

Export Requirements of Pneumolysin in *Streptococcus pneumoniae*

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Streptococcus pneumoniae is a major causative agent of otitis media, pneumonia, bacteremia, and meningitis. Pneumolysin (Ply), a member of the cholesterol-dependent cytolysins (CDCs), is produced by virtually all clinical isolates of *S. pneumoniae*, and ply mutant strains are severely attenuated in mouse models of colonization and infection. In contrast to all other known members of the CDC family, Ply lacks a signal peptide for export outside the cell. Instead, Ply has been hypothesized to be released upon autolysis or, alternatively, via a nonautolytic mechanism that remains undefined. We show that an exogenously added signal sequence is not sufficient for Sec-dependent Ply secretion in *S. pneumoniae* but is sufficient in the surrogate host *Bacillus subtilis*. Previously, we showed that Ply is localized primarily to the cell wall compartment in the absence of detectable cell lysis. Here we show that Ply released by autolysis cannot reassociate with intact cells, suggesting that there is a Ply export mechanism that is coupled to cell wall localization of the protein. This putative export mechanism is capable of secreting a related CDC without its signal sequence. We show that *B. subtilis* can export Ply, suggesting that the export pathway is conserved. Finally, through truncation and domain swapping analyses, we show that export is dependent on domain 2 of Ply.

Streptococcus pneumoniae, a Gram-positive bacterial pathogen and colonizer of the human nasopharynx is one of the leading causes of otitis media, sinusitis, meningitis, pneumonia, and bacteremia throughout the world (15). In developed countries, pneumonia caused by *S. pneumoniae* is a significant contributor to mortality in the elderly. Childhood *S. pneumoniae* infections are generally not fatal but do place a large burden on the medical community and an economic burden on parents and caregivers of children. For example, in the United States, ear infections represent the most frequent cause for a child under 5 years of age to visit a pediatrician, at an estimated cost of \$3 billion to \$4 billion per year (29). In developing countries, particularly in sub-Saharan Africa and southeast Asia, consequences of childhood *S. pneumoniae* infections are dire. Every year, over one million children in the developing world die as a result of pneumonia, bacteremia, and meningitis caused by *S. pneumoniae* (33).

Many virulence factors contribute to the severity and wide variety of disease that *S. pneumoniae* causes. Among these virulence factors is pneumolysin (Ply), a multifunctional protein that is found in virtually all clinical isolates and contributes to both colonization and disease. Ply is a 53-kDa member of a family of proteins known as the cholesterol-dependent cytolysins (CDCs). CDCs bind cholesterol on a eukaryotic membrane surface, oligomerize, and form ~400-Å-diameter pores, causing cell lysis and tissue damage. In addition to its ability to cause direct tissue damage, Ply can modulate the immune system in several ways, including by the stimulation of polymorphonuclear leukocytes and complement activation (6, 21, 25). All of these Ply functions require an extracellular localization, but unlike all other known members of the Gram-positive pathogen CDC family (e.g., perfringolysin O, streptolysin O, and listeriolysin O), Ply lacks an N-terminal signal sequence for secretion through the canonical Sec machinery (32). Moreover, Ply lacks other known signals for any of the previously described export systems (32). Early work on Ply reported that it has a cytoplasmic localization in *S. pneumoniae* (17). That report, along with the finding that Ply lacks a signal sequence and the observation that Ply is not found in culture supernatants until cells have undergone, autolysis suggested a cell lysis-dependent release of Ply (24, 32). The release of Ply into the

host milieu upon autolysis has been a working hypothesis in the field for the past 20 years, and therefore, subsequent studies that have analyzed Ply's activity or presence have considered only a cytoplasmic or lysis-mediated extracellular location.

Several lines of evidence contradicting this model have emerged. Certain strains have been reported to release Ply into the culture supernatant in early stationary phase when autolysis of the culture has not yet occurred (4). Additionally, Ply has been reported to be released in the absence of the major autolysin LytA, indicating an autolysis-independent release of Ply (1). Subsequently, we confirmed the autolysis-independent export and, in addition, showed that the exported Ply localizes primarily to the bacterial cell wall (26). Here we build on these observations and show that Ply which is released by *in vitro* autolysis of stationary-phase cultures does not reassociate with the cell wall of actively growing *S. pneumoniae* when added exogenously, further supporting the autolysis-independent export and cell wall localization model. We identify a domain of Ply that is required for export. Additionally, we show that a signal sequence is not sufficient for Sec-dependent Ply secretion in *S. pneumoniae* but is sufficient in the surrogate host *Bacillus subtilis*. Finally, using the native ply sequence, we show that a signal sequence-less protein export pathway also exists in *B. subtilis*.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. *S. pneumoniae* was grown to an optical density at 600 nm (OD₆₀₀) of 0.3 in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) plus 5 μl/ml Oxyrase (Oxyrase, Inc., Mansfield, OH). These cultures were

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TABLE 1 Strains used in this study

Species and strain	Alternative strain name	Relevant genotype or description	Reference or source
<i>S. pneumoniae</i>			
AC353	TIGR4	TIGR4, Sm ^r derivative	11
AC2394	acaps TIGR4	AC353 Δ <i>cps4A</i> '-4L	R. Iyer
AC4037	2394 Δ <i>ply</i> :: <i>spc</i>	AC2394 Δ <i>ply</i> :: <i>spc</i> , Spc ^r	This study
AC4038	Ply-HA	AC2934, Ply tagged with HA epitope, Cm ^r	This study
AC4039	N-1	AC4038, Ply aa 1-21 removed, Cm ^r	This study
AC4041	N-4	AC4038, Ply aa 1-197 removed, Cm ^r	This study
AC4043	C-2	AC4038, Ply aa 343-471 removed, Cm ^r	This study
AC4044	C-3	AC4038, Ply aa 319-471 removed, Cm ^r	This study
AC4045	SS ^{RrgB} -Ply	AC2394, SS from RrgB fused to <i>ply</i> , Cm ^r	This study
AC4046	PfoNoSS	AC2394, <i>pfo</i> without SS in <i>ply</i> locus, Cm ^r	This study
AC4047		AC2394, Ply with Pfo D2 in <i>ply</i> locus, Cm ^r , HA tag	This study
AC4048		AC2394, Pfo with Ply D2 in <i>ply</i> locus, Cm ^r , HA tag	This study
AC4049	D2	AC2394, Ply's D2 replacement with GGS linkers, Cm ^r	This study
<i>B. subtilis</i>			
AC4050		AC4052, Ply under control of Pspac* at <i>amyE</i> locus, Cm ^r	This study
AC4051		AC4052, SS ^{RrgB} -Ply under control of Pspac* at <i>amyE</i> locus, Cm ^r	This study
AC4052	JH642	<i>trpC2 pheA1</i>	13
<i>E. coli</i>			
AC1000		pAC1000, Cm ^r	12
AC578		pMmeISpc, Spc ^r	19

mixed with glycerol to a final concentration of 20% glycerol, aliquoted into 300- μ l “starter cultures,” and stored at -80°C . Starter cultures were thawed and diluted into THY broth plus Oxyrase and grown to mid-exponential phase ($\text{OD}_{600} = 0.4$ to 0.5).

The *Bacillus subtilis* strains were grown on LB plates overnight and were used to inoculate THY broth to a starting OD_{600} of 0.01 to 0.02. Cultures were grown with shaking at 37°C to an OD_{600} of 0.4 to 0.5.

Strain construction. The *S. pneumoniae* strains listed in Table 1 were constructed using the splicing by overlap extension (SOE) PCR method with the primers listed in Table 2 (14). A marked deletion of *ply* (AC4037, *ply*::*spc*) was constructed in three parts, i.e., “Up,” “Spc,” and “Down,” and transformed into AC2394, an acapsular derivative of TIGR4 (AC353), where it replaced the *ply* allele by double-crossover homologous recombination. All subsequent mutants were transformed into this new background, such that the *spc* marker would be replaced with a mutated version of *ply* and a *cat* chloramphenicol resistance cassette. AC4038 was constructed in four parts, i.e., “Up,” “*ply*-HA,” “*cat*,” and “Down,” using AC1000 as the template for “*cat*” and AC2394 genomic DNA (gDNA) as the template for all other parts. The construct was transformed into strain AC4037 and replaced the *spc* allele. Truncation mutants and domain swaps (AC4039, AC4041, AC4047, AC4048, and AC4049) were constructed in three parts, i.e., “Up,” “*ply*'-HA,” and “*cat*-down,” using gDNA from strain AC4038. AC4046 was constructed in three parts, i.e., “Up,” “*pfo*,” and “Down.” Genomic DNA from AC2394 served as the template for “Up” and “Down”; gDNA from *Clostridium perfringens* (a gift of A. L. Sonenshein) served as the template for “*pfo*.” AC4048 was constructed in four parts, i.e., “Up,” “*pfo* with Ply D2 a,” “*pfo* with Ply D2 b,” and “Down.” Genomic DNA from AC2394 served as the template for “Up” and “Down”; gDNA from AC4046 served as the template for “*pfo* with Ply D2.”

The *B. subtilis* strains expressing the signal sequence (SS) from RrgB fused to *ply* (SS^{RrgB}-Ply) and Ply were constructed by first amplifying the SS^{RrgB}-Ply and Ply sequences from the gDNAs of strains SS^{RrgB}-Ply and AC2394, respectively. These PCR products were cloned into the blunt-ended cloning vector pCRscript (Stratagene) to introduce EcoRV and SacI restriction enzyme sites. The insert was cut from the vector and ligated into the prepared integrative plasmid pAWS8 (28). Plasmid pAWS8 contains the yeast aconitase gene *acoI* under the control of the constitutively active Pspac* promoter between two segments of the nonessential *amyE* gene.

The *acoI* gene was removed from pAWS8 by first digesting with HindIII, blunting the plasmid with Klenow, and then digesting the plasmid with SacI, thus removing *acoI* and creating a blunt end and a SacI overhang. The *ply* insert (with a blunt 5' end and a 3' SacI overhang) was ligated into the cut vector with T4 DNA ligase (NEB) and transformed into *Escherichia coli* DH5 α (for the *ply* construct) and *E. coli* JM107 (for the SS^{RrgB}-Ply construct). Colonies were picked, and plasmids were isolated with the QIAquick miniprep kit (Qiagen). The resulting plasmids were used in a transformation into *B. subtilis* strain JH642 (9, 13). Colonies were tested for double crossover by amylase activity assays, colony PCR, and sequencing.

Fractionation of *S. pneumoniae* and *B. subtilis*. Cells were grown to an OD_{600} of 0.4 to 0.5 and centrifuged at $4,000 \times g$ for 7 min. The culture supernatants were saved, and proteins were precipitated with 10% (vol/vol) trichloroacetic acid (TCA) and resuspended in 70 μ l 50 mM Tris, pH 7.5. The cell pellets were washed once with THY, resuspended in 70 μ l cell wall digestion buffer (1 \times protease inhibitor cocktail [Roche], 300 U/ μ l mutanolysin, and 1 mg/ml lysozyme in a 30% sucrose–10 mM Tris [pH 7.5] buffer), and incubated at 37°C for 2 h with rocking. Protoplasts were separated from the cell wall by centrifugation at $13,100 \times g$ for 10 min. Protoplasts were resuspended in 70 μ l 10 mM Tris buffer, pH 7.5.

Hemolytic assays. Hemolytic assays were performed as described previously (1) with some modifications. Samples of culture supernatants (not TCA precipitated), cell walls, and protoplasts were pooled, and 100 μ l of each fraction was 2-fold serially diluted in assay buffer (10 mM dithiothreitol [DTT] and 0.1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]) in a 96-well V-bottom plate. Fifty microliters of triple-washed 2% sheep red blood cells (RBCs) was added to the dilutions and incubated for 1 h at 37°C . Plates were spun at $233 \times g$ for 10 min, and hemolytic units were determined visually. Hemolytic units are equal to the reciprocal of the highest dilution at which there was 100% lysis. Cell wall and protoplast hemolytic units were divided by 10 to normalize them to the same volume as the culture supernatant.

Western blot analysis. Equal volumes of samples (cell equivalents) were boiled in SDS sample buffer (50 mM Tris–Cl [pH 6.8], 2% SDS, 0.5% bromophenol blue, 10% glycerol, 100 mM β -mercaptoethanol), cooled, loaded onto an SDS-polyacrylamide gel, and run for 25 min at 75 V and

TABLE 2 Primers used in this study

Strain	Primer no.	Primer name	Sequence (5' → 3')	
AC4037	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP101	Up R1	CCGGGCCCCCCTCGAGGTCTTCTACCTCCTAATAAGTTCCTGGA	
	KP102	SpC F2	CTTATTAGGAGGTAGAAGACCTCGAGGGGGGGCCCGGTACCGAGG	
	KP103	SpC R2	GCATTCTCCTCTCTATAAATTTTTTAAATCTGTTATTTAAATAGTTTATAG	
	KP104	Down F3	CAGATTAATAAATTATAAGAGAGGAGAATGCTTGGCACA	
AC4038	KP032	Down R3	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP081	Up R1	TTCTCCCTGATGGGTCATCTTCTACCTCCTAATAAGTTCCTGGA	
	KP082	PlyHA F2	ATTAGGAGGTAGAAGATGACCCATCAGGGAGAAAAGTATTGAA	
	KP034	Cat R2	TTCTCCTCTCTTATAAAAGCCAGTCATTAGGCCT	
AC4039	KP031	Down F3	GCTTTTATAAGAGAGGAGAATGCTTGGCACA	
	KP032	Down R3	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP083	Up R1	TACAGAAATATCACTCATCTTCTACCTCCTAATAAGTTCCTGGA	
	KP084	PlyCatDown F2	ATTAGGAGGTAGAAGATGAGTGATATTTCTGTAAACAGCTACCAAC	
AC4041	KP032	PlyCatDown R2	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
AC4043	KP089	Up R1	GAGATAGACTTGGCGCATCTTCTACCTCCTAATAAGTTCCTGGA	
	KP090	PlyHACatDown F2	ATTAGGAGGTAGAAGATGCGCCAAGTCTATCTCAAGTTGGA	
	KP032	PlyHACatDown R2	GCCCCATTTACGTCCCATTAG	
	KP038	PlyHA F1	CAGTCGCCTCTATCCTGGAG	
	KP039	PlyHA R1	AGATCCTAAGCATAGTCTGGTACATCGTAGGGGTATCTGTAAGCTGTAACTTAGTCTC	
AC4044	KP040	PlyHACatDown F2	AGCTTACAGATACCCTACGATGTACCAGACTATGCTTAGGATCTCGAGGTGACGGTAT	
	KP032	PlyHACatDown R2	GCCCCATTTACGTCCCATTAG	
	KP041	PlyHA F1	CAGTCGCCTCTATCCTGGAG	
	KP042	PlyHA R1	AGATCCTAAGCATAGTCTGGTACATCGTAGGGGTACGCAACTACATTGTCACGTA	
	KP043	PlyHACatDown F2	TGTAGTTGCGTACCCTACGATGTACCAGACTATGCTTAGGATCTCGAGGTGACGGTAT	
AC4045	KP032	PlyHACatDown R2	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP191	rrgB SS R1	TGCTTTATTTGCCATCCAGCCGCAAAAACCTGTTGCAGCTGAAAACAGGCTACTCGCTGCAGTAATA	
			AGGCAGCAAGCATTGTTAAAAATTTGTTGATTGATTTTCATCTTCTACCTCCTAATAAGTTCCTGG	
	KP190	rrgB SS F2	ATTAGGAGGTAGAAGATGAAATCAATCAACAAAATTTTAAACAATGCTTGTGCTGCCTTATTACTGACAGCG	
AC4046			AGTAGCCTGTTTTCAGCTGCAACAGTTTTTGGCGGTGGGATGGCAATAAAGCAGTAATGACTT	
	KP032	Down R2	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP201	<i>ply::noSSPfo</i> R1	ACTTTGATTTCATCTTCTACCTCCTAATAAGTTCCTGG	
	KP200	<i>ply::noSSPfo</i> F2	AGGAGGTAGAAGATGAATCAAAAGTATTGATTCTGGAATATCA	
	KP199	<i>ply::pfo</i> R2	GACCTCGAGATCTTAATTGTAAGTAATACTAGATCCAGGG	
	KP197	<i>ply::pfo</i> F3	ACTTACAATAAGATCTCGAGGTGACGGTAT	
	KP032	Down R3	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP212	Ply with Pfo D2 R1	AAATTTATACCAGCTCTTTTACCTTCCCTTTGGAACAAAACCTTCAATTTTATCTCCATTACTAGCCAA	
AC4047			GAGTTTCTTTTATCGTAATTCA	
	KP213	Ply with Pfo D2 F2	GAAGGTAAAAAGACTGGTAATAAATTTATAGTTGTAGAACGTCAAAAAAGATCCCTTACAACATCACC	
			AAGTGATATTTCTGTAACAGCTACCA	
	KP214	Ply with Pfo D2 R2	AGTTGTTCTATATAATCTGTTTTATTGTGAACAGCCGCAACTACATTGTCACGTA	
	KP215	Ply with Pfo D2 F3	ACAGATTATATAGAAACAACCTTCTACAGAGTATTTCTAACGGAGATTACTGCTGG	
	KP032	Down R3	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP216	Pfo with Ply D2 R1	AAACTCATCGGGTAGCTGATTACCCTCTTTGATGAAACGATTTTCAATACTTTCTCCCTGATGGGTTA	
			AAACTTCATTTCTATTGTAACCTAAGC	
	KP217	Pfo with Ply D2 a F2	ATCAAAGAGGGAATCAGCTACCCGATGAGTTTGTGTTATCGAAAAGAAAGAGCGGAGCTTGTGCA	
AC4048			CAAATACAGTAGATATATCAATAATTGATTCTGTAATGAC	
	KP218	Pfo with Ply D2 a R2	AGCTGTAACCTTAGTCTCAACATAGCTCTGACTACTATTTTGAAGGTAGCAACTGAGTTATCTTTAAGAAAAAC	
	KP219	Pfo with Ply D2 b F3	ACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAGGGAAAAATAAAGTATGATCATAGTG	
	KP229	Pfo with Ply D2 b R3	GTACATCGTAGGGGTAATTGTAAGTAATACTAGATCCAGGG	
	KP228	Pfo with Ply D2 F4	GTATTACTTACAATTAACCCCTACGATGTACCAGA	
	KP032	Down R4	GCCCCATTTACGTCCCATTAG	
	KP064	Up F1	TTCTGGAGTTTCCCGATTTC	
	KP208	Ply domain 2 R1	AGAAATATCACTAGAACCACCAGAACCACCAAGAGTTTCTTTTATCGTAATTCATAG	
	KP209	Ply domain 2 F2	AAGAACTCTTGGGTGGTCTGGTGGTCTAGTGATATTTCTGTAACAGTACC	
	KP210	Ply domain 2 R2	AGTAAATCTCCGTTGAACACCACCAGAACCACCAGAACCACCAGAACCACCAGAACCCCGCAACTA	
AC4049			CATTGTCACGTA	
	KP211	Ply domain 2 F3	AATGTAGTTGGGGTGGTCTGGTGGTCTGGTGGTCTGGTGGTCTGGTGGTCTAACGGAGATT	
			TACTGCTGG	
	KP032	Down R3	GCCCCATTTACGTCCCATTAG	
	KP245	Ply in B.s. F	TAAGGAGGTGTTATATATGGCAAATAAAGCAGTAAATGACTTT	
	KP246	Ply in B.s. R	CTAGTCATTTTCTACCTTATCTTCTACCTG	
	AC4051	KP244	SS Ply B.s. F	TAAGGAGGTGTTATATATGAAATCAATCAACAAAATTTTAAACAATGC
		KP246	Ply in B.s. R	CTAGTCATTTTCTACCTTATCTTCTACCTG

for an additional 90 min at 125 V. Proteins were transferred to a nitrocellulose membrane at 30 V for 75 min. Equal loading of samples on each gel was confirmed by staining the membrane with Ponceau S prior to Western blotting (data not shown). Western blotting was performed using the Snap-ID system (Millipore) according to the manufacturer's directions. Membranes were cut and blocked with NAP blocker (GBiosciences) diluted 1:2 in Tris-buffered saline (TBS). Primary antibody to either Ply

(Statens Serum Institut) at 1:1,000, CodY (a gift of A. L. Sonenshein) at 1:10,000, RrgB (18) at 1:2,500, Pfo (a gift of R. Tweten) at 1:1,000, or hemagglutinin (HA) (Santa Cruz) at 1:2,500 in NAP blocker diluted 1:4 in TBS was applied to the membrane for 10 min at room temperature. Membranes were washed three times with TBS. Cy5-conjugated antibody (Invitrogen) was applied to the membrane at a 1:2,500 dilution in NAP (diluted 1:4 with TBS) for 10 min. Membranes were washed as described

above and scanned with a Fuji FLA-9000 instrument at 635 nm excitation and with the DBR filter.

Reassociation assay. A TIGR4 culture was allowed to grow overnight and autolyze. The supernatant was separated from the cell debris by centrifugation ($4,000 \times g$ for 7 min). The resulting supernatant was filtered through a $0.22\text{-}\mu\text{m}$ filter to remove any remaining cells. This cell-free supernatant was then applied to a *ply* culture and allowed to incubate with *ply* cells throughout growth. Wild-type cultures and *ply* cultures with THY alone served as positive and negative controls for the presence and absence of Ply, respectively. All cultures were grown to an OD_{600} of 0.6, fractionated, and assayed for the presence of Ply and CodY by Western blotting.

Protein purification. Ply-HA and D2-HA proteins were purified from strains AC4038 and AC4049, respectively. Strains were grown to mid-exponential phase, harvested by centrifugation, and resuspended in lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 2 mM MgCl_2 , $1 \times$ Roche complete mini-protease inhibitor cocktail). Each sample was treated with lysozyme (1 mg/ml; Sigma) at 37°C for 1 h, after which the cells were broken apart using a Bead Beater and 0.1-mm zirconia beads. Appropriate samples were pooled and treated with Benzonase (1,250 units; Sigma) at 37°C for 30 min. The resulting lysate was cleared by centrifugation at $100,000 \times g$ for 1 h at 4°C using a Beckman Vti 65.2 rotor. The S100 fractions were collected and used as the source of protein for immunoprecipitation with Pierce anti-HA agarose (Thermo Scientific) as described by the manufacturer. Elution was carried out with Pierce HA peptide (Thermo Scientific), and eluted fractions were run on SDS-PAGE and stained with Coomassie brilliant blue to assess purity. Appropriate fractions were pooled, glycerol was added to a final concentration of 20%, and protein was stored at -20°C .

Limited proteolysis. Approximately equivalent amounts of each purified protein, i.e., Ply-HA, D2-HA, or Ply* (His-tagged Ply toxoid protein, a gift of J. C. Paton), were incubated either alone or in the presence of $0.4 \mu\text{g}/\mu\text{l}$ μg of trypsin. Trypsin alone was incubated under the same conditions as a control. Equal amounts of each reaction were analyzed by Western blotting with an HA antibody.

Model of Ply. The molecular model of Ply was made using the ModWeb program and manipulated in MacPyMol software.

RESULTS

Ply released by autolysis cannot reassociate with *S. pneumoniae*. There are several examples of streptococcal proteins that localize to the cell wall despite lacking a signal sequence or cell wall-anchoring motif (16, 22, 23). Among them, enolase has been shown to reassociate with the surface of *S. pneumoniae* (5). This reassociation leaves open the possibility of an autolysis-dependent release of Ply as opposed to a true mechanism of export from intact, live cells. We have shown previously that Ply localizes to the cell wall in a LytA-independent manner (26), but it is still possible that limited lysis promoted by other, minor lysins releases Ply, which could then reassociate with the cell wall of neighboring, intact cells. To test this possibility, wild-type *S. pneumoniae* strain TIGR4 was grown overnight in broth to a state of complete autolysis. The supernatant was collected by centrifugation and filtered to ensure that there were no cells remaining. The cell-free supernatant containing Ply released by autolysis was added to a *ply* mutant culture at the time of inoculation. Cultures of wild-type and *ply*-deficient strains with no added cell-free supernatant were grown as positive and negative controls for the presence or absence of Ply, respectively. Cultures were grown to mid-exponential phase, fractionated, and assayed by Western blotting for the presence of Ply and CodY, a cytoplasmic control protein. Exogenously added, autolysis-released Ply is detected only in the supernatant and not in the cell wall or protoplast fraction (Fig. 1). This

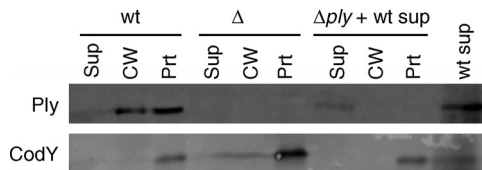


FIG 1 Ply released by autolysis cannot reassociate with intact cells. A wild-type (wt) culture was grown overnight and allowed to autolyze. The supernatant was collected, filtered, and added to a Δ *ply* culture. Wild-type and Δ *ply* cultures without added supernatant from an autolyzed culture were grown as positive and negative controls for the presence or absence of Ply, respectively. All cultures were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. Equal cell equivalents were loaded on the gel. Sup, culture supernatant; CW, cell wall; Prt, protoplast.

result indicates that Ply released by autolysis cannot reassociate with *S. pneumoniae*, further supporting the autolysis-independent model for Ply release and suggesting that there is a Ply export pathway that is coupled to cell wall localization.

In *S. pneumoniae*, Ply is incompatible with an exogenously added signal sequence. Ply is the only known member of the CDC family that lacks a signal sequence. Since Ply shares extensive sequence identity with the mature portions of other, Sec-secreted CDCs (having, for example, 48% identity with perfringolysin O [Pfo] [31]), we hypothesized that we could create a Sec-secreted derivative if we supplied Ply a signal sequence. To test this hypothesis, the strain SS^{RrgB} -Ply, in which a canonical Sec signal sequence was fused to Ply, was constructed (Fig. 2A). The signal sequence is derived from RrgB, an *S. pneumoniae* secreted, cell wall-anchored protein. The SS^{RrgB} -Ply strain was grown, fractionated, and assayed for the presence of Ply and CodY by Western blotting. Surprisingly, SS^{RrgB} -Ply was not detected in any fraction (Fig. 2B). The lack of detectable Ply could be indicative of a defect in transcription or a posttranscriptional defect. To test the former possibility, quantitative reverse transcription-PCR (qRT-PCR) was performed on *ply* in the SS^{RrgB} -Ply fusion strain and in an acapsular derivative of TIGR4, which is known to exhibit wild-type expression and export of Ply (26). The amounts of *ply* transcript detected in the two strains were comparable (Fig. 2C). Thus, the lack of detectable protein is a posttranscriptional effect and may indicate that the SS^{RrgB} -Ply protein, if it is made, is incompatible with the Sec machinery in such a way that leads to its degradation.

Expression of the SS^{RrgB} -Ply fusion in *Bacillus subtilis*. There are two major players in secretion of a protein: the substrate and the export machinery. To examine why the SS^{RrgB} -Ply fusion protein was not detectable, we expressed the fusion in a heterologous host, *B. subtilis*. We hypothesized that if the fusion protein was intrinsically unstable in *S. pneumoniae*, then it would be degraded and undetectable in *B. subtilis* as well. However, if the degradation was instead due to a fault of the Sec machinery of *S. pneumoniae*, then the SS^{RrgB} -Ply fusion may be detectable and possibly secreted in *B. subtilis*. To examine these possibilities, the SS^{RrgB} -Ply fusion was placed under the control of the P_{spac^*} constitutively active promoter in the *amyE* locus on the chromosome of *B. subtilis* via allelic exchange (9, 28). The strain was grown to mid-exponential phase and fractionated into culture supernatant, cell wall, and protoplast; the presence of Ply was detected by Western blotting, and the activity of Ply was measured by hemolysis assay. SS^{RrgB} -Ply was detected in all three fractions, including the culture superna-

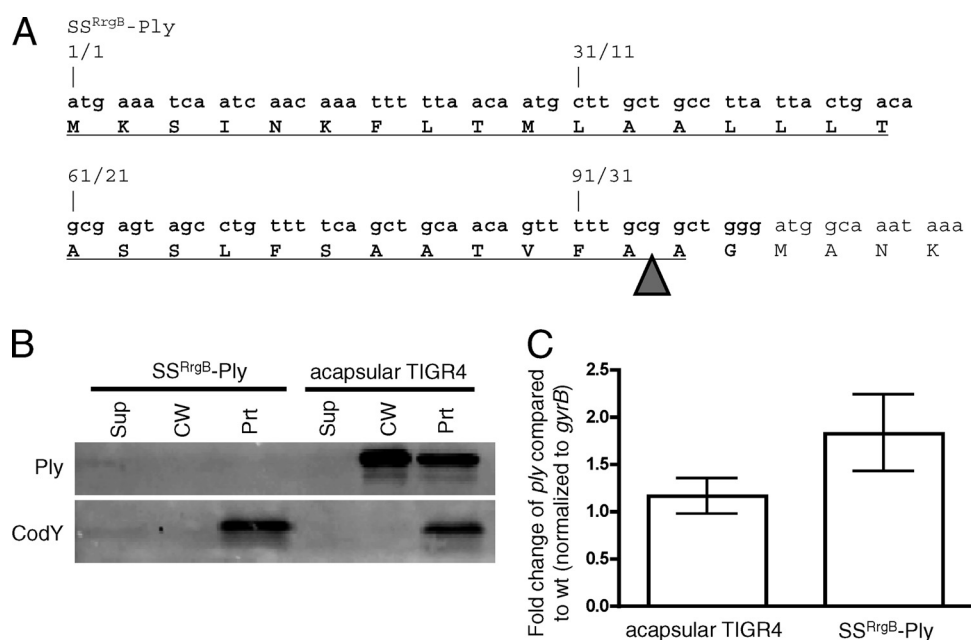


FIG 2 SS^{RrgB}-Ply fusion mRNA is made but protein is not detected in *Streptococcus pneumoniae*. (A) N-terminal DNA (top) and amino acid (bottom) sequences of SS^{RrgB}-Ply. Text that is not in bold is Ply sequence. The RrgB signal peptide is in bold and underlined. The arrowhead indicates the predicted site of signal peptidase cleavage. (B) Western blot of cell wall fractionation of SS^{RrgB}-Ply and acapsular TIGR4 cells. Cultures were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. Equal cell equivalents of each fraction were loaded on the gel. A representative Western blot from two independent experiments is shown. Sup, culture supernatant; CW, cell wall; Prt, protoplast. (C) Quantitative RT-PCR on the *ply* transcript in acapsular TIGR4 and SS^{RrgB}-Ply cells. Shown is the median fold change of the *ply* transcript of three replicates compared to acapsular TIGR4, normalized to *gyrB*; error bars indicate the range.

tant (Fig. 3A), which had the highest level of hemolytic activity (Fig. 3B). In the protoplast fraction, two species of Ply were detected, one at the expected molecular weight of the mature, signal peptidase-cleaved form of the protein and one at the expected molecular weight of the preprotein with its signal sequence still attached (Fig. 3A). In contrast, only the mature, cleaved form was detected in the cell wall and culture supernatant. There was no Ply or cross-reactive species detected in the parent strain *B. subtilis* JH642. Because the SS^{RrgB}-Ply fusion is cleaved, presumably by signal peptidase, and secreted by *B. subtilis* into the culture supernatant, this suggests that the SS^{RrgB}-Ply substrate is compatible with the Sec machinery of *B. subtilis*. Thus, the failure of SS^{RrgB}-Ply

to be secreted or even detected in *S. pneumoniae* is likely due to its Sec machinery being unable to process and secrete the fusion protein.

The Ply homolog Pfo lacking its signal sequence localizes primarily to the protoplast and weakly to the cell wall in *S. pneumoniae*. If our hypothesis that the instability of SS^{RrgB}-Ply in *S. pneumoniae* is due to failure of the Sec machinery to process this substrate is correct, then a closely related homolog of Ply should be similarly incompatible and perhaps even toxic. To test this, we precisely replaced the Ply-coding sequence with that for full-length Pfo. Consistent with our hypothesis, this allele was not tolerated by *S. pneumoniae*, as the only viable transformants recovered had spontaneous

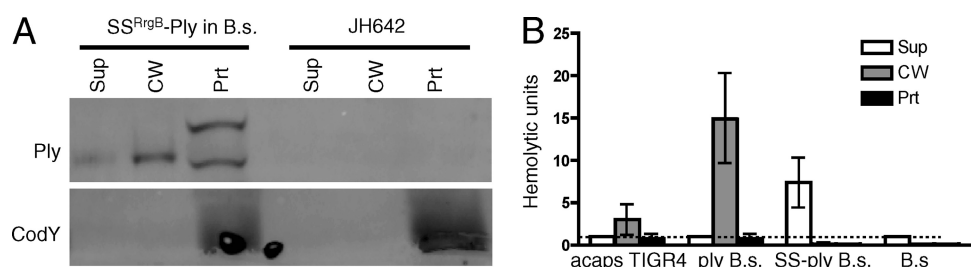


FIG 3 SS^{RrgB}-Ply expression in *Bacillus subtilis*. (A) SS^{RrgB}-Ply can be secreted when expressed in *B. subtilis*. SS^{RrgB}-Ply was placed under the control of the Pspac⁺ promoter in the *amyE* locus on the *B. subtilis* chromosome. All strains were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. SS^{RrgB}-Ply in B.s., SS^{RrgB}-Ply fusion in *B. subtilis*; JH642, wild-type *B. subtilis* parent strain. Equal cell equivalents were loaded on the gel. Sup, culture supernatant; CW, cell wall; Prt, protoplast. (B) Hemolytic assay of culture supernatant, cell wall, and protoplast fractions of acapsular *S. pneumoniae* (acaps TIGR4), *B. subtilis* with *ply* (ply B.s.), *B. subtilis* with SS-*ply* (SS-ply B.s.), and *B. subtilis* parent strain JH642 (B.s.). The *ply* in *B. subtilis* has the same distribution as in *S. pneumoniae*, with the highest hemolytic activity in the cell wall fraction. The supernatant fraction has the highest hemolytic activity in the SS-*ply B. subtilis* strain. No hemolytic activity was detected in the *B. subtilis* parent strain. The dotted line indicates the limit of detection. Bars show the means for three biological replicates; error bars indicate standard errors of the means (SEM).

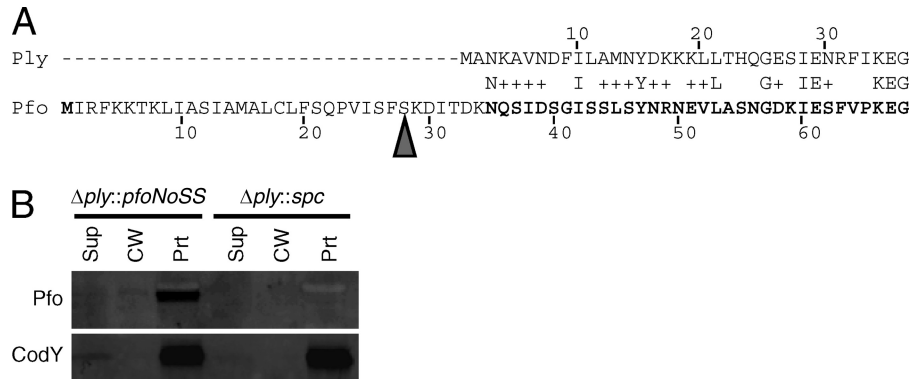


FIG 4 Pfo expression in *Streptococcus pneumoniae*. (A) Amino acid alignment of Ply (top) and Pfo (bottom). Sequence homology does not begin until the third amino acid of Ply; therefore, fusions were made at this amino acid. The arrowhead indicates the site of signal peptidase cleavage in Pfo. The Pfo sequence not in bold was excluded in the Pfo signal sequence-less construct, thus deleting the Pfo signal peptide and six amino acids after the signal peptidase cleavage that share no homology to Ply. (B) Expression of signal sequence-less Pfo in *S. pneumoniae*. Signal sequence-less Pfo is expressed in *S. pneumoniae*, and a small amount localizes to the cell wall. The Pfo signal sequence-less allele (*pfoNoSS*) was used to replace *ply* at the native locus (Δ *ply::pfoNoSS*). This strain and a negative-control strain in which the *ply* deletion was replaced with a spectinomycin resistance gene (Δ *ply::spc*) were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting with anti-Pfo and anti-CodY antibodies. Equal cell equivalents were loaded on the gel.

mutations that destroyed the signal sequence. Together with the inability to detect the SS^{RrgB}-Ply fusion in *S. pneumoniae*, this result suggests that there is a general incompatibility between CDC proteins and Sec in *S. pneumoniae*.

To confirm the incompatibility between Pfo and the *S. pneumoniae* Sec machinery, a signal sequence-less version of Pfo was expressed from the *ply* locus (Fig. 4A and the Fig. 4 legend give a description of the mutation). This mutant was not toxic to *S. pneumoniae* and was detectable with an antibody to Pfo (Fig. 4B). The majority of Pfo localized to the protoplast fraction, and no protein was detected in the culture supernatant. Interestingly, a small portion of Pfo localized to the cell wall fraction (Fig. 4B). This result suggests that the putative Ply export pathway can recognize another mature CDC protein but less efficiently.

Domain 2 is necessary for Ply export. To determine which domain(s) might be important for Ply export and cell wall localization, N- and C-terminal truncation analysis was used. Truncated sequences corresponded to structurally assigned domains of Ply (27). All truncated products have a C-terminal HA epitope tag for detection by Western blotting (a model of Ply structure and a schematic of the truncation mutants are shown in Fig. 5A and B).

Full-length Ply with an HA tag (Ply-HA) was exported and localized to the cell wall like the native Ply (Fig. 5C). The N-1 N-terminal truncation, which lacks a portion of domain 1 (amino acids [aa] 1 to 21), was detected at its predicted molecular mass and was localized to the cell wall and protoplast fractions similarly to the full-length Ply-HA (Fig. 5C). The N-4 mutant, which lacks portions of domains 1, 2, and 3 (amino acids 1 to 197), was detected at its predicted molecular mass but was found only in the protoplast and not in the cell wall or supernatant fraction (Fig. 5C).

For the C-terminal truncations, when domain 4 and a portion of domain 2 (amino acids 343 to 471) were truncated (C-2), the protein was detected at its predicted molecular mass but was found only in the protoplast and not in the cell wall or supernatant fraction (Fig. 5D). Similarly, a further C-terminal truncation of Ply lacking domain 4 and portions of domain 2 and 1 (amino acids 319 to 471) (C-3) was detected at its predicted molecular mass and

was found only in the protoplast and not in the cell wall or supernatant fraction (Fig. 5D). The C-3 mutant was weakly expressed, possibly indicating that the protein is unstable.

In both sets of truncations, when a portion of domain 2 was deleted, the mutant no longer localized to the cell wall, was absent from the culture supernatant, and instead was found in the protoplast. This suggests that domain 2, which comprises a beta-sheet between domains 1 and 4 in Ply (Fig. 5A and B), is necessary for export.

One caveat to the conclusion that domain 2 is necessary for Ply export is that all the truncation mutants lacking a portion of domain 2 are also missing portions of other domains. To more rigorously test this hypothesis, domain 2 was replaced with a flexible linker (Gly-Gly-Ser repeats), leaving the rest of the protein's primary sequence unchanged. The N-terminal portion of domain 2 was replaced with two repeats, while the C-terminal portion of domain 2 was replaced with five repeats. These repeat lengths, which were based on the known length and flexibility of the linker (20), were chosen to maintain the three-dimensional spacing of the remaining domains relative to each other. When analyzed for localization, the domain 2 replacement mutant (D2) was detected at its predicted molecular mass but was found only in the protoplast and not in the cell wall or supernatant fraction (Fig. 6A). Thus, the localization of the D2 mutant recapitulates the truncation analysis, providing further evidence for the role of domain 2 in Ply export.

To ensure that the domain 2 linker replacement did not result in gross alterations to the three-dimensional structure of the protein, purified full-length Ply-HA and D2-HA were subjected to limited proteolysis (Fig. 6B). Approximately equivalent amounts of each purified protein were incubated either alone or in the presence of identical amounts of trypsin. Trypsin alone and Ply* (polyhistidine-tagged Ply toxoid) plus trypsin served as negative controls. Equal amounts of each reaction product were analyzed by Western blotting with an HA antibody. The proteolytic degradation products of both Ply-HA and D2-HA are nearly identical (Fig. 6B, asterisks), which suggests that the trypsin-accessible sites and thus the three-dimensional structures of the proteins are sim-

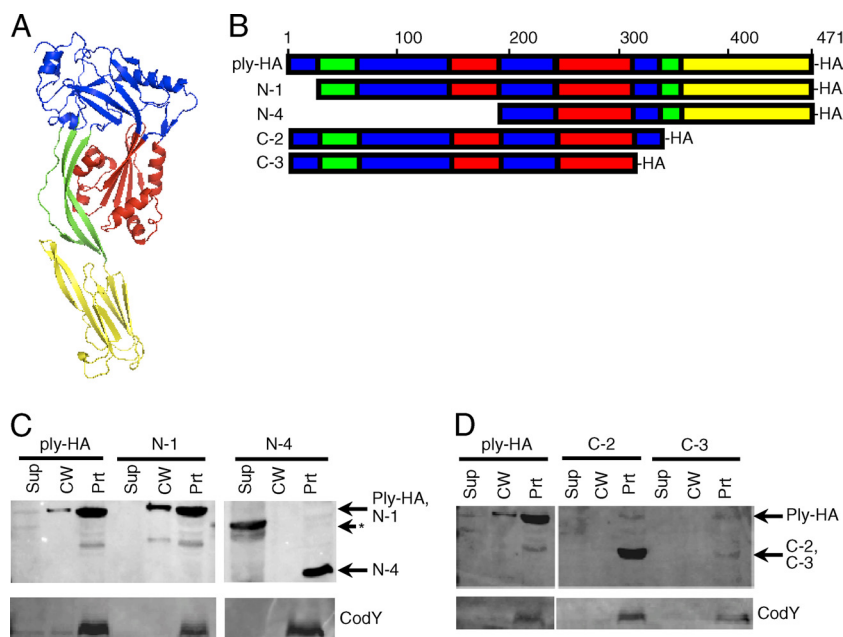


FIG 5 Ply truncation mutants reveal that domain 2 of Ply may be required for export. (A) Model of Ply three-dimensional structure. The model was made with ModWeb software and manipulated with MacPyMol software. Domains are color coded: blue, domain 1; green, domain 2; red, domain 3; yellow, domain 4. (B) Schematic of primary sequences of Ply and truncation mutants. Domains are color coded as in panel A. Truncations of Ply were made from the N and C termini, tagged with an HA epitope at the C terminus of each construct, and crossed into the *ply* locus on the *S. pneumoniae* chromosome. Strains were grown to mid-exponential phase, fractionated into cell wall and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. (C) N-terminal truncation mutants. The band in the supernatant lane for the N-4 truncation (shown by the asterisk) is of incorrect size and is unexplained. (D) C-terminal truncation mutants. Equal cell equivalents were loaded on the gel. Sup, culture supernatant; CW, cell wall; Prt, protoplast.

ilar. The presence of a single unique product in each (Fig. 6B, circles) suggests a local change in surface accessibility that simultaneously exposes one trypsin cleavage site while removing another. The latter is consistent with the removal of domain 2, which

contains several potential trypsin cleavage sites, and its replacement with a GGS linker that lacks trypsin cleavage sites.

Domain 2 swaps between Ply and Pfo show that Ply domain 2 is necessary for Ply export. Given that Pfo localizes to the cell wall

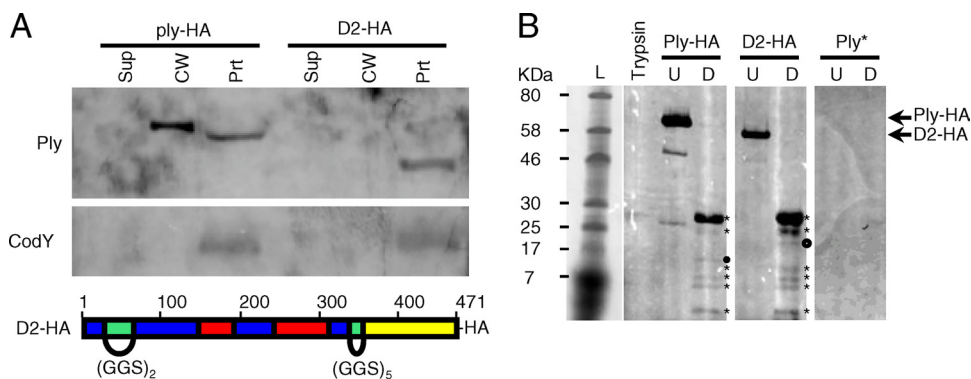


FIG 6 Ply with domain 2 replacement does not localize to the cell wall. Domain 2 was replaced with flexible linkers comprised of GGS repeats of appropriate length to maintain the three-dimensional spatial orientation of the remaining domains, tagged with a C-terminal HA epitope, and crossed into the *ply* locus on the *S. pneumoniae* chromosome. (A) Top, the *ply*-HA and D2 strains were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. Equal cell equivalents were loaded on the gel. Sup, culture supernatant; CW, cell wall; Prt, protoplast. Bottom, schematic of the D2 replacement mutant, color coded by domain structure as in Fig. 5; blue, domain 1; green, domain 2; red, domain 3; yellow, domain 4. (B) Limited proteolysis of Ply with the domain 2 linker replacement reveals a wild-type conformation. Ply-HA and D2-HA were purified from strains Ply-HA and D2, respectively, via immunoprecipitation. His-tagged Ply toxoid protein (Ply*) served as a negative control for the anti-HA antibody. Approximately equivalent amounts of each purified protein were incubated either alone (U) or in the presence of identical amounts of trypsin (D). Trypsin alone was incubated under the same conditions. Equal amounts of each reaction product were analyzed for the presence of HA-tagged trypsin-cleaved peptides by Western blotting. The proteolytic degradation products of Ply-HA and D2-HA (marked with asterisks) are nearly identical. The presence of a single unique product in each (marked with circles) suggests a local change in surface accessibility that simultaneously exposes one trypsin cleavage site while deleting or masking another.

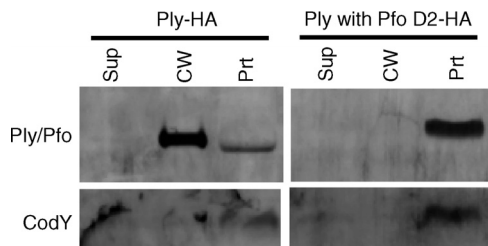


FIG 7 Ply domain 2 is necessary for export to the cell wall in *S. pneumoniae*. Domain 2 of Ply was replaced with domain 2 of Pfo and crossed into the *ply* locus on the *S. pneumoniae* chromosome. The strains carrying *ply*-HA and *ply* with Pfo's D2-HA were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. Equal cell equivalents were loaded on the gel. Sup, culture supernatant; CW, cell wall; Prt, protoplast.

of *S. pneumoniae* with low efficiency and that domain 2 is required for Ply export, we hypothesized that domain 2 of Ply is a signal for, or possibly directly involved in, Ply export in *S. pneumoniae*. To test this hypothesis, domain 2 swaps between Ply and Pfo were constructed and tested. Replacement of Pfo's domain 2 with that of Ply resulted in an unstable protein, as no protein was detected in any fraction using either Pfo or Ply antibodies (data not shown). Additionally, this mutant strain had a low growth rate (data not shown). Taken together these results suggest that the modified Pfo protein is unstable and somewhat toxic to *S. pneumoniae*. On the other hand, replacement of Ply's domain 2 with that of Pfo resulted in a protein that is detected at its expected molecular mass but is found only in the protoplast and not in the cell wall or supernatant fraction (Fig. 7). This result supports the hypothesis that domain 2 of Ply is necessary for, and possibly directly involved in, Ply export.

Expression of *ply* in *Bacillus subtilis*. To test whether the pathway that exports Ply in *S. pneumoniae* is conserved in other Gram-positive bacteria, we generated a *B. subtilis* strain that expresses native *ply* under the control of the Pspac* promoter in the same manner as the SS^{RrgB}-Ply fusion. The *ply*-expressing *B. subtilis* strain was grown to mid-exponential phase, fractionated, and assayed for the presence of Ply and CodY by Western blotting and for the activity of Ply by hemolytic assays. Ply was detected in all fractions, with the strongest band in the cell wall, which also had the highest level of hemolytic activity (Fig. 8 and 3B). This result suggests that the putative Ply export pathway is conserved in *B. subtilis* or, alternatively, that Ply is autosecretory.

DISCUSSION

One commonality to all known functions of Ply is where they take place—outside the bacterial cell (6, 7, 25, 27). Homologs of Ply, which share similar functions, contain signal sequences to direct their secretion through the type II (Sec) secretion system. Ply, in contrast, does not contain a signal sequence (32) and was initially hypothesized to be released by autolysis (24). We have previously shown that Ply is cell wall localized in a *lytA*-independent manner (26); however, the possibility still remained that limited autolysis caused by other lysins may release Ply, which could then reassociate with cells.

There are other proteins lacking a recognizable signal sequence or cell wall anchoring motif that “moonlight” on the bacterial surface, such as enolase and streptococcal surface GAPDH (SDH)

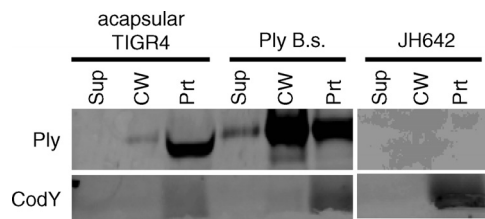


FIG 8 Ply localizes to the cell wall when expressed in *Bacillus subtilis*. The *ply* gene was placed under the control of the Pspac* promoter in the *amyE* locus on the *B. subtilis* chromosome (strain Ply B.s.). The *B. subtilis* parent strain, JH642, served as a control. Strains were grown to mid-exponential phase, fractionated into supernatant, cell wall, and protoplast compartments, and assayed for the presence of Ply and CodY by Western blotting. Equal equivalents were loaded on the gel. Sup, culture supernatant; CW, cell wall; Prt, protoplast.

(5, 16, 22, 23). Enolase has been shown to reassociate with the cell surface (5), leaving open the possibility that a limited number of cells lyse and release their cytoplasmic contents, which then stick to the surfaces of nearby intact, unlysed cells. However, a recent study analyzing the localization of SDH in *S. pyogenes* showed that the protein could be held inside the cell with a hydrophobic tail, lending support to the hypothesis that there is a dedicated export pathway for this protein (16). Here we show that exogenous Ply that has been released by autolysis cannot reassociate with cells. Combined with our previous work showing Ply in the cell wall fraction in a *lytA*-independent manner (26), we conclude that Ply localized to the cell wall is not the result of autolysis and that there must be an autolysis-independent Ply export pathway.

It is striking that Ply is not secreted through the Sec machinery, in contrast to the case for all other known members of the CDC family. This difference in Ply secretion mechanism could simply be due solely to the lack of a signal sequence in *ply* or, in addition, to an unknown incompatibility between Ply and the Sec machinery. To address these possibilities, we expressed Ply containing a known functional signal peptide from RrgB, an *S. pneumoniae* secreted protein (18). When the SS^{RrgB}-Ply fusion was expressed in *S. pneumoniae*, there was no detectable Ply protein. The transcription of this gene was comparable to that of wild-type *ply*, indicating that the defect in protein expression is posttranscriptional in nature. Assuming that the RrgB signal peptide directs the fusion protein to the Sec machinery, we hypothesize that SS^{RrgB}-Ply is incompatible with the Sec machinery of *S. pneumoniae* in such a way that leads to Ply degradation.

This putative signal sequence-dependent degradation of Ply is reminiscent of results with MalE-LacZ fusions from classic studies that identified and defined signal sequences in *Escherichia coli* (2, 3). According to those experiments, the addition of a signal sequence directed LacZ to the membrane, but the Sec machinery was unable to secrete it. Thus, LacZ is incompatible with the Sec machinery. In our case, two possibilities that could explain the SS^{RrgB}-Ply secretion defect are (i) incompatibility between the SS^{RrgB}-Ply fusion protein and the Sec machinery of Gram-positive bacteria in general (i.e., the defect lies with the substrate) or (ii) incompatibility between SS^{RrgB}-Ply and specifically the *S. pneumoniae* Sec machinery (i.e., the defect lies with the machinery). To test the first of these possibilities, we expressed SS^{RrgB}-Ply in the heterologous Gram-positive host *B. subtilis*. Strikingly, the SS^{RrgB}-Ply protein in *B. subtilis* is expressed and secreted. Thus, SS^{RrgB}-Ply is compatible with the Sec machinery of *B. subtilis*. This result

rules out the hypothesis that the defect of SS^{RrgB}-Ply secretion in *S. pneumoniae* lies with the substrate and suggests that the defect of SS^{RrgB}-Ply secretion in *S. pneumoniae* is likely due to components of the *S. pneumoniae* Sec machinery.

Given that the incompatibility between SS^{RrgB}-Ply and the Sec machinery was likely the Sec machinery of *S. pneumoniae*, we reasoned that natural signal sequence-containing CDCs might also be incompatible with the Sec machinery in *S. pneumoniae*. Therefore, we expressed a closely related homolog of Ply, perfringolysin O (Pfo), from the *ply* locus and determined its localization. We expressed two versions of Pfo, one with and one without its native signal sequence. Similar to the SS^{RrgB}-Ply result, Pfo with its signal sequence not only was undetectable but appeared to be detrimental to cell growth, since all viable transformants had frameshift mutations within the signal sequence or lacked the signal sequence altogether. These results indicate that there is an incompatibility between Pfo and the Sec machinery of *S. pneumoniae*. In contrast, a construct of Pfo lacking its signal sequence was tolerated by *S. pneumoniae* and was detected mainly in the protoplast fraction, with a small amount detected in the cell wall fraction. Thus, the putative Ply export pathway appears to be able to recognize and secrete other signal sequence-less CDCs, albeit with lower efficiency. We do not know if this putative Ply export pathway is the same pathway used to export the signal sequence-less proteins enolase and GAPDH. Testing of this possibility awaits identification and inactivation of the putative export pathway.

Though the identity of the hypothesized Ply export pathway remains unknown, we can nonetheless begin to characterize domains of Ply that are required for its export and cell wall localization. To this end, we used a series of terminal truncations and internal domain replacements to deduce that domain 2 is required for proper Ply localization. Domain 2 is a beta-sheet formed by two noncontiguous regions of Ply (amino acids 22 to 57 and 343 to 359). The function of this domain in the biology of Ply is in the prepore-to-pore transition. The alpha-helices of domain 3, which ultimately form the transmembrane hairpins (TMHs) that form the pore in the eukaryotic membrane, are stabilized by domain 2. Once the TMHs have formed, domain 2 is responsible for the vertical collapse of the protein that allows the TMHs to reach the target membrane and pierce it (8, 30). It is therefore possible that in the absence of domain 2, the domain 3 alpha-helices are not stabilized and can form beta-sheets that might be able to insert into the bacterial cell's membrane or result in a conformation not recognized by the putative export pathway, thus preventing its export. However, given that limited proteolyses of Ply-HA and D2-HA yield nearly identical banding patterns, it is unlikely that the D2-HA protein is misfolded, lending more evidence to the hypothesis that domain 2 is directly involved in the export of Ply.

Given that domain 2 is required for Ply export and that a signal sequence-less version of Pfo is somewhat competent for export and localization to the cell wall when expressed in *S. pneumoniae*, we determined whether domain 2 is necessary and sufficient for cell wall localization of CDCs in *S. pneumoniae*. Ply with Pfo's domain 2 is not exported, further suggesting that domain 2 is necessary for Ply export. Unfortunately, Pfo with Ply's D2 is undetectable in *S. pneumoniae*, so we cannot conclude whether Ply's domain 2 is sufficient for export. It may be possible in future studies to identify critical residues in domain 2 by site-specific mutagenesis and thus refine this model.

It appears that there is a domain 2-dependent Ply export path-

way in *S. pneumoniae*. It is possible that this putative export pathway is specific to *S. pneumoniae* or instead might be conserved among other bacteria. To begin testing how conserved this putative pathway is, a *B. subtilis* strain that expresses native *ply* under the control of a constitutively active promoter was constructed. Ply localizes in *B. subtilis* much like it does in *S. pneumoniae*, indicating that the putative Ply export pathway is conserved in *B. subtilis*. However, in the absence of knowledge of the components of this export pathway, we cannot rule out the alternative mechanism of autosecretion of Ply, even though Ply lacks any recognizable autosecretory domain (10).

This work shows that cell wall-localized Ply is exported from the cytoplasm in an autolysis-independent, domain 2-dependent manner. The incompatibility with the Sec machinery exhibited by Ply (and Pfo when expressed in *S. pneumoniae*) is not observed when Ply is expressed in *B. subtilis*. These results indicate that the *S. pneumoniae* Sec machinery cannot accommodate a CDC, which may provide insight as to why Ply, unlike all other CDCs, does not contain a Sec signal sequence.

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