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A *prl* Mutation in SecY Suppresses Secretion and Virulence Defects of *Listeria monocytogenes secA2* Mutants

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The bulk of bacterial protein secretion occurs through the conserved SecY translocation channel that is powered by SecA-dependent ATP hydrolysis. Many Gram-positive bacteria, including the human pathogen *Listeria monocytogenes*, possess an additional nonessential specialized ATPase, SecA2. SecA2-dependent secretion is required for normal cell morphology and virulence in *L. monocytogenes*; however, the mechanism of export via this pathway is poorly understood. *L. monocytogenes secA2* mutants form rough colonies, have septation defects, are impaired for swarming motility, and form small plaques in tissue culture cells. In this study, 70 spontaneous mutants were isolated that restored swarming motility to *L. monocytogenes secA2* mutants. Most of the mutants had smooth colony morphology and septated normally, but all were lysozyme sensitive. Five representative mutants were subjected to whole-genome sequencing. Four of the five had mutations in proteins encoded by the *lmo2769* operon that conferred lysozyme sensitivity and increased swarming but did not rescue virulence defects. A point mutation in *secY* was identified that conferred smooth colony morphology to *secA2* mutants, restored wild-type plaque formation, and increased virulence in mice. This *secY* mutation resembled a *prl* suppressor known to expand the repertoire of proteins secreted through the SecY translocation complex. Accordingly, the Δ*secA2prlA1* mutant showed wild-type secretion levels of P60, an established SecA2-dependent secreted autolysin. Although the *prl* mutation largely suppressed almost all of the measurable SecA2-dependent traits, the Δ*secA2prlA1* mutant was still less virulent *in vivo* than the wild-type strain, suggesting that SecA2 function was still required for pathogenesis.

The essential general secretory (Sec) pathway is responsible for exporting the majority of secreted proteins across the bacterial cell membrane (1–3). Much of what we know about this pathway was discovered in *Escherichia coli* by using genetic screens to identify loss-of-function mutations in components of the Sec system. This led to the identification of components of the SecYEG complex that forms the translocation channel and the SecA ATPase that binds to signal sequences to drive the export of precursor proteins across the channel (3–9). In contrast to loss-of-function *sec* mutations, *prl* alleles are gain-of-function mutations, which expand the repertoire of substrates being exported across the SecYEG channel (6–8, 10, 11). The most dominant *prl* variants are in *secY*, known as *prlA* mutations, which allow for the secretion of proteins with altered signal sequence (7, 9, 11) without significantly altering the secretion of other proteins (6, 12).

In addition to the canonical SecA, an accessory cytosolic ATPase, SecA2, has been identified in *Mycobacterium* and a subset of other Gram-positive bacteria. Unlike SecA, SecA2 is not essential for cell viability but is required for virulence in some pathogenic bacteria (13–15). SecA1 and SecA2 are homologous but are not interchangeable (16). There are two types of SecA2 secretion pathways; some bacteria have an additional SecY homolog, which functions together with SecA2 to form a SecA2-SecY2 system. This system has been extensively studied in streptococci and is highly specialized to export glycosylated proteins that are incompatible with the canonical Sec system (13, 17–20). Other bacteria contain a SecA2-only system, which is thought to interact with the canonical SecYEG channel (16, 21, 22). The repertoire of proteins that require the accessory SecA2 for secretion is relatively small and varies between organisms (13, 17).

Listeria monocytogenes is a saprophytic Gram-positive, facultative intracellular pathogen capable of causing food-borne listeriosis in humans (23, 24). Spontaneous rough mutants of *L. monocytogenes* commonly arise on solid media, and many of these contain mutations in *secA2* (25–27). Mutants in *secA2* exhibit a chaining phenotype and are approximately 1,000-fold less virulent in animal models of infection (14, 25, 28). These mutants secrete a reduced subset of proteins, including two major autolysins, P60 (CwhA) and NamA (MurA), that contribute to septation and pathogenesis (14, 29–32). To better understand SecA2-dependent protein secretion and the contribution that SecA2 makes to *L. monocytogenes* pathogenesis, we undertook a suppressor analysis of a Δ*secA2* mutant. The primary finding reported in this communication is that a *prlA* mutation rescued most of the defects associated with a *secA2* mutation, although it did not lead to full restoration of virulence.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations specified in the *Guide for the Care and Use of Laboratory*

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Animals of the National Institutes of Health (33). All protocols were reviewed and approved by the Animal Care and Use Committee at the University of California Berkeley (Master Animal Use Protocol R235-0813B).

Bacterial strains and growth conditions. All *L. monocytogenes* strains used and generated in this study were in the 10403S background and are listed in the supplemental material (see Table S1 in the supplemental material). The Δ*secA2* strain previously characterized (14, 25) served as a parent strain for generating motility revertants. Unless otherwise stated, all *L. monocytogenes* strains were grown in brain heart infusion medium (BHI; Difco, Detroit, MI). All *E. coli* strains used for generating in-frame deletion and complement constructs were grown in Luria-Bertani (LB) medium (Difco, Detroit, MI).

Swarming motility. A single colony of the Δ*secA2* strain was used to generate a single motility revertant by stab-inoculating semisolid LB 0.4% agar at 30°C for 5 days. Revertant strains were grown overnight in BHI broth at 30°C without shaking; 1 μl of culture was then incorporated into a semisolid LB agar, and the swarming area was evaluated using ImageJ (http://rsbweb.nih.gov/ij/) following incubation at 30°C for 48 h.

L2 plaque assay. Plaque assays using murine L2 fibroblasts were performed as previously described (34). Briefly, overnight 30°C static cultures of *L. monocytogenes* were allowed to infect monolayers of L2 cells for 1 h. Cells were washed and overlaid with 0.7% agarose in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, California) containing 10 μg/ml gentamicin (GM). After 3 days at 37°C, plaques were overlaid with 2 ml of 0.7% agarose in DMEM with GM and 0.3% neutral red (Sigma-Aldrich). Monolayers were stained overnight, and plaque size was evaluated using ImageJ.

DNA isolation and sequencing. Genomic DNA was isolated from stationary-phase cultures of *L. monocytogenes* using the MasterPure DNA purification kit (Epicentre). DNA was then fragmented using Covaris S22 (Covaris Inc.). Libraries were constructed using Apollo 324 (IntegenX Inc.), PCR amplified, and multiplexed at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley. The resulting libraries were sequenced using single-end reads with a HiSeq 2000 Illumina platform. Sequence data were aligned to the *L. monocytogenes* 10403S reference genome (GenBank accession number CP002002.1) using Bowtie 2 (35), and single-nucleotide polymorphisms (SNPs) were identified using SAM tools (36). Approximately 93% of all reads aligned to the reference genome, resulting in >50× coverage.

In-frame deletion and complementation constructs. In-frame deletion mutants of *lmo2769* and *lmo1721* were constructed by splice overlap extension and introduced by allelic exchange using pKSV7 vector and primers JD17 to JD20 and JD30 to JD33, respectively (see Table S2 in the supplemental material), as previously described (37). The pPL2 integration vector was used to complement Δ*lmo2769* mutants and the R57 revertant strain with the *lmo2769-2767* operon and all the endogenous regulatory sequences using the JD46 and JD47 primers (see Table S2 in the supplemental material) as previously described (38).

Transductions. Transductions of the *lmo2768*::Tn and *lmo2637*::Tn donor strains were generated using phage U153 and erythromycin as a selection marker, as previously described (39). Strain R57 containing the *prlA1* allele was used as the recipient strain for *lmo2637*::Tn, and subsequent recipient strains of *secY* mutation were confirmed by Sanger sequencing (Elim Biopharmaceuticals, Hayward, CA).

***In vivo* mouse infections.** CD1 [Crl:CD1(ICR)] mice (Charles River Laboratories) were injected intravenously with 1×10^5 CFU of wild-type (WT) or mutant *L. monocytogenes* strains. Spleens and livers were harvested after 48 h, and bacterial burdens were evaluated as previously described (40).

Disk diffusion assay. A total of 3×10^8 stationary-phase *L. monocytogenes* cells were plated on BHI agar plates. Sterile Whatman paper discs (7 mm in diameter) containing 1 mg of chicken egg white lysozyme (Sigma), 10 μg of vancomycin, or 120 μg of penicillin in a 10-μl volume were added to plates. The area of growth inhibition around each disc was measured using ImageJ following 24 h of incubation at 37°C.

Microscopy. Phase-contrast microscopy was performed using stationary BHI culture at 37°C. Fluorescence microscopy was conducted using an Olympus IX81 TIRF microscope on mid-log cells stained with SYTO9 green fluorescent stain (Invitrogen).

Western blotting. Secreted proteins from mid-log phase (5 h) or for the time course experiment from 1-h, 2-h, and 3-h LB culture supernatants were precipitated with 10% trichloroacetic acid (TCA), as previously described (41), and solubilized in NuPAGE LDS buffer (Invitrogen) containing 5% β-mercaptoethanol at a volume adjusted to an optical density at 600 nm (OD_{600}) for each strain. Samples were fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblotting with polyclonal anti-P60 and anti-LLO antibodies, and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

RESULTS

Isolation and initial characterization of Δ*secA2* suppressor mutants. The inability of bacteria to properly septate during cell division can negatively influence swarming efficiency in semisolid media (42–44). Indeed, the filamentous nature of the *L. monocytogenes secA2* mutant led to a 92% reduction in swarming motility compared to that of the wild-type (WT) 10403S strain (Fig. 1). Seventy spontaneous, independent Δ*secA2* suppressor mutants were generated by identifying swarming bacteria that appeared after 5 days of incubation at 30°C in semisolid LB agar. Prolonged incubation for isolation of motility revertants was used based on the original isolation of spontaneous rough colonies, which facilitated the identification of *secA2* in *Listeria* (25). These suppressor mutants displayed a wide range of swarming phenotypes (11 to 112%) compared to the WT strain (Fig. 1). Motility revertants were further characterized based on their colony and microscopic appearance (see Table S3 in the supplemental material). The majority of suppressor mutants (64%) reverted from a rough to a smooth colony morphology. Rough colony morphology correlated with chaining, with less chaining observed for isolates forming smooth colonies. Whereas the WT (45–47) and the *secA2* mutants of *L. monocytogenes* are lysozyme resistant, all 70 suppressor mutants were susceptible to lysozyme (see Table S3 in the supplemental material).

A critical aspect of *L. monocytogenes* pathogenesis is the capacity to spread from cell to cell and form plaques in monolayers of tissue culture cells, which correlates well with mouse virulence (48). The *secA2* mutant forms plaque sizes that are approximately 30% of those of the WT (Fig. 2) (14). The Δ*secA2* swarming suppressor mutants varied greatly in their ability to form plaques, with sizes ranging from 0% to 87% of those of the WT (Fig. 2). There was no significant correlation between chaining phenotype and plaque size, suggesting that chaining alone does not directly influence cell-to-cell spread, as measured by the plaque assay.

Whole-genome sequencing of five Δ*secA2* suppressor mutants. To identify mutations responsible for the observed phenotypes, five strains, R9, R23, R35, R57, and R60.1, were selected for further analysis (Table 1). Whole-genome sequencing revealed a number of single-nucleotide polymorphisms (SNPs) that differed from the WT strain and confirmed that each lacked the *secA2* gene (Table 2). Most revertants encoded three to four SNPs, while revertant R35 was an outlier with 13 SNPs, one of which mapped to *lmo1403*, a gene encoding the DNA mismatch repair protein MutS. We hypothesize that inactivation of *mutS* led to a hyper-

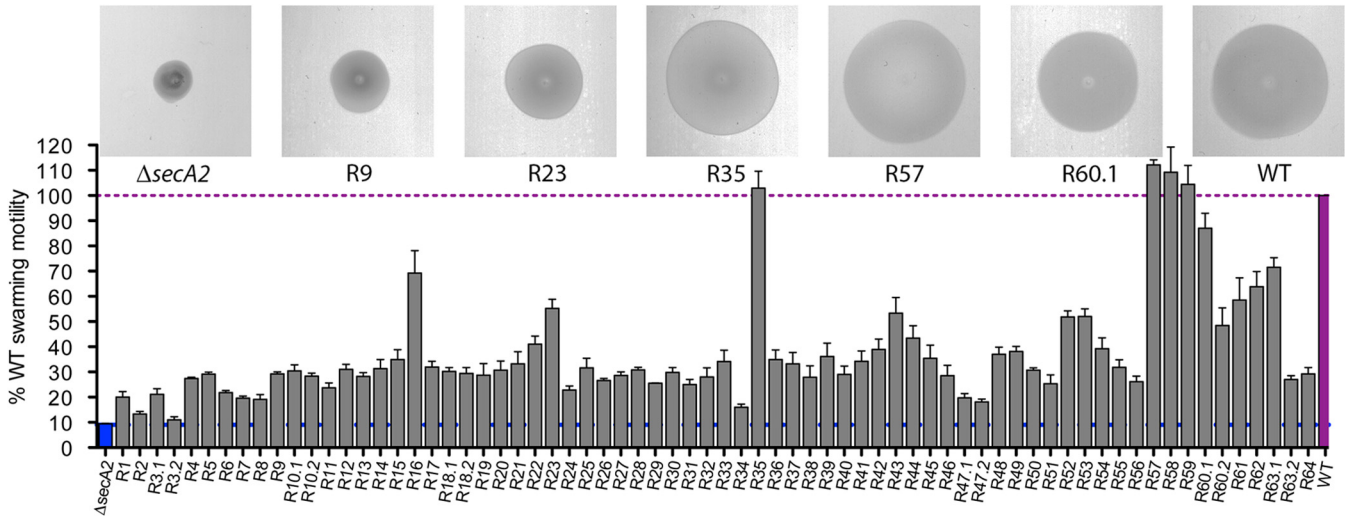

FIG 1 Swarming motility of suppressor mutants. Motility assessed on semisolid LB agar following incubation at 30°C after 48 h. Motility is expressed as a percentage of the swarming area of the WT strain seeded on the same plate. Error bars represent standard deviations of the mean zone ratios from three independent experiments. Images show motility zones for the suppressor mutants indicated.

mutation phenotype (49). The presence of multiple mutations in all suppressors was likely a consequence of extended incubation that allowed for acquisition of multiple mutations, which collectively promoted swarming.

Two revertants, R23 and R35, contained SNPs in genes encoding the flagellar motor switch proteins FliM and FliG, respectively (Table 2). Both of these proteins function in controlling the direction of flagellar rotation (50–53), and single point mutations in FliM have been shown to restore swarming motility in a chemotaxis-deficient mutant of *Salmonella enterica* (52). We hypothesize that SNPs in these genes led to enhanced swarming motility of the corresponding revertants by direct manipulation of the flagellar motor. However, neither of these suppressor mutants formed plaques in tissue culture cells and therefore were not subjected to further analysis.

All suppressor mutants were significantly more susceptible to lysozyme than the WT or the parent *secA2* mutant strain (Fig. 3B; see also Table S3 in the supplemental material). Susceptibility to cell wall-acting antibiotics was less prominent but still evident for R23, R35, and R60.1 (see Fig. S1A and B in the supplemental material). Additionally, four out of five suppressors had mutations in the *lmo2769* operon, previously shown to be required for lysozyme resistance (46). Lastly, mutant R60.1 contained a mutation in *walI*, a regulator of the WalRK two-component system required for autolysin regulation and lysozyme resistance in *L. monocytogenes* (46).

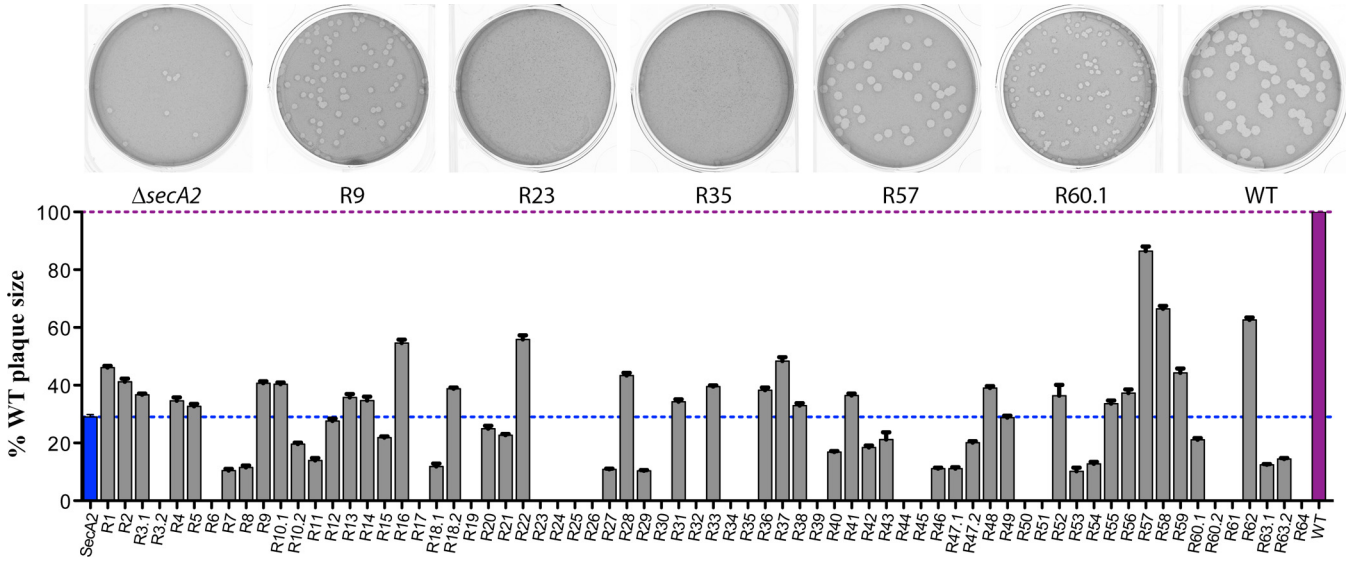

FIG 2 Plaque size of *secA2* suppressor mutants. L2 fibroblasts were infected with *secA2* suppressor mutants, and mean plaque size was measured 3 days postinfection. Plaque area of each mutant is shown as a percentage compared to that of the WT strain. Error bars represent standard deviations of the mean zone ratios from three independent experiments. Images show plaques for the suppressor mutants indicated.

TABLE 1 Strains selected for sequencing and their phenotypes

Strain	Colony	Chaining[b]	% motility (±SD)[c]	% plaque area (±SD)[d]
R9	Rough	+++	29 (±3)	41 (±9)
R23	Smooth	+	55 (±13)	0
R35	Smooth	++	102 (±26)	0
R57	Smooth	+	112 (±15)	87 (±13)
R60.1	Smooth	++	87 (±23)	21 (±6)
Δ*secA2* strain[a]	Rough	++++	9 (±2)	29 (±9)

[a] The parent Δ*sec*A2 strain was included as a reference strain.
[b] Chaining score interpretation by phase microscopy. +, single cells and chains of up to 3 cells/field at ×40 magnification; ++, single cells and chains of up to 4 cells/field at ×40 magnification; +++, chains of up to 6 cells/field at ×40 magnification; ++++, chains of >6 cells/field at ×40 magnification.
[c] Expressed as an average percentage (standard deviation) of the swarming area normalized to that of the WT strain on 0.4% LB agar after 48h at 30°C.
[d] Expressed as an average percentage (standard deviation) of the plaque area compared to that of the WT strain.

Most notably, mutants R57 and R60.1 contained SNPs in genes encoding two essential components of the canonical Sec pathway, SecY and SecA, respectively. These mutations resulted in amino acid substitutions leading to altered protein function, and the SNP in *secY* is analyzed in detail below. The identification of mutations in *secY* and *secA* suggests a link between SecA2 and the general Sec pathway in *L. monocytogenes*.

Improved swarming motility and lack of chaining did not improve cell-to-cell spread in most of the mutants, with the exception of R57. This mutant showed a complete loss of chaining (Fig. 3A) and restored cell-to-cell spread to 87% of the WT strain (Fig. 1). Based on the increased plaque size, we hypothesized that this strain would show increased virulence *in vivo*. However, upon intravenous infection of mice, the R57 mutant was just as attenuated as the Δ*secA2* parental strain (Fig. 3C and D). The lack of even

TABLE 2 Single-nucleotide polymorphisms of strains sequenced

Strain	Position[a]	Reference[a]	Alteration	Change	Lmo no.	Gene name	Encoded protein or function
R9	1104029	C	T	A141V	*lmo1087*		Ribitol-5-phosphate 2-dehydrogenase
	1921584	T	C	V195 silent	*lmo1892*	*pbpA*	Penicillin-binding protein 1
	2812912	A	G	48 bp upstream of *lmo2769* transcription start site	*lmo2769*		Antibiotic transport system ATP-binding protein
R23	714441	A	G	E28G	*lmo0699*	*fliM*	Flagellar motor switch
	2734020	G	C	G122 silent	*lmo2694*		Lysine decarboxylase
	2811145	G	T	T169 stop codon	*lmo2768*		Membrane protein (permease)
R35	728791	G	A	A340T	*lmo0714*	*fliG*	Flagellar motor switch protein G
	736353	A	G	E176G	*lmo0723*		Methyl-accepting chemotaxis protein
	1102328	C	T	P499S	*lmo1085*		Similar to teichoic acid biosynthesis protein B
	1191185	T	C	I486T	*lmo1208*	*cbiP*	Cobyric acid synthase
	1264242	CTTTTTTTT	CTTTTTTT	58 bp upstream of *lmo1281* transcription start site	*lmo1281*		Hydroxybenzoyl coenzyme A thioesterase
	1343106	G	A	R232H	*lmo1360*	*folD*	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase
	1390074	GAAAAAAAA	GAAAAAAA	I309S, premature stop at codon 319	*lmo1403*	*mutS*	DNA mismatch repair protein MutS
	1932076	A	G	S247A	*lmo1901*	*panC*	Pantothenate synthetases
	2137030	A	G	L219W	*lmo2100*		Similar to transcriptional regulator (GntR family)
	2323057	G	A	R264C	*lmo2278*	*lysA*	L-Alanoyl-D-glutamate peptidase
	2447109	CAAAAAAA	CAAAAAAAA	A25C, premature stop at codon 49	*lmo2421*		Similar to two-component sensor histidine kinase
	2468643	CTTTTTTTT	CTTTTTTT	A46Q, premature stop at codon 62	*lmo2444*		Similar to glycosidase
	2812179	ATTTTTTT	ATTTTTTTT	I229N, premature stop at codon 236	*lmo2769*		Antibiotic transport system ATP-binding protein
R57	1738627	TAA	TAAA	L780F, premature stop at codon 791	*lmo1721*	*lacR*	Sigma-54 interaction domain-containing protein
	2658496	C	T	G408R	*lmo2612*	*secY*	Protein translocase subunit SecY
	2812179	ATTTTTTT	ATTTTTTTT	I229N, premature stop at codon 236	*lmo2769*		Antibiotic transport system ATP-binding protein
R60.1	311857	CAAAAAA	CAAAAAAA	G109R, premature stop at codon 279	*lmo0290*	*walI*	Similar to *B. subtilis* YycI protein
	2542503	C	T	D559N	*lmo2510*	*secA*	Protein translocase subunit SecA
	2568428	T	CA	V211G, premature stop at codon 223	*lmo2537*	*mnaA*	UDP-*N*-acetylglucosamine 2-epimerase

[a] Reference genome, CP002002.1.

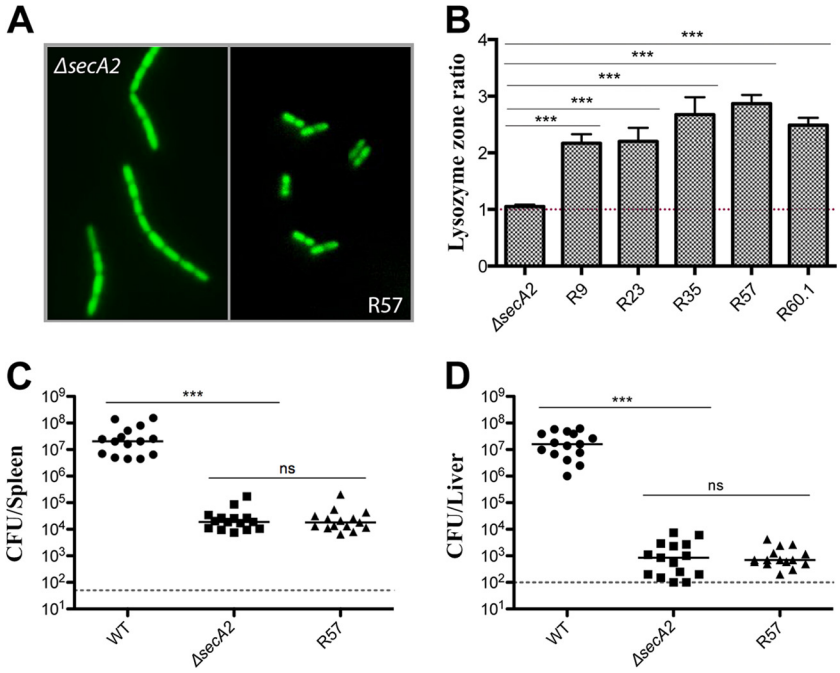

FIG 3 Characterization of the R57 revertant. (A) Fluorescence microscopy of the Δ*secA2* and R57 strains. Mid-exponential-phase cells were stained with SYTO9 green. (B) Disk diffusion susceptibility to 1 mg of lysozyme expressed as a ratio of the WT, where a ratio of >1 indicates increased susceptibility to lysozyme. Error bars represent standard deviations of the mean zone ratios. Student's *t* test was used to analyze statistical significance: ***, $P < 0.0001$; ns, $P \geq 0.05$. Bacterial burdens in spleens (C) and livers (D) 48 h postinfection with 1×10^5 CFU in CD1 mice. The dashed line represents the limit of detection. Results show CFU from three independent experiments. Statistical significance was evaluated using a Mann-Whitney test. ***, $P < 0.0001$; ns, $P \geq 0.05$.

a partial rescue of virulence was somewhat surprising and suggested that either the *secA2* mutation cannot be overcome *in vivo* or that the combination of SNPs resulted in virulence suppression. Because of these prominent phenotypes and a possible link with the SecYEG translocon, R57 was chosen for further analysis.

Mutations in the *lmo2769* operon resulted in lysozyme susceptibility and increased swarming motility but did not contribute to increased plaque formation. Four out of five sequenced revertants had SNPs in the *lmo2769* operon (Table 2). The first gene of this uncharacterized operon, *lmo2769*, encodes an ATP-binding protein, followed by an ABC transporter permease and a membrane protein encoded by *lmo2768* and *lmo2767*, respectively. The function of the ABC transporter is unknown; however, it has been implicated in lysozyme sensitivity (46). It seemed probable that mutations in this operon, in addition to contributing to lysozyme susceptibility, also enhanced swarming motility of Δ*secA2* revertants. Indeed the swarming motility of the Δ*lmo2769* Δ*secA2* mutant was twice that of the parent Δ*secA2* strain (Fig. 4A). The Δ*lmo2769* mutants were also significantly more susceptible to lysozyme than were the WT or Δ*secA2* strains (Fig. 4B; see also Fig. S1 in the supplemental material) (46). There was no change in susceptibility to other cell wall-acting agents tested (see Fig. S1 in the supplemental material); however, *lmo2768*::Tn has been shown to be more susceptible to cationic antimicrobial peptides (CAMPs) and cefuroxime (46). The increase in swarming motility and lysozyme susceptibility was diminished by complementing either the Δ*lmo2769* Δ*secA2* double mutant or R57 with the *lmo2769* operon (Fig. 4A and B).

Lysozyme susceptibility caused by the disruption of the *lmo2769* operon had a negligible impact on virulence (Fig. 4C; see also Fig. S2A and B in the supplemental material). Plaque size of the Δ*secA2* Δ*lmo2769* double mutant increased 10% from that of the Δ*secA2* strain; however, complementation of R57 with the *lmo2769-lmo2767* operon had no effect on plaque formation of the revertant (Fig. 4C). We concluded that disruption of *lmo2769* led to a cell wall defect that increased swarming motility of the mutant but was not responsible for the increased plaque size in the originally isolated R57 suppressor strain.

Deletion of *lmo1721*, a gene harboring the second SNP identified in R57, had a suppressive effect on the swarming motility of the Δ*secA2* strain and the Δ*secA2* Δ*lmo2769* double mutant (see Fig. S3A in the supplemental material) and had no effect on lysozyme susceptibility (see Fig. S1F in the supplemental material). This gene encodes the transcriptional regulator LacR, which has been suggested to suppress virulence in *L. monocytogenes* in response to cellobiose (54, 55) in addition to regulating the phosphotransferase system (56). Plaque formation was unaffected by in-frame deletion of the *lmo1721* gene alone or in combination with a *secA2* deletion (Fig. 4D) but had a suppressive effect on the Δ*lmo1721* Δ*lmo2769* double mutant, suggesting that the combination of the two mutations negatively influences virulence when SecA2 is undisturbed. Collectively, these observations suggest that the SNP in *lmo1721* did not contribute to the revertant phenotype.

The *secY* mutation significantly improved virulence and swarming motility of the Δ*secA2* strain. The third SNP identified in the R57 revertant was in *lmo2612*, encoding SecY, which resulted in the G408R amino acid substitution. The G408 residue corresponded to V411 when aligned to the *E. coli* SecY and mapped to the last transmembrane segment of the protein. Mutations in this region of *E. coli* SecY, such as L407R and I408N,

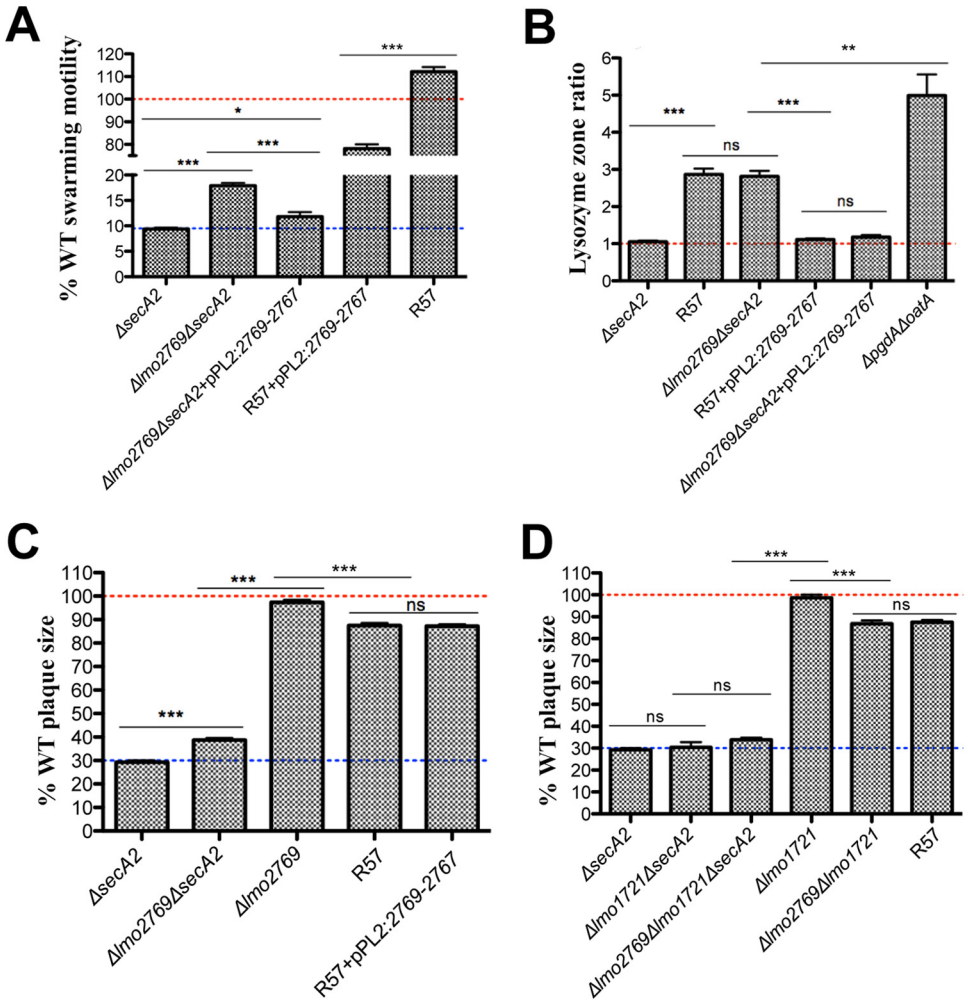

FIG 4 Characterization of an *lmo2769* mutant in a Δ*secA2* background. (A) Swarming motility of mutants and strains complemented with the *lmo*2767 operon was assessed on semisolid LB agar following incubation at 30°C after 48 h. Motility is expressed as a percentage of the swarming area of the WT strain and set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean zone ratios from three independent experiments. (B) Disk diffusion susceptibility to 1 mg of lysozyme expressed as a ratio of the WT. Error bars represent standard deviations of the mean zone ratios. (C and D) Plaque area of each mutant is shown as a percentage of that of the WT strain, set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean plaque size ratios from three independent experiments. Student's *t* test was used to analyze statistical significance: ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$; and ns, $P \geq 0.05$.

have been described as protein localization (*prl*) mutations (7, 8, 57). We refer to the *L. monocytogenes secY* (G408R) allele as *prlA1* in the remainder of the manuscript.

As in other bacteria, *secY* is essential in *L. monocytogenes* (58) and is transcribed with other essential genes in a 28-gene operon. Attempts to introduce the *prlA1* mutation into a WT background by allelic exchange at the native *secY* locus were unsuccessful. Therefore, we used phage transduction utilizing a *Himar1* transposon insertion in a neighboring gene (*lmo2637*) as an antibiotic marker to transduce the *prlA1* allele from the R57 revertant and the WT *secY* into various strains. Disruption of *lmo2637* had a minor effect on swarming motility (see Fig. S3B in the supplemental material) but no effect on plaque formation (see Fig. S3C in the supplemental material) or *in vivo* virulence (see Fig. S2C and D in the supplemental material).

Introduction of the *prlA1* mutation into the Δ*secA2* strain or the Δ*secA2* Δ*lmo2769* double mutant resulted in enhanced motility (Fig. 5A) and restored the smooth colony morphology of these mutants. In addition, motility of R57 diminished significantly (88%) upon replacement of *prlA1* with the WT allele (R57: *lmo2637*::Tn strain) (Fig. 5A), which was coupled with a change in colony morphology from smooth to rough. This suggested that the *prlA1* mutation significantly contributed to the R57 motility and cell morphology phenotypes.

Surprisingly the *prlA1* mutation alone was sufficient to completely restore cell-to-cell spread of the Δ*secA2* strain and the Δ*lmo2769* Δ*secA2* double mutant (Fig. 5B), while no change was observed for *prlA1* alone in a WT background (see Fig. S3C in the supplemental material). Exchanging the *prlA1* mutation for the WT allele in R57 (R57:*lmo2637*::Tn strain) decreased plaque formation from 87% to 39%, the level observed for the Δ*lmo2769* Δ*secA2* double mutant (see Fig. S3C in the supplemental material).

To investigate whether destabilization of the SecY channel in

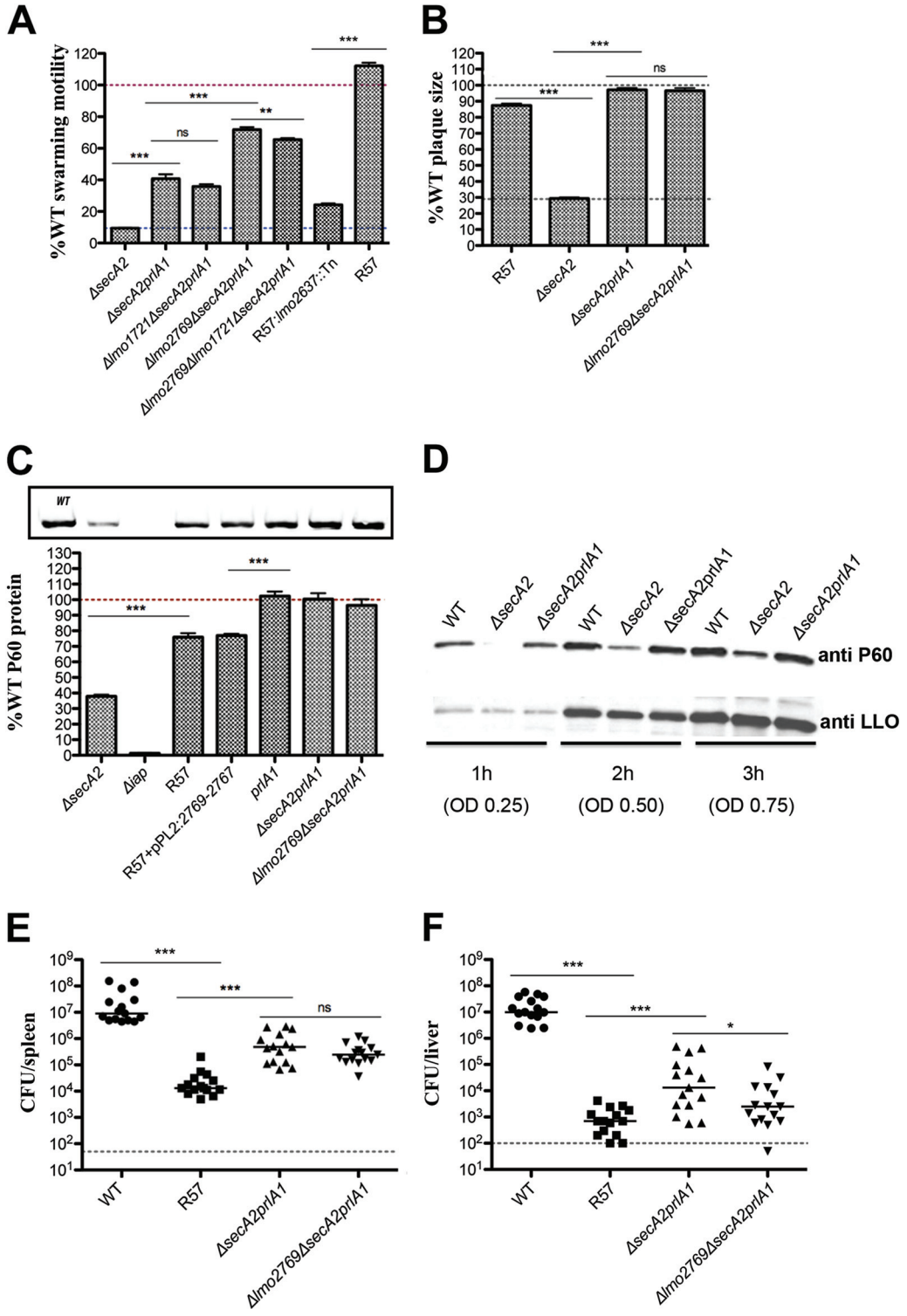

FIG 5 Characterization of the *prfA1* mutation. (A) Swarming motility of mutants and strains complemented with the *lmo*2767 operon was assessed on semisolid LB agar following incubation at 30°C after 48 h. Motility is expressed as a percentage of the swarming area of the WT strain and set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean zone ratios from three independent experiments. (B) Plaque area of each mutant is shown as a percentage compared to that of the WT strain, set at 100% (represented by a dotted line). Error bars represent standard deviations of the mean plaque size ratios from three independent experiments. Student's *t* test was used to analyze statistical significance: ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$; and ns, $P \geq 0.05$. (C) Secreted levels of P60 in supernatants from mid-log (5-h) cultured cells in LB broth expressed as a percentage of protein secreted by the WT strain. (D) Secreted levels of P60 and LLO in supernatants from 1-h, 2-h, and 3-h cultures in LB broth. Bacterial burdens in spleens (E) and livers (F) 48 h post-intravenous infection of 1×10^5 CFU in mice, showing data from three independent experiments. The dashed line represents the limit of detection. Statistical significance was evaluated using a Mann-Whitney test. ***, $P < 0.0001$; *, $P < 0.05$; ns, $P \geq 0.05$.

response to the *prlA1* mutation was sufficient to restore protein secretion in Δ*secA2* mutants, the abundance of P60 in the supernatants of mutant cultures was evaluated as a proxy for other SecA2-dependent proteins. Notably, P60 levels were completely restored to WT levels in the *secA2* constructs expressing the *prlA1* mutation, while the *prlA1* strain maintained normal levels of the secreted protein (Fig. 5C). Additionally, the intracellular accumulation of P60 observed in the Δ*secA2* strain (14) was diminished in the Δ*secA2prlA1* strain (data not shown). Restoration of P60 was entirely PrlA1 dependent, as exchanging *prlA1* for the WT allele in the R57 background (R57:*lmo2637*::Tn strain) diminished the secreted P60 levels to those observed for the Δ*secA2* parent strain (see Fig. S3D in the supplemental material). Further, to address the possibility of differing secretion levels between the WT and Δ*secA2prlA1* strains, we undertook a timed experiment in which secretion levels of P60 were assessed. The introduction of the *prlA1* mutation restored the levels of P60 secretion in the Δ*secA2* strain, while not affecting the secretion of SecA-dependent listeriolysin O (LLO) (Fig. 5D). Interestingly, P60 levels of the R57 mutant were only 80% of those of the WT strain (Fig. 5C) and were not directly linked to the Δ*lmo*1721 (see Fig. S3E in the supplemental material) or Δ*lmo*2769 (Fig. 5C) mutation. Collectively these observations show that a *prl*A1 mutation alone was sufficient to overcome the motility, cell morphology, and cell-to-cell spread phenotypes associated with the Δ*secA2* strain, by restoring secretion of the autolysin P60 and likely other SecA2-depended proteins to WT levels.

Lastly, the effect of the *prlA1* allele on virulence *in vivo* was assessed. Somewhat surprisingly, a *prlA1* mutation alone in a WT background did not affect virulence (see Fig. S2C and D in the supplemental material). However, virulence of both the Δ*secA2* strain and the Δ*lmo2769* Δ*secA2* double mutant was partially restored (Fig. 5E and F; see also Fig. S2E and F in the supplemental material). Whereas the *secA2* mutant and the R57 suppressor, compared to the WT, were 1,000-fold less virulent in the spleen and 10,000-fold less virulent in the liver (Fig. 3C and D and Fig. 5E and F), the *prlA1* mutation partially rescued virulence by approximately 100-fold in the spleen and 20-fold in the liver (Fig. 5E and F). Notably though, it was still considerably less virulent than the WT (20-fold in the spleen and 1,000-fold in the liver).

DISCUSSION

Much of what is known about the general secretory pathway (Sec) in bacteria originates from seminal genetic studies that led to the identification and characterization of the Sec components, including the SecYEG channel and SecA ATPase (3–9). While the *secA* gene is essential in all bacteria, a subset of Gram-positive bacteria harbor an additional, nonessential homologous gene called *secA2* (13–15, 17, 20, 59). Both pathogenic and nonpathogenic species of *Listeria* encode SecA2 (60), which facilitates the secretion of two major autolysins contributing to septation, swarming motility, plaque formation in tissue culture cells, and virulence in mice (14, 25, 28, 60). It remains unclear, however, why certain bacteria carry *secA2* while others do not, and why *secA2* is dispensable for cell viability. In this study, we took advantage of the swarming motility defect to isolate spontaneous mutants capable of swarming motility (Fig. 1). One mutant was phenotypically very similar to the WT and was characterized in depth, with its phenotype being genetically traced directly to a single amino acid mutation in SecY. Similar mutations in *E. coli secY*, called *prlA* alleles, are gain-of-function mutations that expand the repertoire of substrates that can be exported (6–8, 10, 11). We hypothesize that the *secY* mutant identified in this study, here named *prlA1*, rescued the *L. monocytogenes secA2* mutant by allowing for secretion of proteins that are normally SecA2 dependent. Recent work in *Mycobacterium smegmatis* reported that increased SecY levels suppressed the severe phenotypes of a *secA2* mutant (21), suggesting that SecA2-dependent protein secretion occurs through interaction with the canonical SecY. Thus, in both *L. monocytogenes* and *M. smegmatis*, the canonical *secY* is very likely part of the translocation pore for SecA2 substrates, in unlike other pathogenic bacteria, including *Bacillus anthracis* and some *Streptococcus* and *Staphylococcus* species, all of which have SecA2 and SecY2 (13, 17, 20, 59).

Wild-type *L. monocytogenes* is highly lysozyme resistant (45–47). One curious finding reported in this study was that all 70 suppressors were lysozyme sensitive, and in four of the five sequenced suppressor mutants, lysozyme sensitivity was caused by mutations in an uncharacterized ABC transporter encoded by the *lmo2769-lmo2767* operon. Lysozyme susceptibility of the fifth mutant was likely due to a mutation in *walI*, which was previously shown to be required for lysozyme resistance (46). The first gene in the *lmo2769-lmo2767* operon encodes an ATP-binding protein similar to a poorly characterized *Bacillus subtilis* protein (YtrB) whose expression has been observed following exposure to cell wall-acting agents (48). Mutation of either *lmo2769* or *lmo2768* conferred lysozyme susceptibility, suggesting that these mutations altered the cell wall and may have led to decreased chaining and increased swarming. Although the exact function of this operon requires further characterization, these data suggest that the *lmo2769* operon is involved in maintaining cell wall homeostasis (61, 62). The high frequency of mutations detected in this operon, associated with increased swarming, likely provided a growth advantage in semisolid media that increased the probability of acquiring less frequent mutations, such as *prlA1*.

The *prlA1* allele characterized in this study resulted in a glycine substitution for arginine (G408R) in the tenth transmembrane helix of SecY. Variations in this region of SecY in *E. coli*, such as L407R (PrlA301) and I408N (PrlA4), as well as a PrlA-like mutation in the accessory SecY2 of *Streptococcus gordonii* (19), promote translocation of proteins with defective signal peptides by destabilizing the pore ring within the SecYEG channel (7, 8, 57). It is likely that the substitution of glycine for a positively charged hydrophilic arginine residue would also induce a conformational change to the pore ring. Further investigation using more direct approaches is required to explain how the *prlA1* mutation is able to rescue the SecA2-dependent secretion in *L. monocytogenes secA2* mutants. It is possible, however, that the rescue in protein secretion is a result of a more efficient interaction between SecA and SecY induced by the conformational change of the pore ring, similar to that observed in a PrlA4 variant of *E. coli* (10, 63).

Protein secretion is an energetically costly process, and in the absence of SecA2, another ATPase, such as SecA, is required to provide energy for translocation of P60 in the *L. monocytogenes* Δ*secA2prlA1* mutant. Since SecA is dimeric during protein translocation (64), it is possible that SecA2 and SecA form a heterodimer which interacts with SecYEG, thereby modulating substrate specificity of the channel. The interaction between SecA2 and the canonical SecA ATPase has previously been shown to occur in *Streptococcus* (65) and implied in *Mycobacterium* (66)

and more recently in *Listeria* (67). In the absence of a functional SecA2, SecA may drive SecA2-dependent secretion, although at a much lower rate, potentially due to a lower affinity of SecA alone for the specific substrates. This view is supported by the observation that the export of P60 and other SecA2-dependent proteins is not completely abolished in the *secA2* mutant (14, 25).

Although it is not yet characterized, we also identified a suppressor mutation in *secA*, providing further support for a possible interaction between SecA2 and SecA in *L. monocytogenes*. This mutation resulted in the D599N amino acid substitution, corresponding to D649 in the second nucleotide-binding domain of SecA in *E. coli*. Amino acid alterations in this region of SecA, in particular residues 631 to 653, are known as *azi*, as these confer azide resistance resulting from an increased ATPase activity (68, 69) in addition to having an increased affinity for SecY (69). A SecA homodimer with enhanced affinity for SecY either through *prlA1* or *azi* mutations may suffice in overcoming the requirement for SecA2 in a *secA2* mutant. The enhanced interaction of SecA with SecY would indirectly increase the rate of secretion of SecA2-dependent proteins independent of altering the affinity of the ATPase for these substrates.

Another piece of evidence supporting the interaction between the two ATPases comes from a recent proteomics study, which showed that P60 secretion is indistinguishable between WT *L. monocytogenes* EGD-e and the Δ*secA2* strain when grown in a defined medium at 37°C but was diminished in the mutant at 20°C (32), thus suggesting that SecA2-dependent secretion is able to engage an alternative pathway under different environmental conditions. One ATPase or dimer combination may be favored over the other depending on the energetic state of the cell. For example, the affinity of SecA2 for ATP in *Mycobacterium* is higher than that of SecA (70). In order not to compete for substrate, SecA2 is bound to ADP in a dormant state until it is required (71). Considering there is no conservation in the signal peptides of proteins being secreted through the SecA2-dependent pathway in *L. monocytogenes*, it remains unclear how proteins are targeted to SecA2 (14, 32) and how that can be overcome in certain conditions.

It is curious why *Listeria* species require SecA2 for normal septation, while most other bacteria use other, SecA2-independent, mechanisms. One possibility is that *secA2* mutants have an advantage under certain conditions. Indeed, *secA2* mutants arise spontaneously in the lab as rough colonies that emanate from a smooth colony and spread on solid media (25–27). At ambient temperatures, these mutants readily form distinct filamentous biofilms, which are thicker than those formed by the WT strain (26, 72). The spontaneous and reversible (25, 73) morphological conversion to a rough phenotype may provide a potential advantage to *L. monocytogenes* in its saprophytic phase by enhancing the ability to colonize abiotic surfaces. This may also be one of the reasons why the nonpathogenic *Listeria* species have retained SecA2; however, this potential advantage outside the host comes at a significant cost to its pathogenic phase, as *L. monocytogenes secA2* mutants are approximately 1,000-fold attenuated for virulence in mice (Fig. 3C and D) (14, 25, 28), although rough mutants can persist in the gut (73) and the gallbladder (74). Some of this attenuation has been traced to the lower levels of secreted autolysins, primarily P60, and to a lesser extent NamA (14). However, even though the *prlA1* mutation described in the study had normal levels of secreted LLO and restored WT levels of secreted P60 and cell-to-cell spread, it resulted in only a 100-fold rescue in virulence *in vivo* of the attenuated *secA2* mutant. The suppressor mutant was still less virulent than the WT strain, suggesting that a functional SecA2 is still required for full expression of *L. monocytogenes* virulence. SecA2-dependent secretion may have evolved in *L. monocytogenes* to provide a mechanism for switching between the parasitic and the saprophytic phase, thus providing an advantage in its ability to thrive in the environment as well as inside a host.

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