Biochemical Activities of Three Pairs of *Ehrlichia chaffeensis* Two-Component Regulatory System Proteins Involved in Inhibition of Lysosomal Fusion†

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Received 7 May 2006/Returned for modification 12 June 2006/Accepted 23 June 2006

*Ehrlichia chaffeensis*, the etiologic agent of human monocytic ehrlichiosis, replicates in early endosomes by avoiding lysosomal fusion in monocytes and macrophages. In *E. chaffeensis* we predicted three pairs of putative two-component regulatory systems (TCSs) designated PleC-PleD, NtrY-NtrX, and CckA-CtrA based on amino acid sequence homology. In the present study to determine biochemical pairs and specificities of the TCSs, the recombinant proteins of the three putative histidine kinase (HK) kinase domains (rPleCHKD, rNtrYHHD, and MBP-rCckAIHD) and the full-length forms of three putative response regulators (RRs) (rPleD, rNtrX, and rCtrA) as well as the respective mutant recombinant proteins (rPleCHKD244A, rNtrYHHD498A, MBP-rCckAIHD449A, rPleDD53A, rNtrXD59A, and rCtrAD53A) were expressed and purified as soluble proteins. The in vitro HK activity, the specific His residue-dependent autophosphorylation of the kinase domain, was demonstrated in the three HKs. The specific Asp residue-dependent in vitro phosphotransfer from the kinase domain to the putative cognate RR was demonstrated in each of the three RRs. Western blot analysis of *E. chaffeensis* membrane and soluble fractions using antibodies specific for each recombinant protein detected PleC and CckA in the membrane fraction, whereas it detected NtrY, NtrX, and PleD in the soluble fraction. CtrA was found in the two fractions at similar levels. *E. chaffeensis* was sensitive to closantel, an HK inhibitor. Closantel treatment induced lysosomal fusion of the *E. chaffeensis* inclusion in a human monocytic leukemia cell line, THP-1 cells, implying that functional TCSs are essential in preventing lysosomal fusion of the *E. chaffeensis* inclusion compartment.

*Ehrlichia chaffeensis* is an obligatory intracellular, gram-negative bacterium replicating in monocytes/macrophages that are equipped with powerful innate antimicrobial defenses. Thereby *E. chaffeensis* causes human monocytic ehrlichiosis, a potentially fatal emerging infectious disease, which has been reported primarily from the United States and occasionally from other parts of the world (14).

The bacterial two-component regulatory system (TCS) is a ubiquitous signal transduction system that controls response and adaptation to a variety of environmental conditions (15). The TCSs are typically composed of a histidine kinase (HK) and a cognate response regulator (RR). We recently predicted that *E. chaffeensis* has three pairs of TCSs designated PleC-PleD, NtrY-NtrX, and CckA-CtrA (1) based on amino acid sequence homology. We cloned DNA fragments encoding the three pairs of wild-type and mutant kinase domains, nine combinations of phosphotransfer activities from three wild-type kinase domains to three wild-type RRs, and three pairs of phosphotransfer activities from wild-type kinase domains to the mutant cognate RRs in vitro.

The intracellular location of TCS proteins is critical in sensing environmental signals and in linking TCSs to downstream signaling events (15). The second objective of the present study was to determine the membrane and/or cytoplasmic localiza-
tion of these six proteins in *E. chaffeensis* in order to define intracellular sites of action of these proteins. 

For obligatory intracellular bacteria including *E. chaffeensis*, no useful genetic system or no naturally isolated mutant has been available, making the genetic approach to examining functions of TCS impossible. We, therefore, took the pharmacological approach using a histidine kinase inhibitor, closantel, (N-[5-chloro-4-(R,S)-4-chlorophenyl]cyano-2-phenyl-2-hydroxy-3,5-diiodobenzamide). *E. chaffeensis* and the autokinase activities of recombinant kinase domains were found to be sensitive to closantel in vitro, indicating that HK function is essential for *E. chaffeensis* infection (1). *E. chaffeensis* has evolved to modulate vesicular trafficking to avoid its delivery to lysosomes (21). The third objective of the present study was, therefore, to analyze the role of *E. chaffeensis* TCS in preventing ehrlichial inclusions from fusing with lysosomes.

**MATERIALS AND METHODS**

**Bacterial strains and culture.** *E. chaffeensis* Arkansas was propagated in a human acute monocytic leukemia cell line, THP-1 (1) (ATCC, Manassas, VA), in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine at 37°C in 5% CO2 and 95% air. *Escherichia coli* cells NovaBlue (Novagen, Madison, WI), BL21(DE3) (Novagen), and DH5α (Invitrogen, Carlsbad, CA) were cultured in Luria-Bertani broth (23) supplemented with 50 μg/ml of ampicillin or 50 μg/ml of kanamycin when required.

**Infection of blood monocytes with *E. chaffeensis* and RT-PCR analysis.** Monocytes were isolated from a human peripheral blood specimen, and adherent monocytes were infected with host cell-free *E. chaffeensis* as described previously (9). Total RNA was extracted from monocytes at 24 h postinfection. Reverse transcription-PCR (RT-PCR) analysis for six TCS protein genes was performed as described elsewhere (1).

**Construction of plasmids for expressing rPleCHKD294, rNtrYHKDH498A, MBPr-CckAHKD449A, rPleDD53A, rNtrXD59A, and rCtrAD53A.** Mutations were introduced in DNA fragments encoding wild-type proteins (1) using the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions with the primers designed as shown in Table S1 in the supplemental material. The mutations were confirmed by DNA sequencing.

**Protein expression in *E. coli* and purification.** BL21(DE3) and DH5α were transformed with the plasmids encoding rPleCHKD, rNtrYHKDH, MBP-rCckAHKD, rPleD, rNtrX, rCtrA, rPleCHKD294A, rNtrYHKDH498A, MBP-rCckAHKD449A, rPleDD53A, rNtrXD59A, and rCtrAD53A. Proteins were expressed by adding 1 mM isopropyl-thio-β-D-galactosidase (IPTG) when the cells reached an optical density at 600 nm of 0.6, and the cells were continuously cultured at 37°C for 2 h or at lower temperature (20 to 30°C) for 12 h. The *E. coli* cells were harvested by centrifugation at 8,000 × g for 10 min and suspended in binding buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl) supplemented with 10 μg/ml DNase I, 400 μg/ml lysozyme, and 1 mM EDTA. The suspension was sonicated and centrifuged at 18,000 × g for 10 min (4°C). The resulting supernatant or pellet (inclusion bodies) was used to purify soluble and insoluble proteins, respectively.

All His6-tagged soluble proteins were purified as follows. The supernatant was filtered through a 0.45-μm filter and loaded onto a His-Select cartridge (Sigma-Aldrich, Saint Louis, MO) equilibrated with binding buffer. After the column was washed with wash buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 10 mM imidazole), the protein was eluted with elution buffer (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, 250 mM imidazole). The eluted protein was dialyzed against TP buffer (50 mM Tris-HCl, pH 8.0, 10 mM KCl). His6-tagged insoluble proteins purified from the inclusion bodies (1) were refolded by being dialyzed against TP buffer containing 3 M urea, TP buffer containing 1 M urea, refolding buffer (50 mM Tris-HCl, pH 8.0, 240 mM NaCl, 10 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 250 mM arginine, 1 mM glutathione, 0.1 mM oxidized glutathione), and finally against TP buffer.

To further purify soluble His6-tagged NtrX, a heparin column made of Heparion-Sepharose 6 Fast Flow (GE Healthcare, Piscataway, NJ) was equilibrated with 50 mM Tris-HCl, pH 7.5, 10 mM KCl. The protein sample was loaded onto the column, and the column was washed with the same buffer. The protein was eluted with a linear KCl gradient from 10 mM to 1 M in 50 mM Tris-HCl, pH 7.5. An amlose column made of amlose resin (New England Biolabs, Beverly, MA) was used for purification of the maltose binding protein (MBP) fusion protein as previously described (1). All mutant proteins were purified as respective wild-type proteins. Proteins were quantified with the biechinonic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

**Kinase and phosphotransfer assays.** To evaluate the phosphorylation of the HK domain (HKD) of HKs, 10 μg of purified proteins was incubated with 0.1 mM [γ-32P]ATP and 0.5 μCi of [γ-32P]ATP (GE Healthcare) for 15 min at room temperature in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 50 mM KCl, 20 mM MgCl2 supplemented with (rPleCHKD and rNtrYHKD) or without (MBP-rCckAHKD) 1 mM dithiothreitol. The reaction was terminated by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For phosphotransfer analysis, 10 to 30 μg of purified recombinant RR's was added to the phosphorylation reaction mixture and incubated for 15 min at room temperature. For rCtrA, phosphotransfer buffer containing CaCl2 instead of MgCl2 and phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA) were added. The reaction was terminated by adding SDS-PAGE sample buffer. Samples and the molecular weight markers, 14C-methylated proteins (GE Healthcare), were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was dried and exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). The screen was scanned with a PhosphorImager 445Si (Molecular Dynamics).

**E. chaffeensis fractionation.** *E. chaffeensis*-infected THP-1 cells were disrupted by N2 decomposition using a cell disruption bomb (Parr, Moline, IL) according to the manufacturer’s instructions. Briefly, the bomb was pressurized with 1,000 lb/in2 of N2 gas and stirred with a magnetic bar placed inside the bomb for 30 min on ice to allow N2 gas to dissolve and reach equilibrium. Cells were disrupted by N2 decomposition to atmospheric pressure. The suspension was centrifuged at 1,000 × g for 3 min, and the resulting supernatant was centrifuged at 15,000 × g for 10 min. Host cell-free *E. chaffeensis* thus obtained and uninfected THP-1 cells as a negative control were suspended in 2× phosphate-buffered saline (PBS; 273 mM NaCl, 5.4 mM KCl, 20 mM Na2HPO4, 55 mM KH2PO4, pH 7.4), sonicated, and centrifuged at 18,000 × g for 3 min. The supernatant was centrifuged at 100,000 × g for 1 h. The resulting supernatant (the soluble fraction) and the pellet (the membrane fraction) were suspended in 2× PBS.

**Western blot analysis.** Samples of the soluble and membrane fractions were mixed with SDS-PAGE sample buffer, boiled for 5 min, and loaded onto a 12% or an 8% SDS-polyacrylamide gel. Proteins were transferred to a sheet of the nitrocellulose membrane. The membrane was incubated with appropriately dialyzed primary antibodies against six recombinant TCS proteins (1). Antibodies anti-CckA, -NtrX, and -CtrA were preabsorbed with the THP-1 lysate, and antibodies anti-PleC, -NtrY, and -PleD were affinity purified using the respective purified recombinant proteins coupled to N-hydroxysuccinimide-activated Sepharose 4 Fast Flow (GE Healthcare) as ligands. Preimmune rabbit sera were used as a negative control for primary antibodies. The membrane was then incubated with secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:12,000) (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and ECL Western blotting detection reagents (GE Healthcare). Bands were visualized with a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The band intensities were calibrated with Image Gauge software (Fujiﬁlm).

**Closantel treatment and double immunofluorescence labeling.** *E. chaffeensis*-infected THP-1 cells at 3 days postinfection (2 × 105 cells, 80% infected) were treated with closantel (100 μM) in dimethyl sulfoxide (DMSO) (1%), vol/vol at 37°C for 1 h. The same concentration of DMSO was used as a control. Ice-cold PBS was added to stop the reaction. Cells were fixed in 2% paraformaldehyde at room temperature for 15 min, and incubated with dog anti-*E. chaffeensis* mouse anti-LAMP-1 (monoclonal antibody [MAb] H4A3) antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) in PBS containing 0.1% gelatin and 0.3% saponin for 1 h at room temperature. After being washed twice with PBS, cells were incubated with Alexa Fluor 555-conjugated goat anti-mouse (Molecular Probes, Eugene, OR) and fluorescein isothiocyanate-conjugated goat anti-dog (Rockland Immunochemicals, Gilbertsville, PA) secondary antibodies for 30 min. Cells were then washed, cytocentrifuged onto glass slides, and observed under a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments Inc., Melville, NY). Images were captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) coupled to the Nikon fluorescence microscope.
RESULTS

Sequence analysis of HKs and RRs. Our analysis of the *E. chaffeensis* Arkansas genome sequence (2) revealed three candidate HKs with characteristic ATP binding motifs (NG1G2G3 or NG1FG2G3) (Fig. 1) and three potential RRs (1). Each of the three *E. chaffeensis* RRs displayed significant homology over the entire length to *Azorhizobium caulinodans* NtrX (18), *Caulobacter crescentus* CtrA (20), or *C. crescentus* PleD (4), respectively (Fig. 1). Therefore, we annotated them as NtrX, CtrA, and PleD. Like *C. crescentus* PleD (4), *E. chaffeensis* PleD was composed of the D1 domain, the D2 domain, and the C-terminal output GGDEF domain containing the four consensus regions. Domain structure analysis revealed that *E. chaffeensis* NtrX comprised a receiver domain, a G5 binding domain, and a helix-turn-helix motif (Fig. 1). Like *C. crescentus* CtrA (20), *E. chaffeensis* CtrA had a receiver domain and the output domain that shares structural features with the OmpR C-terminal DNA binding domain (Fig. 1).

In *A. caulinodans* (18) and *A. brasilense* (5), the only two known bacteria in which NtrX and NtrY have been studied, NtrY is suggested to be a cognate HK of NtrX. Of the three putative *E. chaffeensis* HKs, the HK encoded by GenBank sequence YP_507120 displayed the highest homology to *A. caulinodans* NtrY including a sensor domain with three putative hydrophobic regions, a HAMP domain, and a histidine kinase domain (Fig. 1). Thus, we annotated the protein as *E. chaffeensis* NtrY. *C. crescentus* CtrA is directly or indirectly phosphorylated by CckA in vivo (8). *C. crescentus* CckA is composed of a sensor domain, a histidine kinase domain, and a receiver domain (8). Since the HK encoded by GenBank sequence YP_507553 was composed of the three homologous domains (Fig. 1), we annotated the protein as CckA. *E. chaffeensis* HK, encoded by YP_507680, displayed the highest homology to *C. crescentus* PleC (28) and DivJ (13). Since the histidine kinase domain showed higher homology to that of *C. crescentus* PleC than that of DivJ, we annotated the *E. chaffeensis* HK as PleC, although the sensor domain had no significant homology to that of *C. crescentus* PleC (Fig. 1). *C. crescentus* PleD has been demonstrated to be phosphorylated by PleC and DivJ in vitro (17). To date no biochemical evidence of phosphotransfer from NtrY to NtrX and from CckA to CtrA has been found in any bacteria, although NtrY-NtrX and CckA-CtrA pairs have been implied through bacterial mutation studies, since mutation of either

FIG. 1. Schematic representation of the domain structures of HKs and RRs in *E. chaffeensis* and reference bacteria. The numbers on each protein are amino acid residues. The conserved histidine (H) in HKs and the conserved aspartate (D) in RRs, GenBank accession numbers, and predicted molecular masses are shown. The bars above HKs and RRs are regions cloned for biochemical analysis. The percentages in the parentheses between the two aligned proteins indicate amino acid identity. HTH, helix-turn-helix.
Expression of six TCS genes by *E. chaffeensis* in human blood monocytes. To confirm the expression observed in a human leukemia cell line in healthy human monocytes, we examined the expression of six genes by *E. chaffeensis* in human peripheral blood monocytes by RT-PCR (Fig. 2). No amplicon was detected without reverse transcriptase, indicating the absence of genomic DNA contamination in the RNA preparation.

Intracellular distribution of HKs and RR in *E. chaffeensis*. The majority of HKs are transmembrane proteins localized in the bacterial inner (or cytoplasmic) membrane with the sensor domain exposed to the periplasm (or extracellular milieu) and the histidine kinase domain present in the cytoplasm (15). Using hydrophathy plot software (DNAStar, Protean program) and the PSORT program (http://psort.ims.u-tokyo.ac.jp/), *E. chaffeensis* PleC and CckA were predicted to be an inner membrane protein with a single transmembrane region in the N terminus of the mature proteins (Fig. 1). *E. chaffeensis* NtrY was predicted to be a nonmembrane protein despite having three hydrophobic regions in the N terminus, suggesting a unique sensory mechanism. RR are generally cytoplasmic proteins, but activation of cognate HKs causes binding of RR to the membrane HKs for phosphotransfer reaction to take place (16). In *C. crescentus* timed redistribution of RR (CtrA and PleD) to the stalked pole is critical for cell cycle progression (7, 8, 11, 22, 25), although there has been no direct evidence for membrane localization of the RR.

We, therefore, examined localization of these six native TCS proteins in the mixed growth phase of *E. chaffeensis* by using the antisera specific to each recombinant protein prepared in our previous study (1). The six antisera/antibodies specifically reacted with *E. chaffeensis* native proteins with the expected molecular mass except for CtrA (Fig. 3). The apparent molecular mass of the native *E. chaffeensis* CtrA on SDS-PAGE was approximately 35 kDa (Fig. 3, aCtrA), which was larger than the calculated size of 30 kDa, presumably because hydrophobic stretches remained unfolded after being boiled with SDS as occurred in other hydrophobic proteins (24). These six antisera/antibodies were each specific to the respective native protein and did not react with any proteins derived from THP-1 cells (Fig. 3), although some THP-1 cell debris remained in the purified *E. chaffeensis* fraction. Preimmune rabbit sera did not react with any proteins in THP-1 cells or *E. chaffeensis* (data not shown).

*E. chaffeensis* membrane and soluble fractions prepared from isolated *E. chaffeensis* organisms were defined with anti-recombinant P28 protein (major outer membrane protein of *E. chaffeensis*) antibody specific to the outer membrane fraction of *E. chaffeensis* (12) (Fig. 3, aP28). Most of PleC was detected in the *E. chaffeensis* membrane fraction, and some was in the *E. chaffeensis* soluble fraction, presumably nascent PleC proteins (Fig. 3, aPleC). NtrY was detected in the soluble fraction, and CckA was found in the membrane fraction (Fig. 3, aNtrY and aCckA) in agreement with in silico prediction. PleD and NtrX were found in the soluble fraction (Fig. 3, aPleD andaNtrX). CtrA was detected in both soluble and membrane fractions (Fig. 3, aCtrA). Based on the band intensities, approximately 50% of CtrA was localized in the membrane and 50% in the soluble fractions. The antibodies did not react with proteins from uninfected THP-1 cell membrane or soluble fractions that were used as negative controls (data not shown).

Purity of soluble recombinant proteins. In order to determine the biochemical activities of these proteins, proteins must be purified free from unknown inhibitors or *E. coli* kinases and be soluble in the assay solution. Although proteins purified from inclusion bodies are more pure than those purified from the soluble fraction, these proteins require a refolding procedure to make them soluble, rPleCHKD and rNtrYHKD were purified from inclusion bodies to near homogeneity (Fig. 4A). Purified His$_6$-tagged rCckAHKD from the inclusion bodies remained insoluble despite the use of several different refolding buffers. To make the recombinant protein more soluble, we constructed a plasmid expressing a fusion protein, MBP-rCckAHKD. About 50% of the expressed MBP-rCckAHKD was soluble, and the soluble protein was purified with an amylose column to near homogeneity (Fig. 4A).

By being cultured at a lower temperature (20 to 30°C), rPleD and rNtrX became slightly more soluble and were purified from the soluble fraction with a Ni column. rNtrX purified with a Ni column required additional heparin column purification to obtain a single band and to increase specific activity (Fig. 4A). All rCtrA expressed in *E. coli* became inclusion bodies; however, a portion of rCtrA purified from the inclusion bodies could be refolded and become soluble. The apparent molecular size (40 kDa) of rCtrA was larger than the calculated size (34 kDa), as with native CtrA (Fig. 4A). The 30-kDa band in Fig. 4A was not an *E. coli* protein contamination but was a degraded rCtrA, as the band was reactive with anti-His$_6$ antibody (data not shown).

Specific His-dependent autokinase activity of the recombinant kinase domains. The autokinase activity of the recombinant HKDs purified as described above was examined by incubation with [$\gamma$-$^{32}$P]ATP as a phosphate donor. rPleCHKD, rNtrYHKD, and rCckAHKD displayed autokinase activity (Fig. 4B). By aligning the three *E. chaffeensis* HKs with other bacterial HKs, we predicted that His244 of PleC, His498 of NtrY, and His449 of CckA could be phosphorylated (Fig. 4A). To confirm this prediction, we expressed and purified mutant HKDs with the conserved His residue replaced by Ala (rPleCHKDH244A, rNtrYHKDH498A, and MBP-rCckAHKD449A) to near homogeneity using the
same methods as for the respective wild-type proteins (data not shown). Autokinase activity was not detected for any of the mutant proteins (Fig. 4B), indicating that the conserved His residues (His244 of PleC, His498 of NtrY, and His449 of CckA) are required for autokinase activity.

Specific phosphotransfer from the recombinant HKDs to cognate recombinant RRs. To demonstrate the putative three pairs of TCS proteins predicted based on amino acid sequences, we performed an in vitro phosphotransfer assay. The D1 domain of *E. chaffeensis* PleD contains the amino acid residues characteristic of the receiver domains of RRs (26), including the predicted phosphorylation site (Asp53), two carboxylate-containing residues (Asp9 and Asp10), Thr83 (a hydroxyl-containing residue), Phe102 (an aromatic residue), and Lys106, while the D2 domain contains four out of the six characteristic amino acids: Asp163 corresponding to Asp9 of the D1 domain, Glu164 corresponding to Asp10, Asp207 corresponding to Asp53, and Tyr254 corresponding to Phe102 (alignment data not shown). The receiver domains of NtrX and CtrA contain the six characteristic amino acids. When the three RRs were added to the three recombinant autophosphorylated HKDs, rPleCHKD phosphorylated only rPleD and rNtrY phosphorylated only rNtrX (Fig. 4C). When rCtrA (10 μg) was mixed with autophosphorylated MBP-rCckAHKD, phosphorylated

FIG. 3. Intracellular localization of HKs and RRs in *E. chaffeensis*. Shown are results of Western blot analysis with rabbit antibodies to PleC, NtrY, CckA, PleD, NtrX, CtrA, and *E. chaffeensis* membrane protein control P28. Ten micrograms (anti-PleC, -PleD, -NtrX, and -CtrA) or 20 μg (anti-NtrY and -CckA) each of *E. chaffeensis* (Ec) lysate and soluble and membrane fractions derived from the same number of *E. chaffeensis* cells as the lysate and 10 μg THP-1 cell lysate were loaded on a 12% (anti-PleC, -PleD, -NtrX, and -CtrA) or 8% (anti-NtrY and -CckA) SDS-polyacrylamide gel. Numbers on the left are molecular masses in kilodaltons.
FIG. 4. His kinase and phosphotransfer activities. (A) Purity of soluble recombinant HKDs and RRs used for biochemical assays. Purified protein samples were loaded onto a 12% SDS-polyacrylamide gel, and the gel was stained with Coomassie brilliant blue. The asterisk indicates a CtrA degradation product. Numbers on the bottom are calculated molecular masses in kilodaltons. (B) Autoradiogram showing the specific histidine residue-dependent autokinase activity of recombinant HKDs. rPleCHKD, rPleCHKD_H244A, rNtrYHKD, and rNtrYHKD_H498A (2 μg each) and MBP-rCckAHKD and MBP-rCckAHKD_H449A (10 μg each) were incubated with [γ-32P]ATP. Only wild-type (wt) HKDs were 32P phosphorylated. (C) Autoradiogram showing phosphotransfer of 32P from rPleCHKD, rNtrYHKD, and MBP-rCckAHKD (arrowheads) to recombinant RRs (arrows). rHKDs (10 μg each) were incubated with [γ-32P]ATP, followed by incubation with wild-type (rPleD, rNtrX, or rCtrA) and mutant (rPleDD53A, rNtrXD59A, or rCtrAD53A) RRs (10 μg each). rCtrA + Ca2+: rCtrA (30 μg) was incubated with 32P-phosphorylated MBP-rCckAHKD in the presence of CaCl2 and the phosphatase inhibitor cocktail. Numbers on the left of each panel are molecular masses in kilodaltons.
rCtrA was not detected despite a reduction in phosphorylated MBP-rCckAHKD (Fig. 4C, from MBP-rCckAHKD to rCtrA). Increasing the amount of rCtrA (to 30 μg) did not yield a detectable phosphorylated rCtrA band (data not shown). However, phosphorylated rCtrA was detected when the phosphotransfer reaction was performed in the presence of Ca2⁺ instead of Mg2⁺ and a phosphatase inhibitor cocktail (Fig. 4C, from MBP-rCckAHKD to rCtrA/Ca2⁺). This result suggests that inability to detect phosphorylated rCtrA in the presence of Mg2⁺ may be due to Mg2⁺-dependent phosphatase activity of rCckAHKD. A similar phenomenon was reported in the E. coli EnvZ-OmpR system where the phosphatase activity of EnvZ was activated upon interaction with OmpR in a Mg2⁺-dependent manner (3). The phosphotransfer from MBP-rCckAHKD was specific to rCtrA (Fig. 4C). Any of the recombinant RRs alone did not have auto-kinase activity (data not shown).

To demonstrate that the conserved Asp residues of the RRs were required for receiving phosphate from cognate HKs, mutant recombinant RRs (rPleDD53A, rNtrXD59A, and rCtrAD53A) were produced by site-directed mutagenesis and purified using the same methods as for corresponding wild-type RRs (data not shown). When the mutant recombinant RRs were mixed with autophosphorylated cognate recombinant HKDs, no phosphotransfer was detected (Fig. 4C), indicating that the conserved Asp resides of RRs (Asp59 of NtrX, and Asp53 of CtrA) were required for phosphotransfer from cognate HKs.

Enhanced lysosomal fusion with E. chaffeensis inclusions by closantel treatment. Incubation of THP-1 cells infected with E. chaffeensis in the presence of 100 μM closantel, an HK inhibitor, for 1 h increased the colocalization of E. chaffeensis and a lysosomal glycoprotein, LAMP-1: 71.4% ± 10.4% (n = 3) of ehrlichial inclusions (morulae) were colocalized with LAMP-1 in closantel-treated cells, whereas 13.1% ± 1.8% (n = 3) of morulae were colocalized in DMSO-treated infected cells (Fig. 5).

**DISCUSSION**

The present study is the first report of specific biochemical activities of TCS pairs in an obligatory intracellular bacterium belonging to the α-proteobacteria. The current study demonstrated direct phosphotransfer from PleC to PleD, from NtrY to NtrX, and from CckA to CtrA and intracellular protein localization in E. chaffeensis. Moreover, a function of E. chaffeensis TCS in inhibition of lysosomal fusion was implied by using an HK inhibitor, closantel.

The NtrY-NtrX system was identified in A. caulinodans (18) and in A. brasilense (5) as a homolog of the NtrB-NtrC system that regulates the expression of nitrogen-regulated (Ntr) genes; however, biochemical activity of the NtrY-NtrX system...
has never been demonstrated. *E. chaffeensis* NtrY-NtrX is the first pair with biochemical evidence of autokinase activity and phosphotransfer for the NtrY-NtrX system. NtrY is predicted to be a transmembrane protein in *A. caulinoeadans* (18) and *A. brasilense* (5). *E. chaffeensis* NtrY was not detected in the membrane fraction, despite the presence of three hydrophobic regions in the sensor domain. *E. chaffeensis* NtrY may be a peripheral membrane protein that loosely associates with the inner membrane, and the protein may have been released from the membrane by sonication. If this is the case, the N-terminal domain of *E. chaffeensis* NtrY might sense (a) membrane-permeant signal(s) or *E. chaffeensis* NtrY might interact with an unknown signal sensor localized in the inner membrane. Alternatively, *E. chaffeensis* NtrY may be a cytoplasmic protein as NtrB is in several bacteria (10). There might also be an unknown protein that modulates cytoplasmic *E. chaffeensis* NtrY activity, as is the case for PIIL, which regulates NtrB activity in *E. coli* (10).

The current study demonstrated direct phosphotransfer from *E. chaffeensis* CcKAHKD to rCtrA. There has been only in vivo evidence for direct or indirect involvement of CcKA in the phosphorylation of CtrA in *C. crescentus* (8). In *C. crescentus*, CtrA phosphorylation is dependent not only on CcKA but also on PleC with involvement of other factors (11, 25). Cross talk among the three *E. chaffeensis* TCSs was not detected in the in vitro phosphotransfer assay.

The conserved *E. chaffeensis* CcKA receiver domain structure suggests phosphorylation of Asp755. HKs composed of a sensor domain, a histidine kinase domain, and a receiver domain employ a multistep phosphorylase: His (on the HK histidine kinase domain)→Asp (on the HK receiver domain)→His (on the histidine-containing phosphotransmitter [HTP] domain)→Asp (on the RR receiver domain) as demonstrated in both the *E. coli* RcsC-YojN-RscB system (27) and the Saccharomyces cerevisiae Sh1-Ypd1-Ssk1 system (19). However, a gene encoding an HPT domain-containing protein was not detected in the *E. chaffeensis* genome by homology search. Since the characteristic amino acid sequences of HPT domains are short and quite variable (6) and the amino acid sequences of HPt domains are short and quite variable (6) and the amino acid sequences of HPt domains are short and quite variable (6), the authors conclude that these genes are not involved in the phosphorylase in the *E. chaffeensis* CcKA-CtrA system.

*E. chaffeensis* CtrA was detected in both the soluble fraction and the membrane fractions. This may be due to the mixed population of *E. chaffeensis* bacteria of various growth phases. In *C. crescentus*, CtrA is dispersed in the swarmer cells and the predivision cells, while CtrA is localized in the stalked pole when the swarmer cells differentiate into stalked cells. However, the membrane localization of *C. crescentus* CtrA has not been shown (22). Although a transmembrane region was not detected in *E. chaffeensis* CtrA by PSORT analysis, hydrophobic stretches of this protein may be involved in its membrane localization. This is consistent with poor solubility of *E. chaffeensis* rCtrA and the abnormal SDS-PAGE mobility of rCtrA and native CtrA. *C. crescentus* PleD has two homologous receiver domains, D1 and D2; however, it has not been determined whether both or either one of the D domains serves as a functional phosphate receiver domain. In the D2 domain of *E. chaffeensis* PleD, two out of the six characteristic amino acids of receiver domains of RR (26) were not found. In agreement with this analysis, the present data showed the lack of phosphorylation of rPleDD53A by rPleCHKD: namely, Asp207 of PleD (corresponding to Asp53 of *E. chaffeensis* PleD D1 domain) was not phosphorylated. Biological functions of the D2 domain of *E. chaffeensis* PleD remain to be elucidated.

The lysosomal fusion with *E. chaffeensis* inclusion was detected within 1 h after addition of closantel, suggesting the active inhibition of lysosomal fusion through functions of TCSs, likely RR output domains. What signals activate TCSs and how and which RR regulates host lysosomal fusion remain to be elucidated.

**ACKNOWLEDGMENTS**

We appreciate the advice of Dr. Robert Tabita regarding purification of TCSs. The HA43 monoclonal antibody developed by J. Thomas August and James E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. This work was supported by the National Institutes of Health grant R01 AI054476. The *E. chaffeensis* genome sequence project was supported by National Institutes of Health grant R01 AI47885.

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Editor: J. T. Barbieri