JOURNAL OF BACTERIOLOGY, Sept. 2010, p. 4388–4394 Vol. 192, No. 17
0021-9193/10/$12.00 doi:10.1128/JB.00578-10

Localization and Cellular Amounts of the WalRKJ (VicRKX) Two-Component Regulatory System Proteins in Serotype 2 *Streptococcus pneumoniae*▽†

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Received 19 May 2010/Accepted 25 June 2010

The WalRK two-component regulatory system coordinates gene expression that maintains cell wall homeostasis and responds to antibiotic stress in low-GC Gram-positive bacteria. Phosphorylated WalR (VicR) of the major human respiratory pathogen *Streptococcus pneumoniae* ($WalR_{Spn}$) positively regulates transcription of several surface virulence genes and, most critically, *pcsB*, which encodes an essential cell division protein. Despite numerous studies of several species, little is known about the signals sensed by the WalK histidine kinase or the function of the WalJ ancillary protein encoded in the $walRK_{Spn}$ operon. To better understand the functions of the $WalRKJ_{Spn}$ proteins in *S. pneumoniae*, we performed experiments to determine their cellular localization and amounts. In contrast to WalK from *Bacillus subtilis* ($WalK_{Bsu}$), which is localized at division septa, immunofluorescence microscopy showed that $WalK_{Spn}$ is distributed throughout the cell periphery. $WalJ_{Spn}$ is also localized to the cell surface periphery, whereas $WalR_{Spn}$ was found to be localized in the cytoplasm around the nucleoid. In fractionation experiments, $WalR_{Spn}$ was recovered from the cytoplasmic fraction, while $WalK_{Spn}$ and the majority of $WalJ_{Spn}$ were recovered from the cell membrane fraction. This fractionation is consistent with the localization patterns observed. Lastly, we determined the cellular amounts of $WalRKJ_{Spn}$ by quantitative Western blotting. The $WalR_{Spn}$ response regulator is relatively abundant and present at levels of ≈6,200 monomers per cell, which are ≈14-fold greater than the amount of the $WalK_{Spn}$ histidine kinase, which is present at ≈460 dimers (920 monomers) per cell. We detected ≈1,200 monomers per cell of $WalJ_{Spn}$ ancillary protein, similar to the amount of $WalK_{Spn}$.

The WalRK (YycFG) two-component regulatory system (TCS) plays critical roles in maintaining cell wall and surface homeostasis and in responding to cell wall stresses in low-GC Gram-positive bacteria (reviewed in references 8, 22, and 43). In *Bacillus*, *Staphylococcus*, and many other species, both the WalR (YycF) response regulator and WalK (YycG) histidine kinase are essential, and they cannot be depleted (Fig. 1) (11, 14, 27). In contrast, the WalR (VicR) response regulator of *Streptococcus* species is essential, whereas the WalK (VicK) histidine kinase is not essential under standard growth conditions (10, 30, 34, 40). WalRK regulons include different sets of genes that mediate peptidoglycan biosynthesis, cell division, and the cell surface in different Gram-positive species (1, 4, 9, 14, 21, 26, 28, 30, 31, 34). The WalK histidine kinases of *Streptococcus* species have sensing domains that are structurally different from those of *Bacillus*, *Staphylococcus*, and most other species (Fig. 1) (8, 32, 43). WalK from *Bacillus subtilis* ($WalK_{Bsu}$), which is typical of one class, contains two transmembrane domains flanking an extracytoplasmic domain (35). The transmembrane domains of $WalK_{Bsu}$ interact with the membrane domains of the auxiliary WalHI (YycHI) proteins to negatively regulate phosphorylation levels of the $WalR_{Bsu}$ response regulator (36–38). In contrast, WalK from *Streptococcus pneumoniae* ($WalK_{Spn}$), which exemplifies the other class, contains only a single transmembrane domain connected to an extracellular peptide of only 12 amino acids (Fig. 1) (24, 32, 40). *Streptococcus* species also lack homologues of WalHI (32). On the other hand, the cytoplasmic domains of the two classes of WalK histidine kinases are highly similar and contain a PAS domain (Fig. 1) (8, 16, 32, 43). The signals that are sensed directly by WalK histidine kinases are not yet known in any species (8, 19, 43). To understand better the functions and signaling of $WalRKJ_{Spn}$ in *S. pneumoniae*, we determined their localization and cellular amounts.

MATERIALS AND METHODS

Localization of $WalRKJ_{Spn}$ and FtsZ by immunofluorescence microscopy (IFM). Unencapsulated *S. pneumoniae* strains IU1824 (D39 non-FLAG negative control), IU1876 [R6 *ftsZ*-(C)-FLAG[3]], IU3816 [D39 $walJ_{Spn}$-(C)-L-FLAG[3]], IU4052 [D39 $walR_{Spn}$-(C)-L-FLAG[3]], IU4062 [D39 $walK_{Spn}$-(C)-FLAG], and IU4099 [D39 Δ$walK_{Spn}$ $walJ_{Spn}$-(C)-L-FLAG[3]] (Table 1) were cultured statically in 5 ml of BD brain heart infusion (BHI) broth in 16-mm glass tubes at 37°C in an atmosphere of 5% CO_2 to an optical density of 0.12 to 0.2 as described previously (39). Four milliliters of cultures was centrifuged at 16,000 × *g* at 4°C for 10 min, resuspended in 1 ml of phosphate-buffered saline (PBS; Ambion; AM9625), and centrifuged at 16,000 × *g* at 4°C for 5 min. The pellets were resuspended in 1 ml of 4% paraformaldehyde (EMS; 157-4) and incubated for 15 min at room temperature and 45 min on ice. Fixed cells were centrifuged, and pellets were washed three times with PBS at 4°C, resuspended in 0.3 ml of prechilled (4°C) GTE (50 mM glucose, 1 mM EDTA, 20 mM Tris HCl, pH 7.5), and stored at 4°C for up to 20 h. Fifty microliters of cell suspensions was

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

▽ Published ahead of print on 9 July 2010.

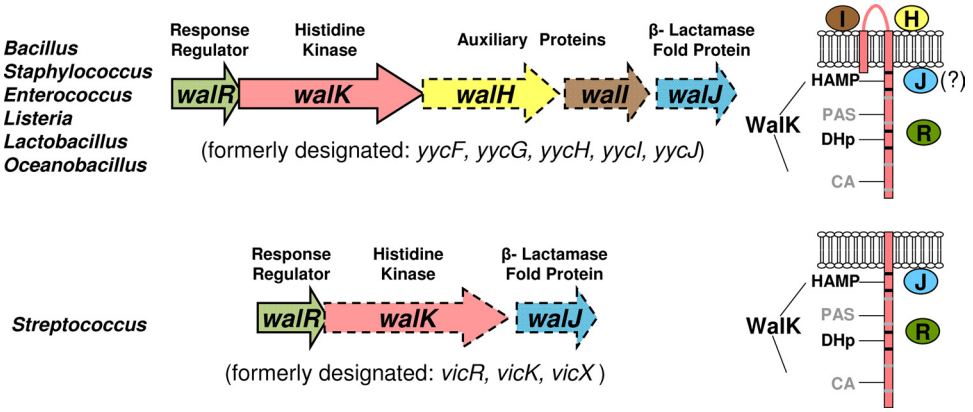

FIG. 1. Organization of the WalRK$_{Spn}$ TCS genes and WalK histidine kinases in low-GC Gram-positive bacteria. In most species (upper row), the operon consists of five genes encoding the essential (solid lines) WalR response regulator and WalK histidine kinase and three nonessential (dashed lines) proteins. In these species, the membrane-bound auxiliary WalHI proteins regulate WalK activity, but the functions and localization of the ancillary WalJ protein, which contains a metallo-β-lactamase fold, are unknown. The WalK histidine kinases of these species possess two transmembrane domains and an extracellular loop. In *Streptococcus* species, the operon contains only three genes encoding essential WalR and nonessential WalK and WalJ. In addition, the streptococcal WalK histidine kinases contain a single transmembrane domain and no extracellular domain, the WalHI proteins are absent, and WalJ is mostly membrane associated as reported here. The WalK histidine kinases of all species contain the HAMP and PAS sensing domains, the DHp dimerization and histidine phosphorylation domain, and the CA (catalytic) ATPase domain. See text for references and additional details.

deposited in 1-cm^2 wells made by a Liquid Blocker Super Pap Pen (EMS; 71310) on precleaned microscope slides and incubated for 5 min at room temperature. Unattached cells were aspirated, and attached cells were treated with 50 μl of 0.2% Triton X-100 (Mallinckrodt; H282) in PBS (PBS-T) for 10 s. PBS-T was aspirated from the wells, and the slides were immediately immersed in prechilled −20°C methanol and kept at −20°C for 10 min and then removed from methanol and air dried completely. The following incubations were carried out with 50 μl of reagents at room temperature: PBS-T for 5 min; 5% skim milk powder (BBL; 11915), 0.2% Triton X-100 in PBS (PBS-T-M) for 1 h; PBS for 10 s twice; 1:200 dilution of primary anti-FLAG polyclonal antibody (Sigma; F7425) in 5% skim milk powder in PBS (PBS-M) for 1 h; PBS twice for 10 s and once for 5 min; 1:100 dilution of secondary anti-rabbit-Alexa 488 (Molecular Probes; A11034), 4′,6-diamidino-2-phenylindole (DAPI; final concentration, 0.2 μg/ml) in PBS-M for 1 h; PBS twice for 10 s and once for 5 min. After the wells were air dried, 7 μl of Slowfade Gold antifade reagent (Invitrogen; S36936) was applied to each well and coverslips were applied and sealed. Cells were examined using a Nikon E-400 epifluorescence phase-contrast microscope equipped with a mercury lamp, a 100× Nikon Plan Apo oil-immersion objective (numerical aperture, 1.40), and filter blocks for fluorescence (DAPI, EX 330 to 380, DM 400, and BA 435 to 485; Alexa 488, EX 460 to 500, DM 505, and BA 510 to 560). Images were captured using a CoolSNAP HQ^2 charge-coupled device (CCD) camera (Photometrics) and processed with NIS-Elements AR imaging software (Nikon).

Fractionation of *S. pneumoniae* cells. Biochemical fractionation of IU3816 [D39 *walJ*$_{Spn}$-(C)-L-FLAG^3], IU4052 [D39 *walR*$_{Spn}$-(C)-L-FLAG^3], and IU4062 [D39 *walK*$_{Spn}$-(C)-FLAG] was performed with modifications from references 6 and 44. Briefly, cells were grown in BHI broth to an optical density at 620 nm (OD_{620}) of 0.2 to 0.4 as described above. One milliliter of cultures was sampled to prepare fractions of culture supernatants (S), cell pellets (P), digested cell wall (CW), protoplasts (Prot), cytoplasm (Cyto), and cell membrane (Memb). Cultures were centrifuged at 13,000 × *g* for 10 min at room temperature to obtain culture supernatants (S), which were stored on ice, and cell pellet fractions (P). Pellets were briefly washed with 0.5 ml 1× SMM buffer (0.5 M sucrose, 20 mM $MgCl_2$, 20 mM morpholineethanesulfonic acid [MES], pH 6.5) at room temperature, collected again by centrifugation, and resuspended in 0.5 ml 1× SMM buffer. To obtain protoplast (Prot) and digested cell wall fractions (CW), 25 μl of 10 mg lysozyme (Sigma) per ml, 2 μl of 1 mg mutanolysin (Sigma; M9901) per ml, and 5 μl of protease inhibitor cocktail III (Calbiochem) were added, and the mixture was incubated at 37°C for 1 h. The protoplast (Prot, pellet) and digested cell wall (CW, supernatant) fractions were separated by centrifugation at 3,000 × *g* for 10 min at room temperature. The CW fraction was stored on ice, and the protoplast pellet was frozen on dry ice for 10 min and resuspended in 0.5 ml of cold buffer H (20 mM HEPES, pH 8.0, 200 mM NaCl, 1 mM dithiothreitol [DTT], and protease inhibitor cocktail III). Five microliters of 0.1 M $MgCl_2$, 5 μl of 0.1 M $CaCl_2$, 1 μl of 5 mg DNase (Sigma; D4527) per ml, and 1 μl of 10 mg RNase (Sigma; R5500) per ml were added, and the mixture was incubated on ice for 1 h. After centrifugation at 16,100 × *g* for 30 min at 4°C, the pellet was collected as the membrane fraction (Memb) and the supernatant was collected as the cytoplasmic fraction (Cyto). The supernatants from each spin (S, CW, and Cyto fractions) were precipitated with cold 10% (wt/vol) trichloroacetic acid (TCA; Sigma; T4885) as described in the work of Barendt et al. (2), and pellets were resuspended in 100 μl lysis buffer (1% [wt/vol] SDS, 0.1% [vol/vol] Triton X-100) and 100 μl 2× SDS sample buffer (Bio-Rad; 161-0737) containing 5% (vol/vol) β-mercaptoethanol (Sigma). The pellets (P, Prot, and Memb fractions) were resuspended in 100 μl lysis buffer (1% [wt/vol] SDS, 0.1% [vol/vol] Triton X-100) and 100 μl 2× SDS sample buffer (Bio-Rad; 161-0737) containing 5% (vol/vol) β-mercaptoethanol (Sigma). Samples were boiled for 5 min, and 40 μl of each sample was resolved by SDS-PAGE (4% stacking, 10% Tris-glycine gels) as described in the work of Barendt et al. (2). FLAG-tagged proteins were detected by Western blotting with primary anti-FLAG polyclonal antibody (1:1,000 dilution; Sigma; F7425), secondary donkey anti-rabbit antibody conjugated with horseradish peroxidase (HRP; diluted 1:10,000; GE Healthcare; NA934), and ECL detection kits (Amersham) as described in reference 2. Autoradiography and quantitation using an IVIS imaging system were done as described in reference 2.

To distinguish protein aggregates from membrane-bound proteins, membrane fractions (Memb) were centrifuged on a sucrose cushion (7). The membrane fraction was resuspended in 0.5 ml buffer H. The suspension was carefully overlaid on 0.5 ml of buffer H containing 2 M sucrose, followed by centrifugation at 150,000 × *g* for 16 h at 4°C. Five equal-volume fractions were collected by removing solution gently along the side of the centrifuge tube from the top. Protein in each fraction was precipitated with 10% (wt/vol) TCA on ice, and the FLAG-tagged proteins were detected by Western blotting as described above.

Determination of cellular amounts of WalRKJ$_{Spn}$ by quantitative Western blotting. Cellular amounts of WalR$_{Spn}$ and WalK$_{Spn}$-(C)-FLAG or WalJ$_{Spn}$-(C)-L-FLAG^3 were determined in strain IU4062 or IU3816, respectively (Table 1). Lysates were prepared from unencapsulated strains IU1473 (R6 P$_c$-*pcsB* Δ*walR*$_{Spn}$), IU1824 (D39 non-FLAG negative control), IU3816 [D39 *walJ*$_{Spn}$-(C)-L-FLAG^3], and IU4062 [D39 *walR*$_{Spn}$$^+$ *walK*$_{Spn}$-(C)-FLAG], which had been cultured in BHI broth to an OD_{620} of 0.35 to 0.45 as described above. One milliliter of cultures was centrifuged at 16,100 × *g* for 10 min at room temperature, and cell pellets were resuspended in 100 μl of lysis buffer as described in reference 41 with the addition of 40 μg mutanolysin (Sigma; M9901) per ml to digest *S. pneumoniae* cell walls. Mixtures were incubated at 37°C for 10 min. One hundred microliters of 2× SDS sample buffer (Bio-Rad; 161-0737) containing 5% (vol/vol) β-mercaptoethanol (Sigma) was then added to sample preparations, and the mixtures were incubated at 95°C for 10 min. Lysates were centrifuged at 3,300 × *g* for 4 min at room temperature, and 30 μl of the supernatants was resolved by 10% SDS-PAGE as described above. Protein standards (N)-His$_{10}$-WalR$_{Spn}$, (N)-Sumo-WalK$_{Spn}$ ΔN35-(C)-FLAG, and WalJ$_{Spn}$-(C)-L-FLAG^3 were purified as described in Table S2 in the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype (description)[a]	Antibiotic resistance[b]	Reference or source
Bacterial strains			
S. pneumoniae			
EL59	R6 (unencapsulated laboratory strain R6 derived from intermediates of strain D39)	None	25
IU1473	R6 Δ*vicR*<>*ermAM kan*-t1t2-P_c-*pcsB*	Kan^r	31
IU1690	D39 (single-colony isolate of serotype 2 strain encapsulated D39 NCTC 7466)	None	25
IU1781	D39 *rpsL1*	Str^r	23
IU1824	D39 *rpsL1* Δ*cps2A′-cps2H′*	Str^r	25
IU1876	R6 *ftsZ*-(C)-$FLAG^3$ intergenic::P_c-*ermAM* (EL59 transformed with fusion amplicon)	Erm^r	This study
IU1885	D39 *rpsL1* Δ$walK_{Spn}$::P_c-[Kan^r-$rpsL^+$]	Str^s Kan^r	19
IU1888	D39 *rpsL1* Δ$walJ_{Spn}$::P_c-[Kan^r-$rpsL^+$] (IU1781 transformed with fusion amplicon)	Str^s Kan^r	This study
IU1896	D39 *rpsL1* Δ$walK_{Spn}$	Str^r	19
IU3126	R6 *pcsB*-(C)-L-$FLAG^3$ intergenic::P_c-*ermAM* (EL59 transformed with fusion amplicon)	Erm^r	This study
IU3150	D39 Δ*bgaA′*::P_c-*kan*1t2-P_{fcsk}-*pcsB* (IU1690 transformed with fusion amplicon)	Kan^r	This study
IU3348	R6 Δ*bgaA′*::P_c-*kan*1t2-P_{fcsk}-*pcsB*-(C)-L-$FLAG^3$ (EL59 transformed with fusion amplicon)	Kan^r	This study
IU3477	R6 Δ*bgaA′*::P_c-*kan*1t2-P_{fcsk}-$walJ_{Spn}$-(C)-L-$FLAG^3$ (EL59 transformed with fusion amplicon)	Kan^r	This study
IU3479	R6 Δ*bgaA′*::P_c-*kan*1t2-P_{fcsk}-$walR_{Spn}$-(C)-L-$FLAG^3$ (EL59 transformed with fusion amplicon)	Kan^r	This study
IU3689	D39 Δ*cps2A′-cps2H′ rpsL1* $walJ_{Spn}^+$ intergenic::P_c-[Kan^r-$rpsL^+$] (IU1824 transformed with fusion amplicon)	Str^s Kan^r	This study
IU3816	D39 Δ*cps2A′-cps2H′ rpsL1* $walJ_{Spn}$-(C)-L-$FLAG^3$ (IU3689 transformed with fusion amplicon)	Str^r	This study
IU3953	D39 Δ*cps2A′-cps2H′ rpsL1* Δ$walJ_{Spn}$ (IU3689 transformed with fusion amplicon)	Str^r	This study
IU4021	D39 Δ*cps2A′-cps2H′ rpsL1* Δ$walK_{Spn}$::P_c-[Kan^r-$rpsL^+$] (IU1824 transformed with Δ$walK_{Spn}$::P_c-[Kan^r-$rpsL^+$] amplicon from IU1885)	Kan^r	This study
IU4052	D39 Δ*cps2A′-cps2H′ rpsL1* $walR_{Spn}$-(C)-L-$FLAG^3$ (IU4021 transformed with fusion amplicon)	Str^r	This study
IU4062	D39 Δ*cps2A′-cps2H′ rpsL1* $walK_{Spn}$-(C)-FLAG (IU4021 transformed with fusion amplicon)	Str^r	This study
IU4092	D39 Δ*cps2A′-cps2H′ rpsL1* Δ$walK_{Spn}$::P_c-[Kan^r-$rpsL^+$] $walJ_{Spn}$-(C)-L-$FLAG^3$ (IU3816 transformed with Δ$walK_{Spn}$::P_c-[Kan^r-$rpsL^+$] from IU1885)	Str^s Kan^r	This study
IU4099	D39 Δ*cps2A′-cps2H′ rpsL1* Δ$walK_{Spn}$ $walJ_{Spn}$-(C)-L-$FLAG^3$ (IU4092 transformed with Δ$walK_{Spn}$ amplicon from IU1896)	Str^r	This study
E. coli			
EL27	BL21(DE3)(pLysS)(pSP001)	Amp^r Cm^r	31
IU4019	BL21(DE3) Rosetta(pLysS)(pIU251)	Kan^r Cm^r	This study
IU4223	BL21(DE3) Rosetta(pLysS)(pIU259)	Kan^r Cm^r	This study
Plasmids (*E. coli*)			
pSP001	pET16b [expression of (N)-His_{10}-$WalR_{Spn}$]	Amp^r Cm^r	31
pIU251	pSumo [expression of (N)-Sumo-$WalK_{Spn}$ ΔN35-(C)-FLAG, where ΔN35 removed the transmembrane domain of $WalK_{Spn}$]	Kan^r Cm^r	This study
pIU259	pET28a [expression of $WalJ_{Spn}$-(C)-L-$FLAG^3$]	Kan^r Cm^r	This study

[a] Primers used to synthesize fusion amplicons are listed in Table S1 in the supplemental material. (N)- or (C)- indicates that fusions were made to the amino- or carboxyl-terminal ends of reading frames, respectively. L refers to a 10-amino-acid spacer linker (GSAGSAAGSG) based on reference 42. $FLAG^3$ indicates three tandem copies of the FLAG epitope (DYKDDDDK) (20). Intergenic means that the resistance gene was cloned into the intergenic region following the indicated gene. BL21(DE3)(pLysS), BL21(DE3) Rosetta(pLysS), pET16b, and pET28a were purchased from Novagen, and pSumo was purchased from LifeSensors.

[b] Antibiotic resistance markers: Kan^r, kanamycin; Str^r, streptomycin, Erm^r, erythromycin; Amp^r, ampicillin; Cm^r, chloramphenicol. Concentrations of antibiotics used for *S. pneumoniae* strains: 250 μg Str per ml (Sigma S6501), 250 μg Kan per ml (Sigma K0254), and 0.3 μg Erm per ml (Sigma E6376); for *E. coli* strains, 34 μg Cm per ml (Sigma C0378) and 30 μg Kan per ml and 100 μg Amp per ml (Sigma A5354).

supplemental material. Standard curves for $WalR_{Spn}$ quantitation were generated by adding 0.03 to 0.5 pmol of (N)-His_{10}-$WalR_{Spn}$ to 30-μl samples of IU1473 lysates before SDS-PAGE. Standard curves for $WalK_{Spn}$-(C)-FLAG and $WalJ_{Spn}$-(C)-L-$FLAG^3$ quantitation were generated by adding 0.016 to 0.45 pmol of (N)-Sumo-$WalK_{Spn}$ ΔN35-(C)-FLAG or 0.03 to 0.45 pmol of $WalJ_{Spn}$-(C)-L-$FLAG^3$, respectively, to 30-μl samples of IU1824 lysate before SDS-PAGE. Detection of the $WalR_{Spn}$ protein on Western blots was achieved using 1:1,000 dilutions of primary anti-$WalR_{Spn}$ rabbit polyclonal antibody (Cocalico Biologicals) (30). FLAG-tagged proteins were detected using primary anti-FLAG polyclonal antibody as described above. Autoradiography and quantitation using an IVIS imaging system were done as described in reference 2.

CFU were determined by serial dilution of cultures used for lysate preparations and plating on Trypticase soy agar II (modified) containing 5% (vol/vol) defibrinated sheep blood agar plates (Becton Dickinson; BBL 221261). Molecules per cell were calculated from CFU, taking into account that these unencapsulated strains form primarily diplococcal cells, which means that there are two cells per CFU (2).

RESULTS AND DISCUSSION

Localization of $WalRKJ_{Spn}$ by immunofluorescence microscopy. $WalK_{Bsu}$ colocalizes with FtsZ at the septa of dividing *B.*

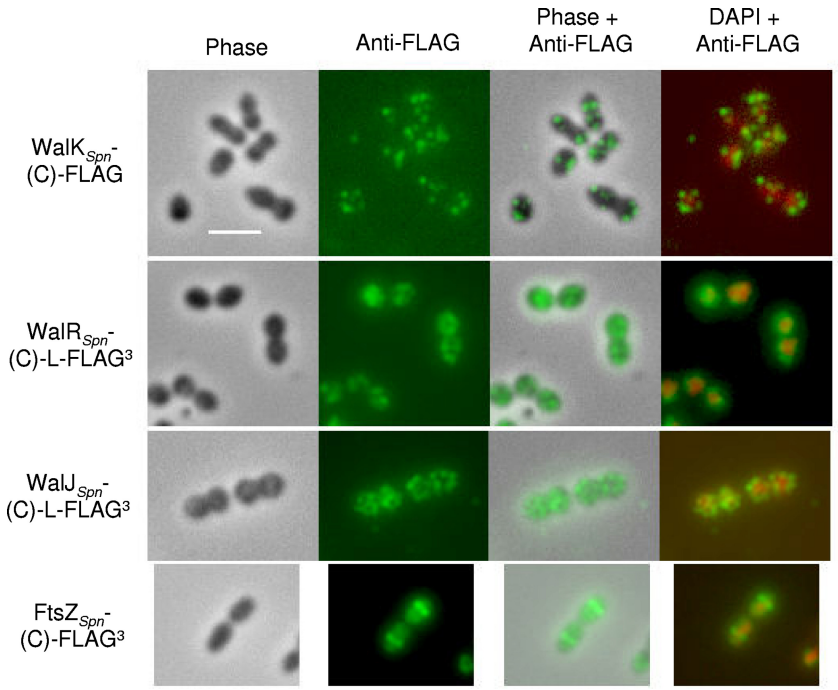

FIG. 2. Localization of WalK$_{Spn}$-(C)-FLAG, WalR$_{Spn}$-(C)-L-FLAG^3, and WalJ$_{Spn}$-(C)-L-FLAG^3 by immunofluorescence microscopy (IMF) in exponentially growing unencapsulated strain D39. Representative phase images of fixed cells and IMF of the indicated FLAG-tagged proteins using anti-FLAG antibody are shown in the first and second columns, respectively. Carboxyl-terminal (C) fusion of the chromosomally expressed proteins to the linker (L) and FLAG epitope tags (Table 1) did not cause any observable changes in cell growth or morphology compared to parent strains (first column; data not shown). IMF of cells lacking a FLAG-tag fusion protein did not show any labeling (data not shown). The third and fourth columns show overlays of the first two columns or overlays of the second column and images stained with DAPI to locate nucleoids, respectively. For comparison and to validate the IMF methods, the bottom row shows localization of FtsZ$_{Spn}$-(C)-FLAG^3 at the equators of exponentially growing cells of R6, which is an unencapsulated laboratory strain originally derived from strain D39. At least three biological replicates were performed for each strain, and multiple fields of cells were observed and analyzed for each replicate. See Materials and Methods for the IMF staining procedure used and the text for additional details.

subtilis cells, and it has been postulated that this interaction allows the WalRK$_{Bsu}$ to sense cell division directly (15). To determine whether WalRK$_{Spn}$ localized similarly in *S. pneumoniae*, we performed immunofluorescence microscopy (IFM) as described in Materials and Methods. Attempts to locate the WalK$_{Spn}$ histidine kinase with purified polyclonal antibodies prepared against the entire polypeptide or a synthetic PAS domain peptide were not successful. Consequently, we fused a FLAG epitope tag (20) to the carboxyl terminus of WalK$_{Spn}$ expressed from its native chromosomal locus (Table 1). The resulting strain (IU4062) did not show several characteristic phenotypes of mutants defective in WalK$_{Spn}$ function (data not shown), indicating that the FLAG tag did not significantly interfere with WalK$_{Spn}$ function. IFM using purified polyclonal anti-FLAG antibody (Sigma Corp.) (Fig. 2) showed that WalK$_{Spn}$-(C)-FLAG was distributed in a random pattern around the periphery of *S. pneumoniae* cells growing exponentially in brain heart infusion (BHI) broth in an atmosphere of 5% CO_2 (see references 33 and 39) and was not localized to division septa, as reported for WalK$_{Bsu}$ (15). Control experiments show no labeling of cells lacking a protein fused to the FLAG tag (data not shown). As another control, FtsZ fused at its carboxyl terminus to three tandem copies of the FLAG tag (FLAG^3) localized normally to cell division sites at equators and septa (Fig. 2; Table 1) (12, 29, 45). No defects in cell division or morphology were observed for this construct (Fig. 2). The tandem FLAG^3 tag amplified detection of FtsZ expressed from its native chromosomal locus, since a single FtsZ-FLAG construct could not be detected.

We also localized WalR$_{Spn}$ and WalJ$_{Spn}$ fused at their carboxyl termini to a 10-amino-acid linker (L) based on reference 42 followed by a FLAG^3 tandem tag (Table 1; Fig. 2). WalJ$_{Spn}$ is a nonessential protein containing a metallo-β-lactamase fold that may play an ancillary role in WalRK$_{Spn}$ TCS signaling (Fig. 1) (30). The resulting constructs (IU4052 and IU3816; Table 1) showed wild-type diplococcal morphology and cell division, consistent with normal WalR$_{Spn}$ and WalJ$_{Spn}$ function (Fig. 2; data not shown). WalR$_{Bsu}$ localized to the nucleoid in growing cells and surrounded the nucleoid in nondividing cells (15). In contrast, WalR$_{Spn}$-(C)-L-FLAG^3 was distributed in the cytoplasm around nucleoids in exponentially growing *S. pneumoniae* (Fig. 2). WalJ$_{Spn}$-(C)-L-FLAG^3 was located throughout pneumococcal cells (Fig. 2) and appeared to be localized near cell surfaces, similarly to WalK$_{Spn}$-(C)-FLAG (Fig. 2). WalJ$_{Spn}$ localization was not affected by the absence of WalK$_{Spn}$ in a Δ*walK*$_{Spn}$ mutant (IU4099, Table 1; data not shown), although Δ*walK*$_{Spn}$ mutants form longer chains (data not shown). On the basis of these data, we conclude that localization of WalK$_{Spn}$ and WalR$_{Spn}$ (Fig. 2) was fundamentally different from that of WalK$_{Bsu}$ and WalR$_{Bsu}$ in growing cells. This difference may reflect the different structures of WalK$_{Spn}$ and WalK$_{Bsu}$ (Fig. 1), and it implies that sensing by WalK$_{Spn}$ of cell

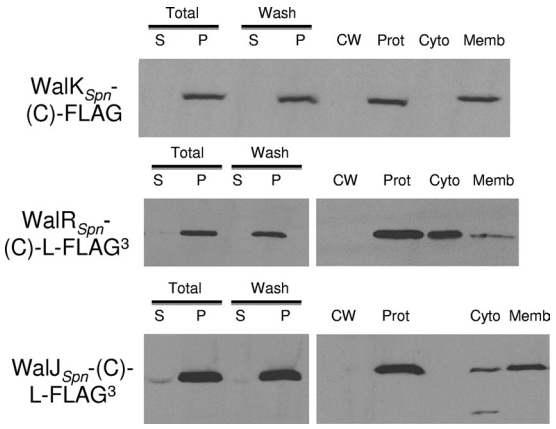

FIG. 3. Biochemical fractionation of WalK$_{Spn}$-(C)-FLAG, WalR$_{Spn}$-(C)-L-FLAG^3, and WalJ$_{Spn}$-(C)-L-FLAG^3 in an exponentially growing unencapsulated derivative of strain D39. Fractionation and Western immunoblot assays using anti-FLAG antibody were performed as described in Materials and Methods. Total, supernatant (S) and pellet (P) of starting cultures. Wash, supernatant (S) and pellet (P) of cells washed with sucrose-containing buffer. Washed pellets were further separated into solubilized cell wall (CW) and protoplast (Prot) fractions. Lysed protoplasts were separated into cytoplasmic (Cyto) and membrane (Memb) fractions. The image shown is a semiquantitative autoradiogram. Quantitation in the text is based on three independent biological replicate experiments in which band luminescence was determined using an IVIS imaging system. See text for additional details.

division likely does not occur through a direct interaction with FtsZ, as proposed for WalK$_{Bsu}$ (15).

Fractionation of WalRKJ$_{Spn}$ correlates with their localization. To gain additional information about these WalRKJ$_{Spn}$ localization patterns, we fractionated cells using methods described in references 6 and 44 with the modifications described in Materials and Methods. As expected, the vast majority of the recovered WalR$_{Spn}$-(C)-L-FLAG^3 response regulator or WalK$_{Spn}$-(C)-FLAG histidine kinase was in the cytoplasmic (88%) or membrane (94%) fractions, respectively (Fig. 3; data not shown). Consistent with its apparent localization to the cell surface (Fig. 2), 79% of the recovered WalJ$_{Spn}$-(C)-L-FLAG^3 was associated with the membrane fraction (Fig. 3). Some of the small amount of WalJ$_{Spn}$-(C)-L-FLAG^3 in the cytoplasmic fraction was degraded at its amino terminus as indicated by a lower-molecular-mass band that retained the FLAG^3 tag. WalJ$_{Spn}$ contains a metallo-β-lactamase fold (Pfam [13]) and at least one extended amphipathic helix predicted by the Heliquest program (17). WalJ$_{Spn}$ does not contain a predicted signal peptide or transmembrane domain, and it seems unlikely that the negatively charged FLAG^3 tag would promote membrane association. Moreover, WalJ$_{Spn}$ and WalJ$_{Bsu}$ have been difficult to purify because of aggregation (data not shown; Bill Burkholder, personal communication). To confirm that WalJ$_{Spn}$ was associated with the cell membrane and not aggregating during the fractionation, we centrifuged membrane fractions on sucrose cushion gradients as described in reference 7. Significant amounts of both WalK$_{Spn}$-(C)-FLAG and WalJ$_{Spn}$-(C)-L-FLAG^3 were found in fractions near the sucrose interface, indicative of membrane association (data not shown). Thus, the majority of WalJ$_{Spn}$ is associated with the cytoplasmic side of the cell membrane in exponentially growing *S. pneumoniae* (Fig. 1 and 3).

WalR$_{Spn}$ is a relatively abundant protein compared to WalK$_{Spn}$ and WalJ$_{Spn}$. We determined the cellular amounts of WalRKJ$_{Spn}$ by quantitative Western blotting as described in references 2 and 41 with the modifications in Materials and Methods. Cellular amounts of cognate histidine kinases and response regulators have not been determined for many TCSs. For the archetypical EnvZ/OmpR TCS, the OmpR response regulator is present in a significant excess over the EnvZ histidine kinase (3,500 monomers versus 59 dimers per cell, respectively) (5). This ratio has important implications for the robustness and response of the system (3, 18). In determinations of WalR$_{Spn}$ amounts, we used polyclonal anti-WalR$_{Spn}$ antibody (30) in the Western blot assays (Fig. 4A). As a standard, we spiked various concentrations of purified (N)-His$_{10}$-WalR$_{Spn}$ protein (19, 31) (see Table S2 in the supplemental material) into an extract of a Δ*walR$_{Spn}$* P$_c$-*pcsB*$^+$ mutant (IU1473, Table 1) containing the same amount of total protein as that used for the *walR$_{Spn}$*$^+$ strain IU4062 (Table 1). Unencapsulated mutants that are predominantly diplococci instead of chains of cells were used in these experiments to simplify estimates of cellular amounts (2). Control experiments showed that similar amounts were present in unencapsulated and encapsulated D39 strains (data not shown). The amount of WalR$_{Spn}$ detected in the *walR$_{Spn}$*$^+$ strain fell within the linear range of the standard curves (Fig. 4B). We found that WalR$_{Spn}$ was relatively abundant at 6,200 ± 1,900 (standard error of the mean [SEM], $n = 3$) monomers per cell in cultures growing exponentially in BHI broth in an atmosphere of 5% CO_2 (Fig. 4C). Parallel determinations of cellular amounts of WalR$_{Spn}$-L-FLAG^3 using anti-FLAG antibodies gave similar results (data not shown) and provided validation for the use of FLAG-tagged proteins for quantitation. Similar amounts of WalR$_{Spn}$ were detected in cells sampled early or later in exponential phase (OD_{620} of 0.1, 0.3, and 0.6). Therefore, as with WalR$_{Bsu}$ (37), there was no growth phase regulation of WalR$_{Spn}$ amount. The abundance of WalR$_{Spn}$ was consistent with its presence throughout the cytoplasm (Fig. 2). Since the shape of an unencapsulated pneumococcus cell approximates a prolate spheroid ($a = 0.62$ μm and $b = 0.82$ μm [2]) with a volume of 1.3×10^{-15} liters, the cellular concentration of WalR$_{Spn}$ in the cytoplasm is ≈8 μM.

We were unable reliably to detect WalK$_{Spn}$ in pneumococcal extracts by using three different polyclonal anti-WalK$_{Spn}$ antibody preparations that did bind to purified WalK$_{Spn}$ protein in Western blots (data not shown). However, we could detect WalK$_{Spn}$-(C)-FLAG used for localization studies (Fig. 2), probably because the commercial anti-FLAG antibody had a higher titer than did the anti-WalK$_{Spn}$ antibody preparations. In this case, a purified (N)-Sumo-WalK$_{Spn}$ ΔN35-(C)-FLAG standard (Table 1; see also Table S2 in the supplemental material) was added to extracts of strain IU1824 (Table 1) in quantitative Western blot assays (data not shown). We found that the cellular amount of the WalK$_{Spn}$ histidine kinase was 920 ± 20 (SEM, $n = 2$) monomers (460 dimers) per cell (Fig. 4C). Thus, similarly to the EnvZ/OmpR TCS, the WalR$_{Spn}$ response regulator mono-

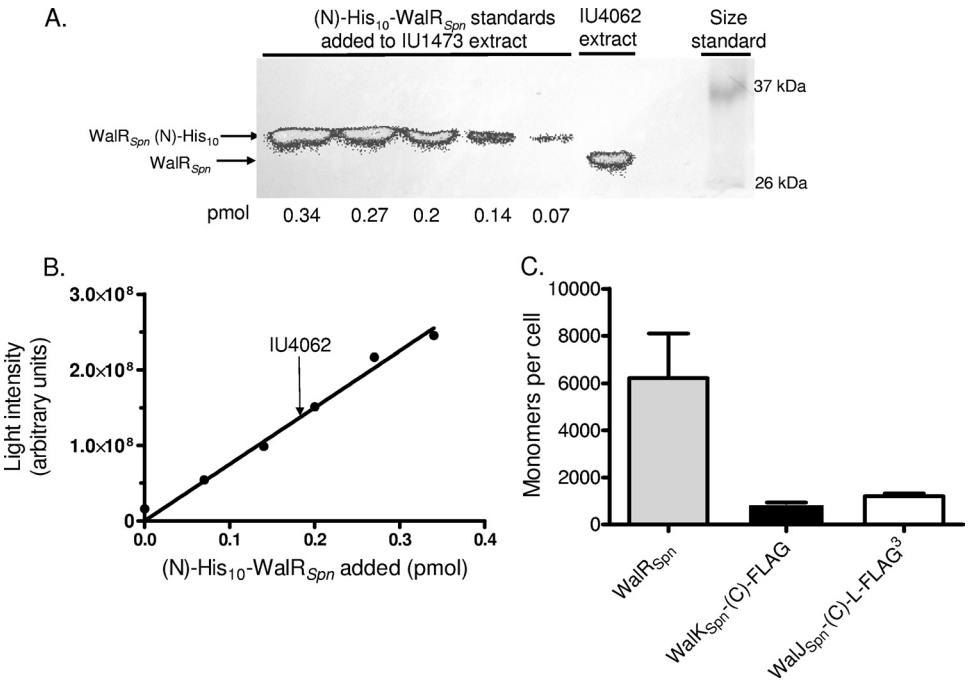

FIG. 4. Quantitative Western immunoblot assays of WalR$_{Spn}$, WalK$_{Spn}$-(C)-FLAG, and WalJ$_{Spn}$-(C)-L-FLAG^3 cellular amounts in exponentially growing unencapsulated derivatives of strain D39. The quantitation procedure is included in Materials and Methods. (A) Representative quantitative Western immunoblot assay used to determine the cellular amount of WalR$_{Spn}$. (Left lanes) The indicated amounts of a purified (N)-His$_{10}$-WalR$_{Spn}$ standard were added to extracts of Δ*walR*$_{Spn}$ P$_c$-*pcsB*$^+$ mutant IU1473. (Right lanes) Amount of native WalR$_{Spn}$ detected in an extract of WalR$_{Spn}^+$ strain IU4062 and protein molecular mass markers. The same amounts of total protein of the IU1473 and IU4062 extracts were loaded into each lane. Band luminescence was determined using an IVIS imaging system. (B) Standard curve (circles) generated from panel A. The arrow shows the light intensity of the IU4062 band from which the cellular amount of WalR$_{Spn}$ was read from the standard curve. (C) Cumulative data showing the amounts in monomers per pneumococcal cell of WalR$_{Spn}$, WalK$_{Spn}$-(C)-FLAG, and WalJ$_{Spn}$-(C)-L-FLAG^3. The amount of WalR$_{Spn}$ in strain IU4062 was from three biological replicates, including the one in panels A and B. The amounts of WalK$_{Spn}$-(C)-FLAG in strain IU4062 and WalJ$_{Spn}$-(C)-L-FLAG^3 in strain IU3816 were from two biological replicates each using anti-FLAG antibody with purified (N)-Sumo-WalK$_{Spn}$ ΔN35-(C)-FLAG and partially purified WalJ$_{Spn}$-(C)-L-FLAG^3, respectively, as standards. See Materials and Methods and the text for additional details.

mer was present in a sizable molar excess (≈14-fold) over the WalK$_{Spn}$ histidine kinase dimer in exponentially growing pneumococcal cells. Since WalK$_{Spn}$ has both autokinase and phosphorylated WalR$_{Spn}$ (WalR$_{Spn}$~P) phosphatase activities (19), this excess implies that WalRK$_{Spn}$ may be a robust signaling system (3, 18). The impact of these cellular amounts and ratios to signal transduction by the WalRK$_{Spn}$ TCS awaits determinations of binding constants, kinetic properties, and cellular phosphorylation levels.

Finally, we approximated the cellular amount of WalJ$_{Spn}$-(C)-L-FLAG^3 used for localization (Fig. 2), where the standard was partially purified WalJ$_{Spn}$-(C)-L-FLAG^3 added to extracts of strain IU1824 (Table 1; see also Table S2 in the supplemental material). Because of a tendency to aggregate, the WalJ$_{Spn}$-(C)-L-FLAG^3 standard could be purified only to ≈40% purity, in contrast to the highly purified (>95%) WalR$_{Spn}$ and WalK$_{Spn}$ standards (Table S2). With this limitation, membrane-associated WalJ$_{Spn}$ was approximated at 1,200 ± 110 (SEM, $n = 2$) monomers per exponentially growing cell, close to the amount of the WalK$_{Spn}$ histidine kinase (Fig. 4C). The functions of WalJ$_{Spn}$ and its relationship to WalRK$_{Spn}$ TCS signal transduction remain to be determined, as does the mechanism that reduces cellular expression of cotranscribed WalK$_{Spn}$ and WalJ$_{Spn}$ compared to WalR$_{Spn}$.

ACKNOWLEDGMENTS

We thank Andre Zapun (CNRS, Grenoble, France) for advice on IMF and Daniel Kearns and members of his laboratory (Indiana University) for advice on microscopy.

This project was supported by grant number AI060744 (to M.E.W.) from the National Institute of Allergy and Infectious Diseases. S.M.B. was a predoctoral trainee on NIH grant number F31FM082090, and K.J.W. was a predoctoral trainee on training grant T32GM007757.

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