Dependency of *Coxiella burnetii* Type 4B Secretion on the Chaperone IcmS

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ABSTRACT Macrophage parasitism by *Coxiella burnetii*, the cause of human Q fever, requires the translocation of proteins with effector functions directly into the host cell cytosol via a Dot/Icm type 4B secretion system (T4BSS). Secretion by the analogous *Legionella pneumophila* T4BSS involves signal sequences within the C-terminal and internal domains of effector proteins. The cytoplasmic chaperone pair IcmSW promotes secretion and binds internal sites distinct from signal sequences. In the present study, we investigated requirements of *C. burnetii* IcmS for host cell parasitism and effector translocation. A *C. burnetii* *icmS* deletion mutant (Δ *icmS*) exhibited impaired replication in Vero epithelial cells, deficient formation of the *Coxiella*-containing vacuole, and aberrant T4BSS secretion. Three secretion phenotypes were identified from a screen of 50 Dot/Icm substrates: IcmS dependent (secreted by only wild-type bacteria), IcmS independent (secreted by both wild-type and Δ *icmS* bacteria), or IcmS inhibited (secreted by only Δ *icmS* bacteria). Secretion was assessed for N-terminal or C-terminal truncated forms of CBU0794 and CBU1525. IcmS-inhibited secretion of CBU1525 required a C-terminal secretion signal whereas IcmS-dependent secretion of CBU0794 was directed by C-terminal and internal signals. Interchange of the C-terminal 50 amino acids of CBU0794 and CBU1525 revealed that sites within the C terminus regulate IcmS dependency. Glutathione S-transferase-tagged IcmSW bound internal sequences of IcmS-dependent and -inhibited substrates. Thus, the growth defect of the *C. burnetii* Δ *icmS* strain is associated with a loss of T4BSS chaperone activity that both positively and negatively regulates effector translocation.

IMPORTANCE The intracellular pathogen *Coxiella burnetii* employs a type 4B secretion system (T4BSS) that promotes growth by translocating effectors of eukaryotic pathways into host cells. T4BSS regulation modeled in *Legionella pneumophila* indicates IcmS facilitates effector translocation. Here, we characterized type 4B secretion by a *Coxiella* Δ *icmS* mutant that exhibits intracellular growth defects. T4BSS substrates demonstrated increased, equivalent, or decreased secretion by the Δ *icmS* mutant relative to wild-type *Coxiella*. Similar to the *Legionella* T4BSS, IcmS dependency in *Coxiella* was determined by C-terminal and/or internal secretion signals. However, IcmS inhibited secretion of some effectors by *Coxiella* that were previously shown to be translocated by *Legionella*. Thus, *Coxiella* has a unique IcmS regulatory mechanism that both positively and negatively regulates T4BSS export.

KEYWORDS *Coxiella*, Dot/Icm, IcmS, Q fever, chaperone, effector proteins, regulation, type 4B secretion

Central to disease pathogenesis by the human Q fever bacterium *Coxiella burnetii* are invasion and replication within mononuclear phagocytes, such as alveolar macrophages (1, 2). *C. burnetii* has a unique intracellular lifestyle that involves acid

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activation of metabolism within a phagolysosome-like compartment called the *Coxiella*-containing vacuole (CCV). An important virulence determinant of *C. burnetii* is the ability to resist degradation in the CCV. Another critical factor for pathogen growth is the manipulation of host cell functions by proteins with effector functions translocated directly into the host cell cytoplasm by the *C. burnetii* Dot/Icm type 4B secretion system (T4BSS) (3–9). Genetic tools have aided functional analysis of *C. burnetii* Dot/Icm substrates with ~140 identified to date (10). Characterized *C. burnetii* effectors are known to modulate apoptotic and pyroptotic cell death pathways (11–14), mitogen-activated protein kinase signaling (15), endosomal trafficking through manipulation of clathrin machinery (16, 17), and autophagy/phosphoinositide metabolism (18, 19).

Although progress has been made in characterizing *C. burnetii* effectors, considerable gaps in knowledge remain regarding regulation of T4BSS export. For example, how is effector translocation controlled to temporally orchestrate events specific to different stages of *Coxiella*'s lengthy infectious cycle? A general physiological requirement for effector delivery is metabolic activation of *C. burnetii*, a process that only occurs after CCV acidification (20, 21). Consequently, translocation of effector-reporter chimeras is not detected until 4 to 8 h postinfection when the nascent CCV has matured to an endolysosomal state (22). Early inhibition of transcription impedes effector translocation, but it is unclear whether this effect is directly related to Dot/Icm function or due to a general decline in metabolic fitness (22). Nonetheless, these data indicate the Dot/Icm T4BSS is not required for early infection events, such as internalization or CCV acidification (4).

A C-terminal secretion signal of ~30 amino acids controls the export of some proteins by the Dot/Icm system (23–31). C-terminal secretion signals exhibit an E block of negatively charged amino acids located between positions –20 to –10 that is flanked by a regular distribution of serine/threonine residues and amino acids with bulky hydrophobic side chains (26, 27). Efficient export of *L. pneumophila* Dot/Icm substrates can also require the chaperone complex IcmSW, which binds to substrates on sites exclusive from signal sequences (28, 32–37). IcmSW, along with DotL, DotM, and DotN, forms a T4BSS coupling protein complex (T4CP) on the cytoplasmic surface of the inner membrane that promotes export of T4BSS effectors by an unknown mechanism (32, 37–41).

L. pneumophila *icmS* and/or *icmW* mutants exhibit moderate growth defects in human macrophage-like cells (36). Similarly, *C. burnetii* *icmS* transposon mutants have a moderate growth defect, whereas mutagenesis of *icmW* results in moderate to severe defects (7, 8, 42). Functional redundancy within the effector pool and IcmSW-independent translocation of some substrates likely mitigate impaired T4BSS function (24, 28, 43). *C. burnetii* *icmS* and *icmW* complement *L. pneumophila* mutants with corresponding gene deletions (44, 45). These results suggest that IcmSW functions are similar between *C. burnetii* and *L. pneumophila*. However, there are discrepancies in substrate translocation between the two pathogens (46, 47), and T4BSS activity and IcmS function have not been directly investigated in *C. burnetii*. Here, characterization of a *C. burnetii* Δ *icmS* mutant reveals that intracellular growth defects correspond with both positive and negative regulatory roles for IcmS in T4BSS export. These data suggest that IcmS regulation of T4BSS activity in *C. burnetii* is distinct from that of *L. pneumophila*.

RESULTS

***C. burnetii* Δ *icmS* mutant has an intracellular growth defect.** *C. burnetii* *icmS* is downstream of *icmT* in a predicted two-gene operon within the ~23-kbp *dot/icm* locus (46) (Fig. 1A). To evaluate the role of *icmS* in *C. burnetii* effector translocation and host cell colonization, we constructed an *icmS* deletion mutant by allelic exchange (5). Deletion of *icmS* was confirmed by PCR (Fig. 1A). Growth of wild-type *C. burnetii*, the Δ *icmS* mutant, and the Δ *icmS* mutant complemented with a single copy of *icmS* under the control of its native promoter was measured in the synthetic medium ACCM-2 or Vero cells. Six-day genome equivalent (GE) increases for the three strains were indis-

FIG 1 *C. burnetii* $\Delta icmS$ has a moderate intracellular growth defect. (A) Schematic depicting the arrangement of *icmS* and adjacent genes in wild-type *C. burnetii* (NMII) and the $\Delta icmS$ mutant. Replacement of *icmS* with a Kan^r cassette by allelic exchange resulted in the $\Delta icmS$ mutant. Deletion of *icmS* was confirmed by PCR using oligonucleotides specific to *icmS*. (B) Replication of wild-type *C. burnetii*, the $\Delta icmS$ mutant, and the complemented $\Delta icmS$ mutant ($\Delta icmS$ Scomp) grown in ACCM-2 or Vero cells. Histograms show fold change in bacterial genome equivalents (GE) at 6 days postinfection relative to the day of infection. ACCM-2 results are expressed as the means from three independent experiments. Vero cell results are expressed as the means of two biological replicates from three independent experiments. Error bars indicate the standard deviations from the means, and asterisks indicate a statistically significant difference ($P < 0.001$) from cells infected with wild-type *C. burnetii*. (C) Fluorescence micrographs of Vero cells infected for 4 days with wild-type *C. burnetii*, $\Delta icmS$, or $\Delta icmS$ Scomp strains. Cells were immunostained for *C. burnetii* (red) and the lysosomal marker CD63 (green). Scale bar, 10 μ m.

tinguishable in ACCM-2. However, in Vero cells, the $\Delta icmS$ mutant had a statistically significant ($P < 0.001$) growth defect, with 10-fold less GE than wild-type *C. burnetii* or the complemented $\Delta icmS$ mutant (Fig. 1B). Deficient replication by the $\Delta icmS$ mutant correlated with defective CCV development. By immunofluorescence, mutant bacteria occupied aberrantly small CD63-positive vacuoles, whereas both wild-type bacteria and the complemented mutant resided in single large and spacious CD63⁺ vacuoles (Fig. 1C).

***C. burnetii* Dot/Icm substrates display three profiles of IcmS dependency.** IcmS control over T4BSS effector translocation was assessed for 50 *C. burnetii* proteins previously reported as Dot/Icm substrates (Table 1). Due in part to past limitations of *C. burnetii* genetics, 44 of the Dot/Icm substrates selected were originally identified using *L. pneumophila* to produce candidate effectors fused to the reporter tags CyaA or BlaM. Fourteen substrates were subsequently validated in *C. burnetii* (3, 6, 8, 15, 16, 28, 30, 31, 46–49). Substrates with defined host cell effector functions included CBU0021 (CvpB, Cig2), CBU0041 (CirA), CBU0072 (AnkA), CBU0077 (MceA), CBU0665 (CvpA),

TABLE 1 Dot/Icm substrates tested in this study^a

ORF	Name(s)	Size (kDa)	Translocation results (reference[s]) ^b
CBU0021	CvpB, Cig2	93.1	Lp CyaA (28), Cb CyaA (47)
CBU0041	CirA	82.7	Lp BlaM [80] (48)
CBU0072	AnkA	45.5	Lp CyaA (WT < IcmS) (31), Lp CyaA (49)
CBU0077	MceA	29.6	Lp CyaA, Cb BlaM [29] (3), Cb BlaM 38% (8)
CBU0080		18.7	Lp CyaA (3), Cb BlaM [1] (6)
CBU0129		11.1	Lp BlaM 70% (48)
CBU0145	AnkB	38.5	Lp CyaA (WT > IcmS) (31), Lp CyaA (49)
CBU0295		56.8	Lp CyaA (3), Lp BlaM [2] (6)
CBU0329		23.3	Lp CyaA (3)
CBU0410		63.3	Lp BlaM [45] (48)
CBU0414		30.4	Lp BlaM [45] (48)
CBU0425	CirB	51.9	Lp CyaA (3)
CBU0447	AnkF	21.3	Lp CyaA (WT = IcmS) (31), Lp CyaA (49)
CBU0534		46.1	Cb CyaA (47)
CBU0635		55.3	Lp CyaA, Cb BlaM [7.7] (3)
CBU0665	CvpA	38.0	Cb CyaA (47)
CBU0781	AnkG	38.6	Lp CyaA (WT = IcmS) (31), Lp CyaA (49)
CBU0794		53.1	Lp BlaM [80] (48)
CBU0885		44.9	Lp CyaA (28), Lp BlaM (6), Cb CyaA (47)
CBU1024	AnkH	88.1	Lp CyaA (WT > IcmS) (31), Lp CyaA (49)
CBU1045		40.0	Lp BlaM [40] (48)
CBU1107		35.5	Lp CyaA (3)
CBU1108		21.8	Lp CyaA (3)
CBU1217		57.8	Lp BlaM [10] (48), Cb CyaA (47)
CBU1460		29.6	Lp BlaM [40] (48)
CBU1461		67.1	Lp BlaM [2] (48)
CBU1493		64.2	Cb CyaA (47)
CBU1524	CaeA	25.1	Lp CyaA (3), Cb BlaM [10], Cb BlaM [38] (8)
CBU1525		43.9	Lp CyaA (3)
CBU1532	CaeB	16.5	Lp CyaA (3)
CBU1556	CvpC	64.6	Lp BlaM [50] (48), Cb CyaA (47)
CBU1569		63.1	Lp BlaM [90] (48)
CBU1599		12.5	Lp BlaM [10] (48)
CBU1614		16.2	Cb CyaA (46)
CBU1676		41.8	Lp CyaA (15), Cb CyaA (47)
CBU1751	Cig57	48.8	Lp BlaM [60] (48)
CBU1776		24.0	Lp CyaA (3)
CBU1790		73.8	Cb CyaA (46)
CBU1818	CvpD	53.9	Lp CyaA (15), Cb CyaA (47)
CBU1819		42.3	Cb CyaA (47)
CBU1823	IcaA	40.3	Lp CyaA, Cb BlaM [10] (3), Cb BlaM [13] (8)
CBU1825		13.3	Lp CyaA (3)
CBU1863	CvpE	66.2	Cb CyaA (47)
CBU1963		72.9	Lp CyaA (3)
CBU2052	CirD	34.0	Lp CyaA (3)
CBU2056		25.4	Lp CyaA (3)
CBUA0013	CpeB	28.0	Lp CyaA (30), Cb CyaA and BlaM (30)
CBUA0014	CpeC	8.7	Lp CyaA (30), Cb CyaA and BlaM (30)
CBUA0015	CpeD	26.3	Lp CyaA (30), Cb CyaA and BlaM (30)
CBUA0016	CpeE	39.5	Lp CyaA (30), Cb CyaA and BlaM (30)

^a*C. burnetii* Nine Mile (RSA 493) open reading frames (ORFs) of proteins tested for secretion by the *C. burnetii* Dot/Icm T4BSS are indicated, along with names and predicted molecular weights.

^bTranslocation results with beta-lactamase (BlaM) and adenylate cyclase (CyaA) reporter assays in *L. pneumophila* (Lp) or *C. burnetii* (Cb) are indicated for the referenced studies. Where reported, the percentage of BlaM-positive cells is indicated in brackets, as well as the relative secretion efficiency in wild-type *L. pneumophila* (WT) versus the *icmS* mutant (IcmS).

CBU0885, CBU1524 (CaeA), CBU1532 (CaeB), CBU1679 (Cem9), CBU1751 (Cig57), and CBU1823 (IcaA) (3, 6, 16, 28, 31, 46–51). THP-1 macrophages were infected for 48 h with wild-type *C. burnetii* or the Δ *icmS* mutant producing effectors fused at their N termini with CyaA, a reporter tag that, when delivered to the host cell cytoplasm, catalyzes the production of cyclic AMP (cAMP). Tested substrates were deemed secreted by *C. burnetii* if they produced ≥ 2.5 -fold more cAMP versus the negative control (CyaA only) (4, 16, 46, 47). Secretion results from wild-type and Δ *icmS* bacteria were separated into

FIG 2 *C. burnetii* Dot/Icm substrates display three profiles of IcmS dependency. THP-I cells were infected for 48 h with wild-type *C. burnetii* or the *icmS* mutant ($\Delta icmS$) producing the indicated Dot/Icm substrates fused to CyaA. Histograms depict the fold change in cytosolic [cAMP] relative to cells infected with *C. burnetii* producing CyaA alone (CyaA). (A) Ten substrates were secreted only by wild-type *C. burnetii* (IcmS dependent); (B) 29 substrates were secreted by both wild-type *C. burnetii* and the $\Delta icmS$ mutant (IcmS independent); (C) 11 substrates were secreted only by the $\Delta icmS$ mutant (IcmS inhibited). The cutoff for positive secretion is indicated by a dotted line at 2.5-fold change [cAMP]. Error bars indicate the standard deviations from the means for two independent experiments conducted in duplicate. Asterisks indicate substrates with significantly different translocation efficiencies by wild-type versus $\Delta icmS$ bacteria ($P < 0.01$, Student *t* test).

three groups (Fig. 2). Ten substrates secreted by only wild-type *C. burnetii* were classified as IcmS dependent (Fig. 2A), 29 substrates secreted by both the wild type and the $\Delta icmS$ mutant were classified as IcmS independent (Fig. 2C), and 11 substrates secreted by only the $\Delta icmS$ mutant were classified as IcmS inhibited (Fig. 2B). Within the IcmS-independent group the cAMP levels were significantly greater for four and seven substrates secreted by wild-type bacteria and the $\Delta icmS$ mutant, respectively. Here and elsewhere in this study, immunoblots demonstrated that differences in cAMP production between strains were not due to inconsistent expression of CyaA fusion proteins (see Fig. S1 and S2 in the supplemental material). Translocation assays were also conducted with Vero cells infected for 48 h with *C. burnetii* expressing CBU0794 or CBU1525 fused to the β -lactamase (BlaM) fluorescent reporter tag. Measurement of BlaM translocation within individual cells by fluorescence confocal imaging coupled with computational image analysis confirmed the IcmS-dependent and IcmS-inhibited secretion phenotypes of CBU0794 and CBU1525, respectively (Fig. S3). Together, CyaA and BlaM translocation assays indicate that expression of IcmS by wild-type *C. burnetii* promotes the secretion of CBU0794 but inhibits the export of CBU1525, a substrate that was previously shown to be translocated by the wild-type *L. pneumophila* T4BSS (3, 30, 48, 49).

IcmS dependency is consistent throughout infection. To examine whether *C. burnetii* IcmS dependency changes from early to late infection, cAMP assays were conducted at 8, 24, 48, 72, and 120 h postinfection with substrates that were IcmS dependent (CBU0794 and CBUA0013), IcmS independent (CBUA0016 and CBU1863), or IcmS inhibited (CBU0072 and CBU1525) (Fig. S4). Translocation signals at 8 h postinfection were consistently lower than signals at the later time points, a result that likely

IcmS-dependent

IcmS-independent

IcmS-inhibited

FIG 3 Effectors translocated by the *C. burnetii* Δ icmS mutant exhibit C-terminal signal sequences enriched in acidic residues. The probability of a particular amino acid at each position in the 35 C-terminal residues of IcmS-dependent, IcmS-independent, and IcmS-inhibited substrates. Amino acids are colored according to their physiochemical and structural properties: negatively charged E and D (red); positively charged K and R (orange); bulky hydrophobic F, I, L, and V (blue); helix breaker P (green); small polar S and T (magenta); and other residues (black).

reflects *C. burnetii* in the early stages of metabolic activation (22). Interestingly, the greatest fold changes in cAMP concentration ([cAMP]) occurred at 24 h postinfection, which is earlier than the maximal growth of *C. burnetii* observed at 48 to 72 h postinfection. Overall, the translocation phenotypes of IcmS-dependent, -independent, and -inhibited substrates remained consistent from 24 to 120 h postinfection as *C. burnetii* is transitioning from the exponential growth to the early stationary phase (4). This behavior suggested that IcmS control of T4BSS translocation is not temporally regulated for substrates constitutively expressed during *C. burnetii* growth in cultured macrophages.

Differences in C-terminal amino acid composition distinguish IcmS-inhibited and IcmS-dependent substrates. A subset of *L. pneumophila* effectors that harbor C-terminal sequences enriched in acidic residues (D and E) at E-block positions -20 to -10 exhibit less dependence on IcmS for export (28). To determine whether specific physiochemical properties in the C-terminal secretion signals of *C. burnetii* substrates correlated with IcmS-related secretion phenotypes, the probability of specific amino acids at positions -35 to -1 was calculated (Fig. 3 and Table S3). For this analysis, substrates were grouped according to statistically different or similar levels of secretion by *C. burnetii* wild type versus the Δ icmS mutant ($P < 0.01$). Based on these criteria, several substrates secreted by both *C. burnetii* wild-type and the Δ icmS mutant strains, but at different levels (Fig. 2B), were removed from the IcmS-independent group and included with the IcmS-dependent or IcmS-inhibited substrates. Overall, IcmS-inhibited substrates exhibited a typical E block of negatively charged amino acids within the -20 to -10 region of the C terminus. These sequences were flanked immediately upstream by amino acids with hydrophobic side chains and downstream by serine/threonine and hydrophobic residues. By comparison, E-block residues were not prominent in C-terminal 35 amino acids of IcmS-dependent substrates that instead exhibited greater enrichment of serine and proline residues. IcmS-independent secretion signals were a hybrid of IcmS-inhibited and -dependent signals, with both E-block residues and

FIG 4 IcmS dependency is regulated by sites within the C-terminal 50 amino acids. THP-I cells were infected for 48 h with wild-type *C. burnetii* or the *icmS* mutant producing CyaA fused to the indicated amino acids. Histograms depict the fold change in cytosolic [cAMP] for cells infected with *C. burnetii* producing N-terminal truncated forms of CBU0794 (IcmS dependent) (A) or CBU1525 (IcmS inhibited) (B) or C-terminal truncated forms of both proteins (C). The cutoff for positive secretion is indicated by a dotted line at 2.5-fold change [cAMP]. Error bars indicate the standard deviations from the means for two independent experiments conducted in duplicate.

C-terminal prolines. C-terminal sequences of CBU0080 and CBU1024 were clear outliers among IcmS-dependent and -inhibited secretion signals, respectively, suggesting that factors other than C-terminal amino acid composition can also influence IcmS dependency (Table S3). However, overall trends indicated that, similar to *L. pneumophila*, *C. burnetii* effectors with strong E blocks exhibit less dependence on IcmS for export (28, 31, 32). Conversely, deficient export of substrates harboring strong E blocks by wild-type bacteria is unique to *C. burnetii*.

IcmS dependency is regulated by sites within the C-terminal 50 amino acids.

Direct binding of IcmS to *L. pneumophila* effectors has been demonstrated with the binding sequence mapped to sites internal to the C terminus of SidG (32, 34, 35, 37, 40). To map the region mediating *C. burnetii* IcmS-dependent and -inhibited translocation, export was tested for deletion derivatives of CBU0794 (IcmS dependent) and CBU1525 (IcmS inhibited) by wild-type *C. burnetii* or the $\Delta icmS$ mutant. CyaA fusions of these proteins were made with progressive N-terminal deletions. The smallest truncated forms of CBU0794 and CBU1525 contained amino acids 434 to 464 (CBU0794₄₃₄₋₄₆₄) and 353 to 382 (CBU1525₃₅₃₋₃₈₂), respectively. N-terminal deletion derivatives containing at least 50 C-terminal amino acids were secreted similar to full-length protein, but larger N-terminal truncations perturbed the IcmS dependency of substrate secretion (Fig. 4A and B). CBU0794₄₂₄₋₄₆₄ and CBU1525₃₄₃₋₃₈₂ were secreted by both wild-type *C. burnetii* and the $\Delta icmS$ mutant, whereas CBU0794₄₃₄₋₄₆₄ and CBU1525₃₅₃₋₃₈₂ were only secreted by the $\Delta icmS$ mutant. These data suggest that residues encompassed within the region from positions -50 to -40 control IcmS dependency of CBU0794 and CBU1525 secretion. Secretion of CBU0794₄₃₄₋₄₆₄ and CBU1525₃₅₃₋₃₈₂ by only the $\Delta icmS$ mutant indicated that both constructs harbor a secretion signal that is inhibited by IcmS. To further investigate how the C-terminal 30 amino acids contribute

FIG 5 Exchange of C-terminal secretion signals alters *lcmS* dependency. Histograms depict the fold change in cytosolic [cAMP] for THP-1 cells infected for 48 h with wild-type *C. burnetii* or the *lcmS* mutant producing the indicated CyaA fusion proteins. (A) Secretion of CBU0072 and CBU1525 fused to the C-terminal 50 amino acids of CBU0794 (CBU0072 Δ CT:CBU0794 or CBU1525 Δ CT:CBU0794, respectively). (B) Secretion of CBU0794 fused to the C-terminal 50 amino acids of CBU0072 or CBU1525 (CBU0794 Δ CT:CBU0072 or CBU0794 Δ CT:CBU1525, respectively). The cutoff for positive secretion is indicated by a dotted line at 2.5-fold change [cAMP]. Error bars indicate the standard deviations from the means for two independent experiments conducted in duplicate. The asterisk indicates a statistically significant difference ($P < 0.01$).

to *lcmS* dependency, translocation was assessed for CBU0794_{1–435} and CBU1525_{1–353} (Fig. 4C). Similar to the full-length protein, CBU0794_{1–435} was secreted by wild-type bacteria but not by the *lcmS* mutant, which, together with the N-terminal truncation data, suggests that *lcmS*-dependent export of CBU0794 by wild-type *C. burnetii* can be directed by a C-terminal and/or an internal secretion signal. In contrast, CBU1525_{1–352} was not secreted by the $\Delta lcmS$ mutant, thereby confirming that its C-terminal secretion signal is required for *lcmS*-inhibited export. These data suggest that CBU1525 has a C-terminal secretion signal whereas CBU0794 has at least two signal sequences, a C-terminal signal and an internal signal, similar to SidJ (37).

Exchange of C-terminal secretion signals changes *lcmS* dependency. To further explore how Dot/*lcm* secretion signals affect *lcmS* regulation, CyaA translocation assays were conducted with C-terminal domain swap constructs. The C-terminal 50 amino acids from the *lcmS*-inhibited substrates CBU0072 and CBU1525 were removed and replaced with the C-terminal 50 amino acids from the *lcmS*-dependent substrate CBU0794. Conversely, two different CBU0794 constructs were generated fused to the C-terminal 50 amino acids of CBU0072 or CBU1525. THP-1 cells were infected for 48 h with *C. burnetii* producing the CyaA fusions of the native substrate or domain swap constructs, and cytosolic [cAMP] was measured (Fig. 5). The CBU0072 and CBU1525 chimeras were exported by wild-type *C. burnetii*, which was in opposition to the *lcmS*-inhibited export of the native substrates. This result indicated C-terminal secretion signals contribute to export of CBU0072 and CBU1525 by the $\Delta lcmS$ mutant. The CBU0794 Δ CT:CBU0072 chimera exhibited *lcmS*-dependent secretion similar to native CBU0794. Interestingly, the CBU0794 Δ CT:CBU1525 chimera was secreted by both *C. burnetii* strains, but at significantly greater levels by wild-type bacteria. Thus, only a subset of secretion signals within *lcmS*-inhibited substrates are sufficient for protein translocation by the $\Delta lcmS$ mutant. These results illustrate that the C-terminal secretion signal is one of several factors that can influence *lcmS* dependency.

***lcmS*-dependent and -inhibited effectors bind *lcmSW*.** Affinity tag fusion to *lcmS* disrupts interactions with *lcmW* required for chaperone recognition of synthesized effectors (39). Therefore, pulldowns with *lcmW* N-terminally tagged with glutathione S-transferase (GST) were conducted to investigate effector engagement by the *lcmS*-containing chaperone complex. *In vitro* transcription and translation (IVTT) was used to simultaneously produce GST-*lcmW*, *lcmS*, and selected *lcmS*-dependent, -independent,

FIG 6 IcmSW binds IcmS-dependent and -inhibited substrates. Immunoblots of GST-tagged IcmW and XpressT-tagged CBU0077, CBU0794, CBU0665, or CBU1525 with or without their C-terminal 50 amino acids. GST-IcmW, IcmS, and individual substrates were produced together by IVTT. After incubation of cell extracts with GST-affinity beads, unbound (Flow through) and bead-bound (GST pulldown) proteins were immunoblotted with antibodies against the GST or XpressT tags.

or -inhibited substrates N-terminally tagged with XpressT (52). IVTT extracts were incubated with GST-affinity beads to collect proteins complexed with GST-IcmSW. Immunoblots of GST pulldown samples demonstrated IcmSW binding to CBU0794 (IcmS dependent) and CBU1525 (IcmS inhibited) but not CBU0665 (IcmS independent) (Fig. 6). For reasons that remain unclear, only an ~30-kDa N-terminal fragment of CBU0794 was produced by IVTT. Therefore, we performed pulldowns with CBU0077, a second IcmS-dependent substrate that also bound GST-IcmSW. Deletion of substrate C-terminal 50 amino acids did not alter binding to GST-IcmSW, suggesting that both substrates have internal IcmS binding sites. Together, these data indicate that IcmSW recognition of internal sites within IcmS-dependent or -inhibited effectors can positively or negatively regulate T4BSS translocation.

DISCUSSION

C. burnetii encodes a virulence-associated T4BSS that exhibits conserved functions with the *L. pneumophila* Dot/Icm secretion apparatus. Historical limitations of *C. burnetii* genetics hindered direct investigations of T4BSS function. Thus, *L. pneumophila* was largely used to gain an understanding of the *C. burnetii* T4BSS and to identify the effector cohort. In this study, we used current techniques for *C. burnetii* genetic manipulation to investigate the role of the T4BSS chaperone protein IcmS in intracellular growth of *C. burnetii* and effector secretion. Gene deletion by allelic exchange produces a *C. burnetii* Δ icmS mutant with a partial intracellular growth defect that correlates with production of a small CCV. The ~10-fold replication defect of the Δ icmS deletion mutant in Vero cells is consistent with published reports of growth by icmS transposon mutants in different cell lines (7, 8, 42). Overall, the moderate growth defects observed for icmS mutants here and elsewhere (7, 8, 42) may reflect a low number of IcmS-dependent effectors (20% in this study) and/or functional redundancy in the effector repertoire. One poorly growing transposon mutant occupied multiple, small CCVs, indicating defective homotypic fusion of *Coxiella* phagosomes. However, the transposon insertion eliminated only two amino acids from the C-terminal end of IcmS; thus, it is unclear how this small deletion results in the multivacuolar phenotype (19).

Studies of *L. pneumophila* T4BSS function have employed gene deletion or point

mutations to identify lcmSW-binding and C-terminal signal sequences required for secretion. These reports indicate that *L. pneumophila* T4BSS effector translocation can be directed by a C-terminal secretion signal, by both C-terminal and internal secretion signals, or by an internal secretion signal (24, 27, 28, 32, 34, 37, 40, 53). Of the 50 *C. burnetii* proteins tested in this study, 36 are secreted by the Δ lcmS mutant at levels significantly greater than or equal to wild-type *C. burnetii*. For this group of effectors, C-terminal sequence alignments show enrichment of acidic E-block residues in the region from -20 to -10 . Strong E-block motifs containing ≥ 3 acidic residues are most prominent within the set of lcmS-inhibited substrates, including CBU1525 and CBU0072. CBU1525 encodes a C-terminal secretion signal typical of *L. pneumophila* Dot/lcm substrates exported in the absence of lcmS. Replacement of the C-terminal 50-amino-acid peptide of lcmS-dependent CBU0794 with that of CBU1525 results in a hybrid protein that is secreted by the *C. burnetii* Δ lcmS mutant. CBU0072 encodes an atypical C-terminal secretion signal with acidic residues (ELEEE, amino acids -25 to -21) located outside the normal E-block location (28). The CBU0794 Δ CT:CBU0072 chimera is not secreted by the Δ lcmS mutant, indicating that C-terminal sequences of lcmS-inhibited effectors are functionally diverse. Indeed, eight substrates that exhibit positive or equivalent secretion by the Δ lcmS mutant relative to wild-type *C. burnetii* do not exhibit prominent E-block motifs (CBU0072, CBU0129, CBU0534, CBU0665 [CvpA], CBU0781, CBU1024, CBU1818 [CvpD], and CBU1819).

Early secretion of *L. pneumophila* SidJ is mediated by a conventional C-terminal signal sequence, while late secretion is orchestrated by an internal signal (37). N-terminal truncation constructs of CBU0794 and CBU1525 with ≥ 50 C-terminal amino acids exhibit the same lcmS-dependent and -inhibited secretions, respectively, consistent with the lcmS dependency of full-length proteins. However, CBU0794 and CBU1525 constructs that encode fewer C-terminal residues exhibit altered secretion. Specifically, the C-terminal 40 amino acids of CBU0794 and CBU1525 are secreted by both wild-type bacteria and the Δ lcmS mutant, whereas 30-amino-acid constructs of both substrates are only secreted by the Δ lcmS mutant. This indicates that the -50 to -40 sequences of CBU0794 and CBU1525 contain sites that regulate lcmS dependency. Additionally, both substrates harbor a secretion signal within the C-terminal 30 amino acids that is inhibited by lcmS. The *L. pneumophila* effector SidJ contains multiple lcmS binding sites distinct from the C-terminal 40-amino-acid peptide that encodes a secretion signal (37). Thus, it is unclear how lcmS represses the secretion of CBU0794 and CBU1525 C-terminal 30-amino-acid peptides by *C. burnetii*. Interestingly, the CBU0794₁₋₄₃₅ construct that lacks only the C-terminal 30 amino acids is translocated by wild-type *C. burnetii* at levels comparable to the full-length protein. Reminiscent of the *L. pneumophila* effectors SidG and SidJ (32, 37), these characteristics of CBU0794 are suggestive of both C-terminal and internal T4BSS secretion signals. In contrast, CBU1525 and CBU0072 are not translocated by wild-type *C. burnetii*, and their C-terminal 30-amino-acid peptides are required for export by the Δ lcmS mutant. We were unable to identify infection conditions that derepressed secretion of full-length CBU1525 or CBU0072 by wild-type *C. burnetii*. However, swapping out the endogenous C-terminal 50-amino-acid sequences from CBU1525 or CBU0072 with the C-terminal 50 amino acids of CBU0794 produces chimeric proteins that are secreted by wild-type *C. burnetii* but not by the Δ lcmS mutant. Together, the domain swap experiments indicate that sites within the C-terminal 50 amino acids promote secretion of CBU0072 and CBU1525 by the Δ lcmS mutant while inhibiting secretion by wild-type bacteria. However, C-terminal signals from CBU0072 and CBU1525 do not abrogate lcmS-mediated secretion of CBU0794 by wild-type *C. burnetii*. Thus, lcmS-dependent secretion of CBU0794 that is mediated by an internal secretion signal(s) exerts greater control over substrate translocation than the lcmS-inhibited C-terminal signals from CBU0072 or CBU1525. Along with pulldown experiments that show that lcmSW binds both lcmS-dependent and -inhibited substrates, these data indicate a regulatory hierarchy involving lcmS controls export of Dot/lcm substrates in *C. burnetii*. Indeed, lcmSW chaperone activity both positively

and negatively regulates substrate translocation in *C. burnetii*. To our knowledge, inhibition of substrate translocation in the presence of IcmS is unique to the *C. burnetii* T4BSS.

All of the 11 IcmS-inhibited substrates described in this study were initially identified in screens that used *L. pneumophila* as a surrogate for validating Dot/Icm substrate translocation (3, 30, 48, 49), including five ankyrin-repeat (Ank) family effectors. AnkA, -B, -F, -G, and -H are secreted at greater levels by the Δ icmS mutant than by wild-type *C. burnetii*. However, when expressed in *L. pneumophila*, AnkB, -F, -G, and -H are secreted more efficiently by wild-type *L. pneumophila* than by an Δ icmS mutant, whereas AnkA secretion increases in an Δ icmS mutant (31). We interpret these differences in substrate translocation to mean that a subset of *C. burnetii* effectors are controlled by IcmS regulatory mechanisms that are distinct from the *L. pneumophila* Dot/Icm system.

In pilot immunoblot experiments we were unable to detect IcmS or IcmW in *C. burnetii* protein lysates with monospecific sera against *L. pneumophila* IcmS or IcmW. However, a previous microarray study indicates stable expression of *icmS* and *icmW* throughout the *C. burnetii* infection cycle (54). *C. burnetii* IcmS and IcmW proteins exhibit 56 and 60% identities to the respective *L. pneumophila* proteins. Impaired growth of *L. pneumophila* Δ icmS or Δ icmW mutants is restored by genetic complementation with respective *C. burnetii* homologs (45), indicating the chaperone complex functions similarly in the two pathogens. Consequently, discrepancies in substrate translocation between *C. burnetii* and *L. pneumophila* observed here and previously (46, 47) likely result from species-specific factors that modulate IcmSW activity. Several recent *L. pneumophila* studies indicate IcmS binding is regulated, in part, by its interactions with other components of the T4CP, including IcmW, DotL, and LvgA (38–40). Notably, *C. burnetii* lacks a homolog of LvgA, an adaptor protein unique to *L. pneumophila* that binds hydrophobic residues on IcmS (39). *C. burnetii* encodes a known homolog of DotL, but an adaptor protein that binds IcmS has not been identified.

Our data suggest that IcmS dependency in *C. burnetii* is regulated according to specific sites within Dot/Icm substrates that function as secretion signals or IcmS binding sites. Overall, substrates secreted in the absence of IcmS, including both IcmS-inhibited and -independent substrates, often exhibit C-terminal signals containing an acidic E-block motif, whereas IcmS-dependent substrates typically lack E-block secretion signals. Exceptions to this trend, exemplified by CBU0080 and CBU1024, indicate that the E-block motif is not the only factor controlling IcmS dependency. Indeed, the observation that IcmS-inhibited secretion of CBU1525 is abrogated by deletion of the –50 to –40 region suggests that IcmS dependency can be regulated by sites distinct from the C-terminal 30 amino acids that contains the secretion signal. Additionally, interactions between IcmS and other proteins within the *C. burnetii* T4CP, for example, a functional analog to LvgA, could alter IcmS activity. In this model, interactions between IcmS and different T4CP adaptor proteins modulate which substrate sites are bound by IcmS and whether IcmS positively or negatively regulates specific types of secretion signals. Detailed characterization of the *C. burnetii* T4CP is critical for determining the molecular basis of Dot/Icm substrate translocation by the *C. burnetii* and how it differs from the *L. pneumophila* T4BSS.

In summary, this study shows a complex interplay between the chaperone IcmS and the *C. burnetii* T4BSS effector pool. Three export behaviors based on IcmS dependency were identified in addition to an internal IcmS binding site. Several intriguing questions remain unanswered. For example, what are the precise physiochemical properties of internal sites engaged by IcmSW? Further, does IcmSW target effector subsets with common functions and/or temporal constraints that modify host cell functions at specific stages of *C. burnetii*'s lengthy infectious cycle? A better understanding of regulation of *C. burnetii* T4BSS secretion will provide important insight into the pathogen-host cell relationship.

MATERIALS AND METHODS

***C. burnetii* and mammalian cells.** The *C. burnetii* Nine Mile, phase II (NMII; clone 4, RSA439) strain was utilized throughout this work. Wild-type *C. burnetii* and genetic transformants were grown microaerobically in ACCM-2, as previously described (55). For storage, bacteria were pelleted after 6 days of growth, washed three times in phosphate-buffered saline (PBS; 1 mM KH_2PO_4 , 155 mM NaCl, 3 mM

Na₂HPO₄ [pH 7.4]), suspended in cell freezing medium (RPMI 1640 medium containing 10% dimethyl sulfoxide and 10% fetal bovine serum [FBS]; Invitrogen), and then frozen at -80°C . *Escherichia coli* Stellar (BD Clontech) and PIR1 cells (Invitrogen) were used for recombinant DNA procedures and cultivated in Luria-Bertani (LB) broth. *E. coli* transformants were selected on LB agar plates containing 50 $\mu\text{g}/\text{ml}$ of kanamycin or 10 $\mu\text{g}/\text{ml}$ of chloramphenicol. African green monkey kidney (Vero) cells (CCL-81; ATCC) were maintained in RPMI 1640 medium containing 10% FBS at 37°C and 5% CO₂. THP-1 monocytes (TIB-202; ATCC) were differentiated into macrophage-like cells by incubation overnight in RPMI 1640 medium containing 10% FBS and 200 nM phorbol myristate acetate (Sigma-Aldrich). *C. burnetii* replication in host cells or ACCM-2 was measured by quantitative PCR of genome equivalents (GE) as previously described using a probe specific to *dotA* (56, 57).

Generation and complementation of an *icmS* mutant. The plasmids and oligonucleotide primers used in this study are listed in Tables S1 and S2, respectively, in the supplemental material. Restriction enzymes were obtained from New England Biolabs. PCR was performed using Accuprime *Pfx* or *Taq* polymerase (Thermo Fisher Scientific). PCR primers were obtained from Integrated DNA Technologies. All cloning procedures were conducted using an In-Fusion PCR cloning system (BD Clontech), and DNA was transformed into either *E. coli* Stellar or PIR1 competent cells. For targeted inactivation of *C. burnetii icmS* (*cbu1642*), the 5' and 3' flanking regions of the gene were amplified from NMII genomic DNA by PCR using the upstream and downstream oligonucleotide pairs CBU1642-5'-F/CBU1642-5'-R and CBU1642-3'-F/CBU1642-3'-R, respectively. The 5' and 3' fragments were cloned into BamHI/Sall-digested pJC-CAT (5) by In-Fusion, resulting in the formation of an internal AgeI site between the 5' and 3' regions and the creation of pJC-CAT::CBU1642-5'3'. The 1169^{bp}-Kan cassette was amplified from pJB-Kan (55) by PCR with P1169-Kan-AgeI-KO-rev-F and P1169-Kan-AgeI-KO-rev-R oligonucleotides and cloned into AgeI-digested pJC-CAT::CBU1642-5'3' to create pJC-CAT::CBU1642-5'3'-Kan. For complementation of the *C. burnetii* $\Delta icmS$ mutant, the *cbu1642* promoter and gene region were amplified from NMII genomic DNA by PCR using the oligonucleotides CBU1642comp-F and CBU1642comp-R. The *cbu1642*comp fragment was cloned into EcoRI-digested pMiniTn7T-CAT (5) by In-Fusion to create pMiniTn7T-CAT::*cbu1642*comp.

Electroporation of *C. burnetii* and selection of allelic-exchange mutants were conducted as previously described (5, 55). The *C. burnetii* $\Delta icmS$ strain was subsequently expanded by culture in ACCM-2 containing kanamycin, and clones were isolated by limiting dilution in ACCM-2. Deletion of *icmS* and clonality were verified by PCR. PCR validation was accomplished by amplifying an internal *icmS* gene fragment with specific primer pairs, using wild-type or mutant genomic DNA as the template. Generation and verification of a Tn7-based genetic complement of the *C. burnetii* $\Delta icmS$ strain were performed as previously described (5).

Indirect immunofluorescence. Vero cells infected at a multiplicity of infection (MOI) of ~ 25 were fixed for 20 min in 4% paraformaldehyde plus PBS, followed by permeabilization for 5 min in 0.1% saponin in PBS. Cells were stained for indirect immunofluorescence as previously described (52, 58). Rabbit anti-*C. burnetii* serum and a mouse monoclonal antibody directed against the lysosomal marker CD63 (LAMP3) (clone H5C6, BD Biosciences) were used as primary antibodies. Alexa Fluor 488- and 594-IgG (Invitrogen) were used as secondary antibodies. Coverslips were mounted using ProLong Gold (Thermo Fisher Scientific). Microscopy was conducted using a modified Perkin-Elmer UltraView spinning-disk confocal system connected to a Nikon Eclipse Ti-E inverted microscope. Images were obtained using Metamorph software (Molecular Devices, Inc.) and processed with ImageJ software (National Institutes of Health).

Translocation assays. Full-length, partial, and chimeric genes encoding published Dot/Icm substrates were amplified by PCR from NMII genomic DNA using the oligonucleotide primers listed in Table S2. PCR products were cloned by In-Fusion into the unique Sall site of pJB-CAT-CyaA or pJB-CAT-BlaM to create the translocation reporter plasmids listed in Table S1. CyaA and BlaM translocation assays were performed using THP-1 macrophage-like cells (5×10^5 per well) in 24-well plates. Cells were washed once with RPMI medium plus 10% FBS, infected with *C. burnetii* transformants expressing CyaA or BlaM fusion proteins at an MOI ~ 50 , and incubated in RPMI medium plus 10% FBS for 48 h. For CyaA translocation assays, the concentration of cAMP in lysates from infected cells was determined using the cAMP enzyme immunoassay (GE Healthcare) as previously described (4). Positive secretion of CyaA fusion proteins by wild-type *C. burnetii* or the $\Delta icmS$ mutant was scored as ≥ 2.5 -fold more cytosolic cAMP than the negative control (wild-type *C. burnetii* expressing CyaA alone) in accordance with the previously validated threshold for secretion (4, 16, 30, 46, 47). For BlaM translocation assays, infected cells in glass bottom plates were loaded for 1 h with CCF4-AM (LiveBLAzer-FRET B/G loading kit; Invitrogen) in solution with 15 mM probenecid (30). Cells were replenished with fresh medium and immediately imaged on a LSM710 confocal laser-scanning microscope (Carl Zeiss Micro Imaging) with a 405-nm diode laser and detectors at 520 to 630 nm for CCF4-AM substrate (green) and 410 to 488 nm for CCF4 product (blue), respectively. Image segmentation and signal quantitation were performed using CellProfiler as previously described (59). Positive translocation of BlaM fusion proteins was scored as a ratio of blue to green signal ≥ 2.5 -fold more than the mean of uninfected cells.

Protein binding assays. Full-length *C. burnetii icmS*, *lcmW*, *cbu0077*, *cbu0665*, *cbu0794*, and *cbu1525*, and respective gene fragments encoding proteins with 50-amino-acid C-terminal deletions were amplified by PCR using the oligonucleotide primers listed in Table S2. PCR products were cloned by In-Fusion into pEXP1-DEST (Invitrogen) digested with BsrGI/HindIII (New England Biolabs) to generate plasmids encoding proteins with N-terminal Xpress tags. For production of the *lcmSW* fusion tagged with GST on the N terminus of *lcmW*, *icmS* was amplified by PCR and fused downstream of an amplicon encoding *lcmW*. The resulting gene fusion was inserted into pDEST15 (Invitrogen) digested with BsrGI/BamHI. Effector-encoding plasmids were mixed with the *lcmSW*-encoding plasmid in equal amounts for in vitro

reactions using the RTS 100 *E. coli* HY kit (Roche) to achieve cell-free production of protein. IVTT reaction mixtures were incubated at room temperature for 1 h with glutathione-Sepharose 4B (GE Healthcare). Glutathione-Sepharose was pelleted in Pierce spin cups (Thermo Fisher) by centrifugation at $1,000 \times g$ for 1 min and washed five times in Tris-buffered saline (pH 7.2) containing 1% Triton X-100. Glutathione-Sepharose was resuspended in SDS-PAGE loading buffer and boiled for 10 min. Proteins were separated by SDS-PAGE on a 10% gel and then immunoblotted as described above using mouse monoclonal antibodies directed against the GST tag (EMD Millipore) to detect IcmW or the Xpress tag (Invitrogen) to detect effector proteins.

Statistical analysis. Statistical analyses were performed using a one-way analysis of variance or a Student *t* test and Prism software (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00431-19>.

SUPPLEMENTAL FILE 1, PDF file, 5.8 MB.

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