane domains (34). The *Francisella* CyoA protein has a lysine at postion +2 and

is predicted to have two transmembrane domains, similar to that of *E. coli*. There are no other lipoproteins in *Francisella* with a lysine at position +2. By this reasoning, of the 69 putative lipoproteins in the SchuS4 genome, 52 can be predicted to be sorted to the OM by their +2 amino acid being the same as the known OM Lpps described above. Thus, most of the Lpps in *Francisella* are probably located in the OM and, therefore, one would expect that the immature Lpps would still need to be sorted to the OM in the Δ*lnt* mutant. Alternatively, there may be no requirement to sort, since there may be no essential functions carried out by OM lipoproteins in *Francisella*. We think the latter case is unlikely, given that in other bacteria, some OM lipoproteins (BamD and LptE) are essential for OM biogenesis as well as the sorting machinery: LolCDE, LolA, and the OM lipoprotein LolB. Furthermore, all these genes appear to be essential to *F. novicida*, since no transposon mutants with insertions in these genes are present in the transposon library mentioned above (24). However, we decided to formally confirm this by performing an essentiality test of LolCD, which comprise part of the ABC transporter in the Lol sorting system, and BamD, which is involved with the insertion of proteins into the OM.

LolCD and BamD are essential in LVS. We used a simple plasmid stability-based assay to test the essentiality of *lolCD* and *bamD* in LVS. First, we performed a standard two-step allelic exchange with a *sacB* suicide plasmid bearing an unmarked, in-frame deletion of *lolCD* (pMP902) or *bamD* (pMP942). After recovering kanamycin-resistant, sucrose-sensitive primary recombinants that carried both a wild-type and deletion allele for each gene, we transformed each primary recombinant with an unstable hygromycin resistance vector (35) carrying either a copy of the wild-type *lolCD* (pMP992) or *bamD* (pMP1003) genes. These plasmids also contained *lacZ*, encoding β-galactosidase, driven by the *Francisella rpsL* promoter. The primary recombinant strains were allowed to undergo secondary recombination, with antibiotic selection maintained for the complementing plasmid. We screened the sucrose-resistant, kanamycin-sensitive secondary recombinant population by PCR and selected a wild-type clone and a deletion clone for each gene. These strains were then grown in the absence of hygromycin selection overnight, then subcultured 1:10, grown for an additional 24 h, and then plated on MMH containing 20 μg ml^{-1} X-Gal. The reaction product of X-Gal cleavage by β-galactosidase decreases *F. tularensis* colony growth (36). Thus, on a plate containing X-Gal, a clone carrying a complementing plasmid appears as a small blue colony after 2 days, whereas a clone lacking the complementing plasmid is normal sized and white. We used this phenomenon to easily check for loss of plasmid in the population, which was confirmed by picking and patching a subset to check for loss of hygromycin resistance carried on the complementing plasmid. The rationale here is that the complementing plasmids are unstable in the absence of antibiotic selection and thus would be lost from the wild-type population, but if the genes were essential, this selective pressure would ensure that the unstable complementing plasmids were maintained in the mutant population in the absence of antibiotic selection. We found that 100% of the colonies in the Δ*lolCD* and Δ*bamD* strains retained the complementing plasmids, while only 71% ±7% and 6% ± 4% (standard deviations from the averages of triplicate determinations) of the wild-type *lolCD* and *bamD* strains, respectively, maintained the complementing plasmids.

Lipoproteins Tul4A (LpnA) and Tul4B (LpnB) still sort to the OM in the Δ*lnt* mutant. Since the Δ*lnt* mutant appears to produce immature lipoprotein, yet must be able to sort lipoproteins to the OM, we next determined the location of the two OM lipoproteins Tul4A (LpnA) and Tul4B (LpnB). We fractionated wild-type LVS and the Δ*lnt* mutant by using a sucrose gradient ultracentrifugation protocol specifically developed for the separation of the IM and OM of *Francisella* spp. (22). Fractions across the gradient were separated by SDS-PAGE and immunoblotted using a panel of antibodies against Tul4A and Tul4B, the OM protein FopA, and the IM protein SecY. As shown in Fig. 3, the Tul4A and Tul4B lipoproteins localized to the same three OM fractions, spanning densities of 1.18 to 1.16 g/ml, in both the wild type and the Δ*lnt* mutant. The distribution of the lipoproteins (and FopA) was skewed to the 1.18-g/ml fraction in the mutant, whereas in the wild type these proteins were evenly distributed across the three fractions.

Loss of Lnt has little effect on cell envelope integrity. Of the various functions of lipoproteins, one of the most important is stabilization and integration of components in the OM. A group of OM lipoproteins that function in this capacity are BamB (YfgL), BamD, LptE (RlpB), and Pal, all of which are encoded in the *F. tularensis* genome (37). To explore the possibility of a defective OM in the absence of fully mature lipoprotein, we tested the sensitivity of the LVS Δ*lnt* mutant to a variety of chemical agents by using a disc-diffusion assay. There was little change in the susceptibilities of the mutant to these challenges, with the exception of globomycin and rifampin (Table 3). The complemented strain PM2006/pMP874, which has lnt^{+} expression driven by the *Francisella blaB* promoter on a multicopy vector, showed decreased susceptibility to globomycin compared to the parental LVS. In addition, wild-type LVS containing an extra copy of *lsp* on plasmid pMP882 also had decreased susceptibility to globomycin.

The LVS Δ*lnt* mutant can survive and replicate intracellularly. Since *Francisella* spp. are facultative intracellular bacteria and lipoproteins have been shown to play a role in tularemia pathogenesis (11, 38, 39), we wanted to examine whether the loss of *lnt* had an effect on intracellular survival. Human monocyte THP-1 cells were treated with PMA and allowed to differentiate into macrophage-like cells for 3 days. We infected the cells at an MOI of 100 with either WT LVS or the Δ*lnt* mutant, allowed bacterial uptake to take place for 4 h, and then treated the cells with gentamicin to eliminate any extracellular bacteria. We then lysed the THP-1 cells and plated at two time points, 6 h and 24 h postinfection. As shown in Table 4, there were no differences in uptake or replication between the two strains, indicating no intracellular growth defect for the Δ*lnt* mutant.

Essentiality of Lnt in other Gram-negative species. We wanted to understand whether the ability to delete *lnt* in *Francisella* was unique. We found one report of a Tn*5* insertion in the *actA* gene of the Gram-negative bacterium *Sinorhizobium meliloti*, and a BLAST analysis of the amino acid sequence indicated that ActA is homologous to Lnt (40). Recently, it was found that the genome of the Gram-negative *Wolbachia* endosymbiont of the *Brugia malayi* nematode lacks an *lnt* gene and appears to produce only diacylated lipoproteins (41). Based on these observations, we then compared the Lol sorting pathways of *F. tularensis*, *S. meliloti*, and *Wolbachia* sp. with those of *E. coli* and *Salmonella enterica* serovar Typhimurium. One major difference that we noticed was that in *E. coli* and serovar Typhimurium, the ATP-binding cassette transporter genes encode two different membrane components,

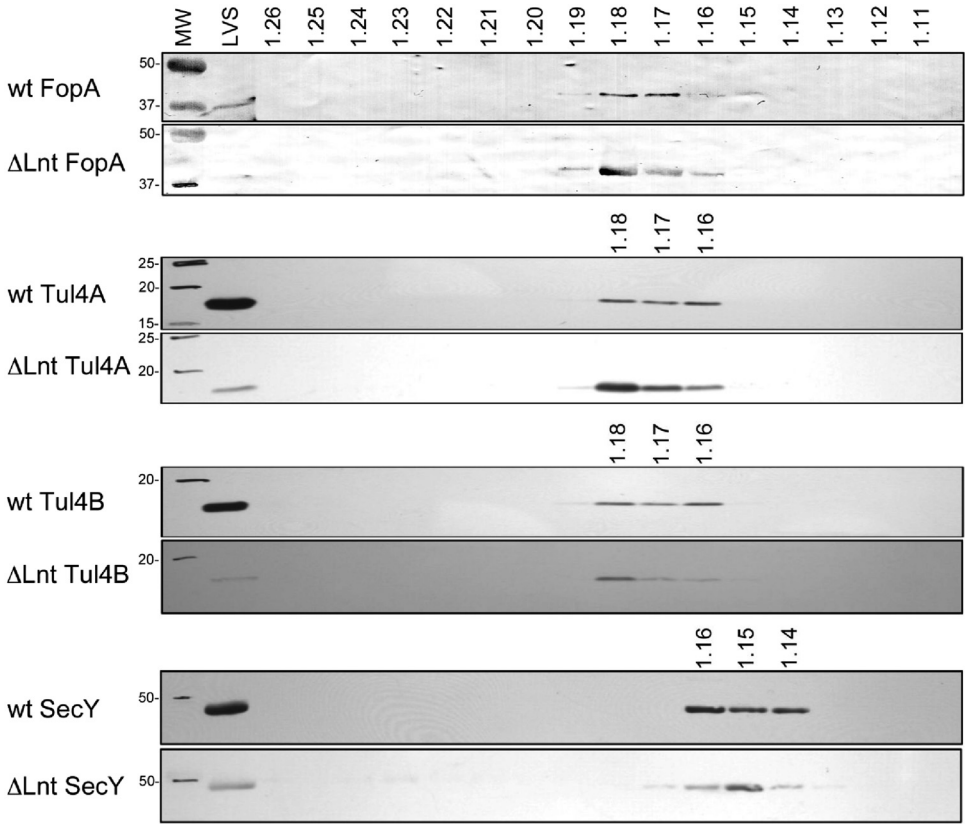

FIG 3 Immunoblotting of *F. tularensis* LVS wild-type and Δ*lnt* sucrose density gradients. Sequential fractions were collected from gradients and densities (in grams per milliliter) were calculated based upon refractive indices and are noted above their respective lanes. Proteins were separated by SDS-PAGE and immunoblotted to detect proteins in OM protein and IM fractions. FopA (OM control), Tul4A (LpnA), and Tul4B (LpnB) (OM lipoproteins), as well as SecY (IM control), were detected with polyclonal, monospecific antisera, as noted in the left margin. MW, prestained molecular mass standards, with sizes (in kilodaltons) noted on the left side of each blot.

LolC and LolE, while *F. tularensis*, *S. meliloti*, and *Wolbachia* sp. apparently lack an *lolE* gene and most likely utilize a homodimer of LolC as the membrane component. However, it is clear from comparing membrane subunits based on amino acid homology that it is impossible to clearly state that a membrane subunit is either LolC or LolE. In *E. coli*, the ABC transporter is encoded by an operon with an *lolCDE* gene order, and so for organisms lacking the third gene, the remaining genes are often annotated *lolCD* by virtue of operon organization. In many genomes, however, the single membrane component gene is annotated *lolC*/*lolE*. Clearly, for organisms lacking both subunits, the remaining membrane protein subunit must act as a homodimer in the ABC transporter and thus may function differently than a transporter with both LolC and LolE. We also looked at a range of Gram-negative genomes to identify whether *Francisella*, *Sinorhizobium*, and *Wolbachia* species were unique in only having one subunit (Table 5), and we found that the LolCD arrangement was present in all members of the alpha- and betaproteobacteria, with a mix found among the gamma-, delta-, and epsilonproteobacteria (see Tables S2 and S3 in the supplemental material).

The *E. coli* LolC and LolE proteins have been the most extensively studied and have similar membrane topologies, based on PhoA fusion analysis (Fig. 4) (42). Both proteins have 4 transmembrane helices each, and LolE has a region between transmembrane helices 3 and 4 that dips slightly back into the cytoplasmic membrane. We performed amino acid alignments of LolC, LolE, and the single LolC/LolE protein of many bacterial species representing a range of proteobacteria by using Clustal W (43) (see

TABLE 3 Drug susceptibilities of LVS, PM2006 (Δ*lnt*), and complemented strains

Drug[a]	Dose (μg/disc)	Zone of inhibition (mm)[b]				
		LVS	PM2006	PM2006/pMP822	PM2006/pMP874	LVS/pMP882
Globomycin	150	20 ± 1	24 ± 1[#]	25 ± 0	16 ± 1	15 ± 0
Rifampin	5	28 ± 2	36 ± 1[##]	38 ± 2	27 ± 1	ND

[a] Other compounds tested that did show any difference between LVS and PM2006 strain included the following (with dose in micrograms per disc in parentheses): tetracycline (30), vancomycin (30), sodium dodecyl sulfate (750), polymyxin B (300 IU), ethidium bromide (5), amoxicillin-clavulanic acid (30/10).
[b] The average diameter of the zone of inhibition (including filter disc) in millimeters, ± the standard deviation. ND, not determined. Statistical significance compared to result with LVS: #, $P < 0.008$; ##, $P < 0.003$. pMP822, vector control; pMP874, *lnt*$^+$; pMP882, *lsp*$^+$.

TABLE 4 Survival and replication of the Δ*lnt* mutant in THP-1 cells

Strain	THP-1 cells (CFU/ml) after[a]:	
	6 h	24 h
LVS	$6.0 \times 10^3 \pm 0$	$4.7 \times 10^5 \pm 0.6 \times 10^5$
PM2006 Δ*lnt*	$5.3 \times 10^3 \pm 1.1 \times 10^3$*	$4.0 \times 10^5 \pm 2.0 \times 10^5$**

[a] Each experiment was performed in triplicate, and the results are averages ± standard deviations. Compared to the wild type, LVS, results with the *lnt* mutant showed no significant differences: *, $P < 0.332$; **, $P < 0.592$.

Table S2 in the supplemental material), and we discovered some sequence motifs that can be used to differentiate LolC from LolE and provide some insight into the relationship of these proteins to the LolC/LolE found in *Francisella*. From this analysis, it is clear that LolC has a C-terminal signature consisting of RYE, located at the end of the protein, which is replaced with SGQ (or SSH/K) in LolE proteins (see Fig. S2 in the supplemental material). In LolE, we found a 16-amino-acid signature that is proximal to the region that dips back into the membrane between transmembrane helices 3 and 4 and is present in all LolE proteins but absent in LolC proteins (see Fig. S3 in the supplemental material). Notably, the *Francisella* LolC/E protein has signatures from both LolC (the C-terminal RYE sequence) and LolE (the 16-amino-acid sequence between TM3 and TM4). The results of a comparison of these signatures in the LolC and LolE proteins in *E. coli* with that of the LolC/LolE protein of *Francisella tularensis* are shown in Fig. 4. This analysis suggested that the membrane component of the Lol system in *Francisella*, and other genera with the same organization, is neither LolC nor LolE but is a hybrid protein, which we propose should be named LolF. We hypothesize that for bacteria that only have LolF, the *lnt* gene is not essential, because the LolFD machinery can recognize diacylated lipoproteins for sorting to the OM.

We examined this hypothesis by attempting to delete *lnt* in *N. gonorrhoeae*, which has the same LolFD organization as *Francisella* (Table 5; see also Fig. S2 and S3 in the supplemental material). We transformed independent cultures of the gonococcal strain F62 with a Δ*lnt* allele bearing a kanamycin resistance marker and successfully isolated Δ*lnt*::*aphA1* mutants (see Fig. S1B in the supplemental material). This confirmed our hypothesis that *lnt* would not be essential; however, it did not indicate that lipoproteins were sorting to the OM. We selected one F62 Δ*lnt*::*aphA1* mutant (PM2475) for further study. *N. gonorrhoeae* can grow anaerobically in the presence of nitrite and relies on an OM lipoprotein, AniA, for nitrite reductase activity (44). We saw no defects in its ability to grow anaerobically (data not shown). We then performed sensitivity assays on PM2475 and the parental strain F62, and we saw increases in susceptibilities to globomycin and rifampin that were similar to that seen with the LVS Δ*lnt* mutant (Table 6). We also saw small increases in susceptibility to vancomycin and polymyxin B, which were not seen in the LVS mutant (Table 6).

DISCUSSION

We have demonstrated that the final step in lipoprotein processing is not required for viability for *F. tularensis* strains Schu and LVS or for *N. gonorrhoeae* F62. The ability to delete the *lnt* gene in these species was surprising, since the full lipoprotein processing pathway has been considered essential for Gram-negative bacteria

TABLE 5 Lol ABC transporter organization in Gram-negative bacteria

Proteobacteria division and species	Presence of gene in species[a]		
	lolC	*lolD*	*lolE*
Alphaproteobacteria			
Brucella suis	→	→	–
Sinorhizobium meliloti	→	→	–
Rickettsia rickettsii	→	→	–
Wolbachia sp.	→	→	–
Betaproteobacteria			
Bordetella pertussis	→	→	–
Burkholderia mallei	→	→	–
Neisseria gonorrhoeae	→	→	–
Gammaproteobacteria			
Escherichia coli	→	→	→
Salmonella enterica serovar Typhimurium	→	→	→
Yersinia pestis	→	→	→
Pseudomonas aeruginosa	→	→	→
Coxiella burnettii	→	→	–
Francisella tularensis	→	→	–
Legionella pneumophila	→	→	–
Vibrio cholerae	→	→	→
Acinetobacter baumannii	→	→	–

[a] Arrows indicate directions of genes, and minus signs indicate missing genes.

(4). In *E. coli*, loss of the Lnt enzyme results in incomplete maturation of lipoproteins and subsequent retention in the cytoplasmic membrane of apolipoproteins normally destined for the OM (45). Experiments using a conditionally lethal *E. coli lnt* mutant showed that loss of Lnt leads to IM retention of Braun's lipoprotein which, when cross-linked to the peptidoglycan (PGN), causes cell lysis (45). This observation helps explain earlier research findings that showed that disruption of the *lpp* gene encoding Braun's lipoprotein suppressed a temperature-sensitive mutation in either the *lnt* or *lgt* gene of *Salmonella enterica* and that mutation of *lpp* also protected *E. coli* from globomycin, an inhibitor of lipoprotein processing (46, 47). Furthermore, the conditional *E. coli lnt* mutant can be partially protected via deletion of the *lpp* gene or by mutation of *lpp* to a form a protein that cannot be cross-linked to the PGN (45). A null mutant of *lnt* cannot be rescued by mutation of Braun's lipoprotein because of the mislocalization of essential OM lipoproteins to the IM. The *F. tularensis* genome lacks a homolog of Braun's lipoprotein, but this loss is not the reason why Lnt is dispensable in this organism. Our results are consistent with the view that the ability to delete *lnt* in *Francisella* is not due to an alternative Lnt activity or that lipoprotein sorting to the OM is dispensable, since we demonstrated that our reporter lipoprotein Tul4A (LpnA) appeared to be diacylated and lipoproteins were still sorted to the OM. Thus, the variability in Lnt essentiality among Gram-negative bacteria is probably a function of the sorting apparatus.

In *E. coli*, the key to sorting to the OM is recognition of the mature lipoproteins by the LolCDE system, with subsequent transfer to the LolA and LolB proteins (48). It was recently shown that deletion of *lnt* is possible in *E. coli* if the *lolCDE* genes are overexpressed, but only in the absence of Braun's lipoprotein (49). This indicates that immature lipoproteins are still present in the IM of the mutant, but enough of the essential proteins get shuttled

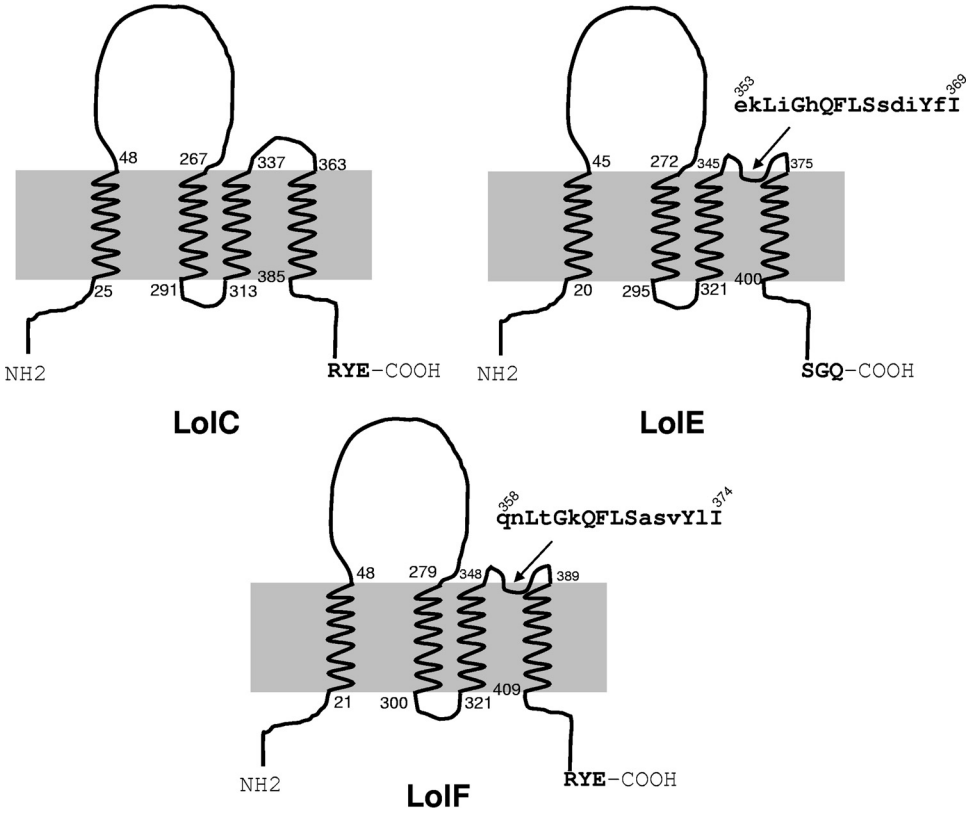

FIG 4 Lol membrane component comparison. The topologies of the *E. coli* LolC and LolE proteins are shown and are based upon the work of Yasuda et al. (42), along with the putative topology of the LolF protein of *F. tularensis*, compared to LolC and LolE and predicted by TMHMM (57). The motifs within the proteins are shown in bold type. The homologous residues of the LolE signature between the *E. coli* LolE protein and the *Francisella* LolF protein are shown in capitalized letters.

to the OM by the overexpressed ABC transporter to maintain viability. These results suggest that the *E. coli* LolCDE ABC transporter has a higher affinity for mature triacylated lipoproteins than for immature diacylated lipoproteins. This idea helps explain our observations that *Francisella* and *Neisseria* have different Lol ABC transporter architectures and how this could allow for loss of Lnt. We propose that the LolFD transporter may have a looser specificity for acyl chains and can recognize both with almost equal affinity. There is the possibility that differences between the LolB and LolA proteins of *E. coli* versus *Francisella* may also be important, but we favor the LolFD hypothesis due to the altered ABC arrangement and the hybrid nature of LolF. These ideas could be tested by performing lipoprotein release assays using reconstituted membranes and by cross-complementation studies with *E. coli* Lol mutants using the corresponding genes from *Francisella* or *Neisseria*.

TABLE 6 Drug susceptibilities of *N. gonorrhoeae* F62 and the Δ*lnt* mutant PM2475

Drug[a]	Dose (disc^{-1})	Zone of inhibition (mm)[b]	
		F62	PM2475
Globomycin	150 μg	24 ± 0	29 ± 0
Rifampin	5 μg	30 ± 1	34 ± 1#
Polymyxin B	300 IU	9 ± 0	12 ± 0##
Vancomycin	30 μg	14 ± 1	17 ± 1##

[a] Compounds showing no difference between F62 and PM2475 included the following (with the dose, in micrograms per disc, in parentheses): tetracycline (30), sodium dodecyl sulfate (750), ethidium bromide (5), amoxicillin-clavulanic acid (30/10).
[b] Average diameter of the zone of inhibition (including the filter disc) ± the standard deviation. Significance of difference from result with F62: #, $P < 0.008$; ##, $P < 0.020$.

The loss of mature lipoproteins in the Lnt mutants has only a small defect on cellular physiology, chiefly a slight sensitivity to hyperosmotic sucrose conditions for the *Francisella* mutant, increased susceptibility to globomycin and rifampin for both species, and a small increase in susceptibility of the *N. gonorrhoeae* mutant to vancomycin and polymyxin B. The *Francisella* sucrose phenotype may be due to slight perturbations in the cell envelope resulting from abnormal amounts of immature lipoproteins. We think that there are subtle changes in the overall envelope composition, as we noted that protein localization blots (Fig. 3) showed a distinct skewing of protein quantity across the gradient fractions for each cellular compartment. While the compartments did not shift in location within the gradient, the distribution of protein within each compartment shifted in the Δ*lnt* mutant. For example, the OM proteins moved toward the OM fractions and away from the fractions closer to the IM fractions. This may be the result of an alteration in the buoyant densities of the OM, since the inner leaflet will contain diacylated lipoproteins in place of the normal triacylated lipoproteins.

The Lnt mutants showed changes in susceptibilities to a limited number of antibiotics and chemical compounds of the several tested. The globomycin susceptibility phenotype was expected,

since this antibiotic inhibits prolipoprotein signal peptidase (Lsp), which catalyzes the second step of lipoprotein processing (50). We presumed that loss of Lnt would feed back on the pathway and possibly slow it to the point where the additional burden of inhibition of Lsp would have a greater effect than the wild type. The rifampin and vancomycin susceptibility phenotypes may be due to increased permeability of the OM, although only *N. gonorrhoeae* showed an increased susceptibility to polymyxin B, which is often an indication of OM perturbation. The lack of a polymyxin B susceptibility phenotype in the *Francisella* sp. mutant may have been due to the inherently high resistance of this organism to the antibiotic (51).

We sought to determine if the loss of Lnt had any effect on the intracellular survival of *Francisella*, as we thought that any perturbations of the envelope might reduce the fitness of the mutant. In addition, lipoproteins are one of the few microbial molecular patterns of *Francisella* that are recognized by the mammalian innate immune system (38). However, we saw no defect for uptake or survival and replication in THP-1 cells. It is possible that the mutant elicits an altered immune response or would have intrinsically defective virulence in a whole-animal infection model.

We speculate that perhaps the nonessential nature of the *lnt* gene in certain bacteria indicates that it is regulated, which may be reflected in the conflicting TLR2 data in the *F. tularensis* field of study. Lipoproteins are sensed via TLR2, depending upon the type of heterodimer formed with either TLR1 or TLR6. Other groups have investigated the role of TLR2 in *Francisella* infections, with varying results. One would expect that recognition would be through TLR2/TLR1 heterodimers, since these detect triacylated lipoproteins, the hallmark of Gram-negative bacteria. However, the work of Katz et al. (39) showed that mouse-derived dendritic cells detect *F. tularensis* LVS via TLR2/TLR6 but not TLR2/TLR1, resulting in a proinflammatory response. In contrast, another group who used reporter cells expressing recombinant receptors showed that proinflammatory sensing of LVS occurred through both TLR2/TLR6 and TLR2/TLR1 heterodimers (52). The same group demonstrated that *F. tularensis* lipoproteins TUL4A (LpnA) and FTT1103 signal via TLR2/TLR1, while another type of ligand, possible one or more nonlipidated proteins, might signal through TLR2/TLR6 (11). Mouse studies performed by Collazo et al. showed that $TLR2^{-/-}$ knockout mice were not any more susceptible to LVS infection than WT mice (53); however, other work by Malik et al. showed that $TLR2^{-/-}$ knockout mice infected with LVS exhibited faster mortality, increased bacterial burdens, and increased histopathology in the lung compared to WT mice, although these effects were seen with higher doses of bacteria (54). Other studies have also yielded conflicting results. Cole et al. determined that the inflammatory response to infection is dependent upon TLR2 and requires live bacteria capable of protein synthesis (55). This is in contrast to the results of Li et al., who demonstrated that the proinflammatory responses mediated by TLR2/TLR6 and TLR2/TLR1 do not require live bacteria (52). These controversies could potentially result from alterations in Lnt expression during infection. Regulation of the lipoprotein processing pathway was seen in *Listeria monocytogenes*, in which Lsp expression is highly induced in bacteria residing within the phagosomes of macrophages (56). It was recently reported that *Staphylococcus aureus* shifts from making diacylated to triacylated lipoproteins in response to growth stage and pH (17). There is yet no evidence that *lnt* expression is regulated in bacteria in which it is nonessential, but we now have the tools to investigate this possibility.

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