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Supplemental Information

IcmQ in the Type 4b Secretion System Contains an NAD^+ Binding Domain

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Inventory of Supplemental Information

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Supplemental Methods

Binding assays with NAD+

For NMR binding studies, double labeled Qcl (^{15}N and ^{13}C) was made as described (Marley et al, 2001). For the NAD+ titration the concentration of Qcl was 330 uM in 100 mM NaCl, 20 mM phosphate buffer, pH 7.5 in 10% D_2O with ~500 uM trimethylsilyl propionate as a reference (Wishart et al., 1995). Freshly prepared NAD+ or NADH was added from 50 mM stock solutions (or serial dilutions) in the same buffer without D_2O. After each addition, 2D, ^{15}N-heteronuclear single quantum coherence (HSQC) spectra were acquired on a Bruker DMX500 spectrometer using a sensitivity enhanced pulse sequence (Palmer et al., 1991; Kay et al., 1992; Schleucher et al., 1994) at 20° C. Spectra were acquired as 128 t1 increments that were the average of 16 scans of 2040 data points. The NMR data was processed using NMRPipe (Delaglio et al., 1995) and analyzed with NMRView (Johnson and Blevins, 1994).

For filter binding titrations, 20 μM Qcl was incubated in increasing concentrations of a 1:2,000 mixture of ^{32}P-NAD+:NAD+ (150-2,400 μM) for 3 minutes at 30°C. To prevent dilution-associated unfolding or aggregation of Qcl, 1.5 μM BSA (New England Biolabs) was also added to each reaction mixture. To account for non-specific binding, equivalent amounts of ^{32}P-NAD+ were tested with 50 mM NAD+, 20 μM Qcl, and 1.5 μM BSA present in the reactions. For competition binding assays, 20 μM protein, as well as 1.5 μM BSA was incubated at 30°C for 5 minutes with 5 μCi of ^{32}P-NAD+ and either 5 mM NAD+ or NADH. Fifteen μL of each sample were deposited in quadruplicate on Whatman GF/B filters that were pre-moistened with 20 mL of 10 mM Tris, pH 7.5 (wash buffer). Filters were washed with 40 mL of wash buffer, and then placed in scintillation vials. Counting was performed using a scintillation counter and the data analyzed with Microsoft Excel. Non-specific binding was subtracted from total binding to give a specific binding curve and the Kd was determined by calculating a Lineweaver-Burk plot.

Membrane binding and permeation assays

L-α-phosphatidylcholine (PC, chicken egg) and 1,2-dimyristoyl-sn-glycerol-3-[phospho-rac-1(glycerol)] (DMPG) were acquired from Avanti Polar Lipids. PC and DMPG were mixed in a 3,1 (w/w) ratio and aliquoted into Corex glass vials in 1 mg quantities and dried under a

nitrogen stream for 20 min at room temperature. Tubes were then placed under vacuum overnight to remove trace organic solvent. Lipid aliquots were resuspended in 250 µl of 20 mM Hepes pH 7.2, 25 mM NaCl and 1 mM EDTA (HNE buffer) by gentle vortexing in a Corex tube. Resolubilized lipids were frozen and thawed several times by immersion of the tube in liquid nitrogen and then a water-bath at 50°C. Next, the suspension was passed 11 times through a membrane with a pore size of 1 um using a mini-extruder (Avanti Polar Lipids). The final concentration of vesicles averaged between 3-4 mg/ml. Membrane binding and calcein release assays were performed as described (Duménil et al., 2004).

To calculate the number of lipids/vesicle, we assumed each lipid molecule had a surface area of 70 $Å^2$ (White and King, 1985). We used a 0.1 micron filter to make vesicles; so the total vesicle surface area was calculated and divided by the average surface area of a lipid molecule to give ~110,000 lipid molecules per vesicle. Next, when vesicles were prepared their lipid concentration after all the steps was determined, (usually between 3-4 mg/ml) and we diluted vesicles to 1.2 ng/uL for calcein release assays. We used this concentration and the given parameters to calculate the approximate number of vesicles in the assay. We then added IcmQ at various concentrations, measured calcein release and calculated protein/vesicle ratios. For this method, we did not have to determine the concentration of protein attached to membranes. However, IcmQ added to the vesicles in our conditions reached a binding saturation point around 1500 IcmQ per vesicle. Interestingly, at this point in our calcein release assay, we stopped seeing an increase in calcein release, as vesicles were probably coated at this point.

Preparation of *Legionella pneumophila* with a mutant *IcmQ*

To mutate the *icmQ* gene in the Lp01 strain, a tri-parental mating assay was employed utilizing the pSR47s "suicide plasmid" (Huang et al., 2011). First, the *icmQ* gene was cloned from the Lp01 strain with 1000 bp excess on each side of the desired mutation site. To introduce mutations into *icmQ*, Site-Overlap PCR was employed. Briefly, in the first round of PCR, two sets of products were obtained. The first set contained the 5' upstream region of *icmQ* up to the desired mutations, and the second set contained the desired mutants and the 3' downstream region of *icmQ*. The final round of PCR combined these two products in an overlapping reaction.

Next, the newly created PCR product with IcmQ mutant as well as pSR47s were treated with BamHI and NotI (Promega), purified on a 1% agarose gel and ligated using DNA ligase (Promega). Ligated plasmids were transformed into DH5α (λ pir) *E. coli* and grown overnight on LB agar plates with 100 µg/mL kanamycin. Single colonies were selected and sequenced to ensure that the desired mutants were present. Upon sequence confirmation, DH5α (λ pir) cells were transformed with pSR47s plasmid. A stable glycerol cell stock was then prepared and stored at -80°C. The tri-parental assay used this cell line along with an MT600 *E. coli* "helper strain," and the Lp01 *Legionella* cell line. The MT600 helper strain carries a plasmid translocation system. This strain will transport a DNA plasmid encoding this translocation system into the DH5α (λ pir) pSR47s-mutant cell line. Once in this cell line, the assembled translocation system will transport the pSR47s plasmid into the Lp01 cell line. After the pSR47s-mutant plasmid is in the Lp01 cell line, genomic recombination replaces the native *IcmQ* with the mutated copy. A double selection process was then used to select colonies that have successfully incorporated the mutation and lost the exogenous plasmid. The appropriate region of the chromosome was then sequenced. The new stable cell line was called Lp01-IcmQtr and a glycerol cell stock was prepared and stored at -80°C.

Matched expression of IcmQ and IcmQ-D151A in *Legionella*

Bacterial Strains
LP02 (*thyA, hsdR, rpsL*) (Berger and Isberg, 1993)
GD59 (*thyA, hsdR, rpsL, icmQ*) (Duménil and Isberg, 2001)

Plasmids
pKB25 (Roy *et al*., 1998)
pKB26 (Roy *et al*., 1998)
pMMB207Δ267 (*mobA*$^-$) (Creasey and Isberg, 2012)
pKB26IcmQ (pKB26 *dotA*$^-$ *icmQ*)
pKB25IcmQD151A (pKB25 *dotA*$^-$ *icmQ* D151A)
pIcmQ (pMMB207Δ267 *rrnB* T1 transcriptional terminator (2X) *icmQ*)
pIcmQD151A (pMMB207Δ267 *rrnB* T1 transcritpional terminator *icmQ D151A*)

Oligonucleotides
IcmQF GGGAGCTCTCCCCTAATTCTTGGTTCCCATAAGT
IcmQR GGGTCGACAACGGCCTATGCATTTTT

Plasmid Constructions

PCR was used to amplify *icmQ* from LP01 and LP01 (*icmQ* D151A) genomic DNA using *Sac*I and *Sal*I tails on the primers. PCR products were then digested with *Sac*I and *Sal*I, ligated into similarly digested pKB25 (single transcription terminator plasmid) and pKB26 (double transcription terminator plasmid) to generate pKB25IcmQD151A and pKB26IcmQ respectively. These plasmids were digested with *Apa*I and *Sal*I and the fragments containing P_{tac}, *rrnB* T1 transcriptional terminators upstream regions and *icmQ* coding sequence were ligated into *Apa*I and *SalI* digested pMMB207Δ267 to generate pIcmQ and pIcmQD151A.

Bacterial Growth and Cell Culture

L. pneumophila strains were grown on CYET plates or in AYE broth, as described previously (Berger *et al.*, 1994). Chloramphenicol was added to a concentration of 5 μg/ml for *L. pneumophila* and 30 μg/ml for *E. coli*. To induce IcmQ expression in *L. pneumophila* strains IPTG was added to a concentration of 1 mM.

Mouse (A/J) bone marrow derived macrophages were grown in RPMI containing 10% FBS (Invitrogen). Macrophages were plated on cover slips in 24 well plates at $2x10^5$ cells/well. Prior to challenge, media was replaced with RPMI supplemented with thymidine to a concentration of 0.2 mg/ml and 1 mM IPTG. Macrophages were challenged at an MOI=1 with post exponential *L. pneumophila* cultures grown overnight in the presence of IPTG. To initiate infection, plates were spun at 1000 RPM for 5 min and then incubated at 37°C with 5% CO_2 for 1 hr. Wells were washed 3X with warm media containing thymidine and IPTG. At 14 hr post infection wells were washed 3X with PBS then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Fixed cover slips were washed 3X with PBS.

Intracellular growth was detected by immunofluorescence microscopy as described previously (Conover, *et al*, 2003). To stain extracellular bacteria, cover slips were probed with rat anti-*L. pneumophila* at 1:5000 followed by AlexaFluor 594 goat antibodies to rat Abs. After Triton X100 permeabilization, intracellular bacteria were detected by probing with rabbit anti-*L. pneumophila* at 1:5000 and AlexaFluor 488 goat anti-rabbit Abs. Nuclei were stained with Hoechst, and intracellular bacteria in each vacuole were enumerated for 100 vacuoles/slide.

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Table S1, related to Figure 6. Membrane binding and permeabilization by IcmQ.

Concentration of IcmQ (nM)	Percentage of Calcein Released	Protein: Vesicle Ratio
1	0	60
5	7	300
10	26	555
25	32	1,340
50	45	2,800
60	48	3,333
100	48	5,600
500	42	29,000
1000	48	57,000

Supplemental Figures

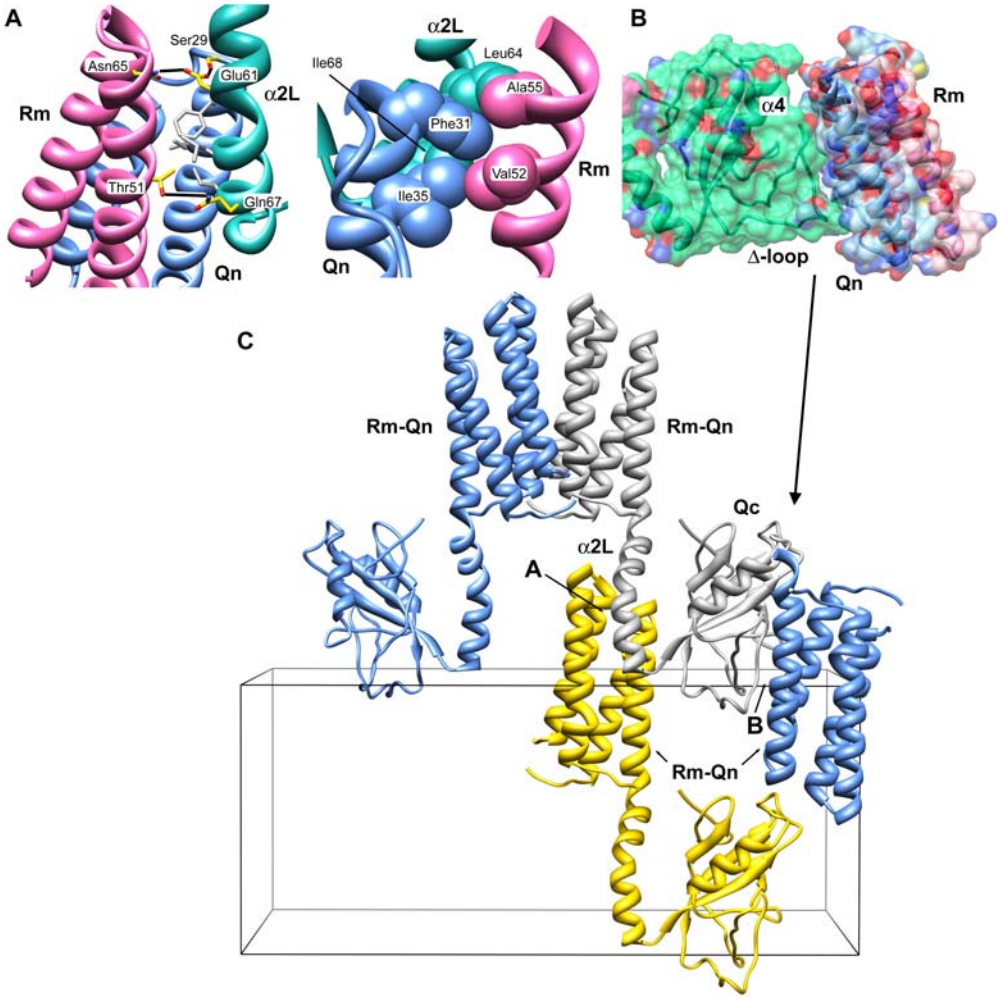

Figure S1, related to Figure 1. Lattice contacts in the Rm-IcmQ crystal.

A. (left) Contacts between the linker helix (shown in green) and the Rm-Qn 4 helix bundle of another molecule in the lattice. Glutamine 67 hydrogen bonds with Thr51 and Glu61 makes a hydrogen bonded network with Ser29 and Asn65. Van der Waals contacts are also present. The replacement of arginine and lysine side chains with the shorter glutamine side chains facilitated the crystallization. (right) van der Waals contacts in this crystal contact.
B. The Δ-loop of Qc has extensive interactions with Qn in an adjacent molecule.
C. An overview is shown of the crystal packing. The extended a2L helix (A) and Qc (B) make extensive interactions with symmetry mates which may have immobilized these domains.

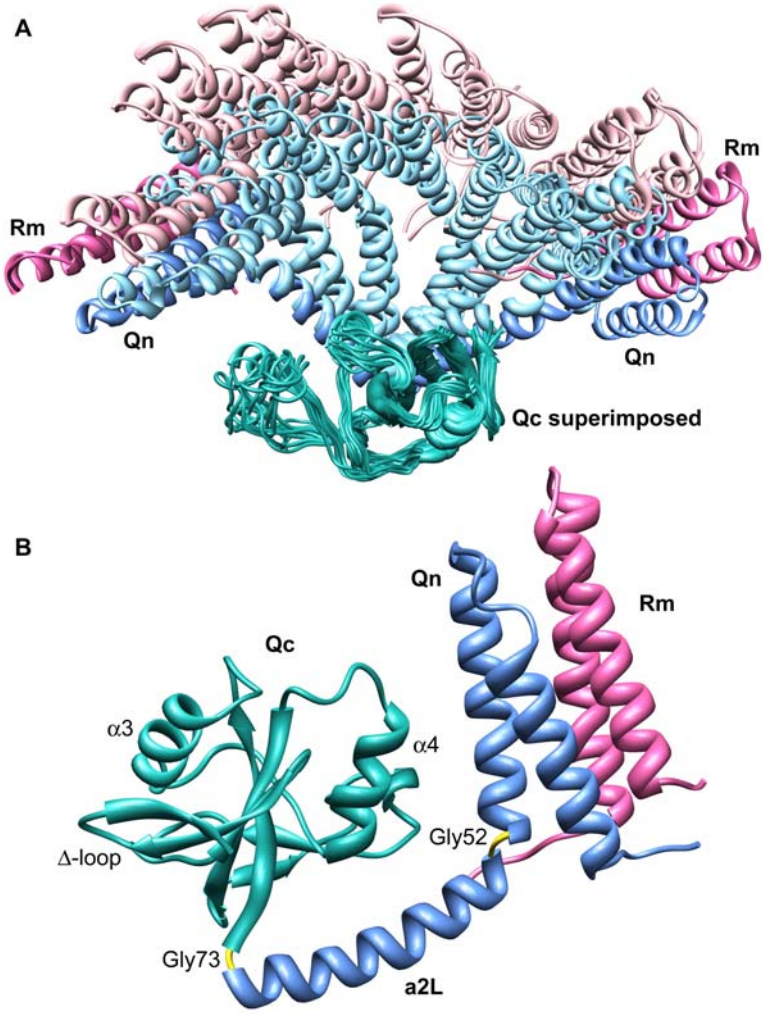

Figure S2, related to Figure 1. Molecular dynamics simulation of inter-domain flexibility in IcmR-IcmQ.

A. A range of IcmR-IcmQ conformations is shown based on a 50 ns molecular dynamics simulation with conformers aligned on Qc. **B.** A calculated structure is shown with the closest approach of Qc and Rm-Qn. Glycines 52 and 73 (shown in yellow) may act as hinges.

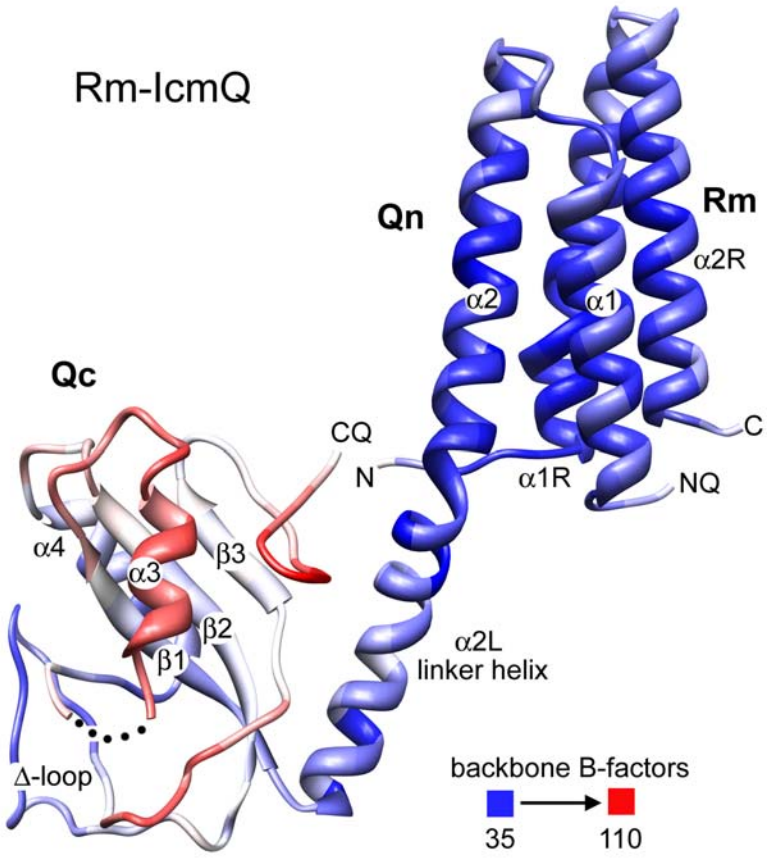

Figure S3, related to Figure 1. The B-factor distribution of the main chain of Rm-IcmQ in the crystal is shown as a gradient from blue to red with Chimera.

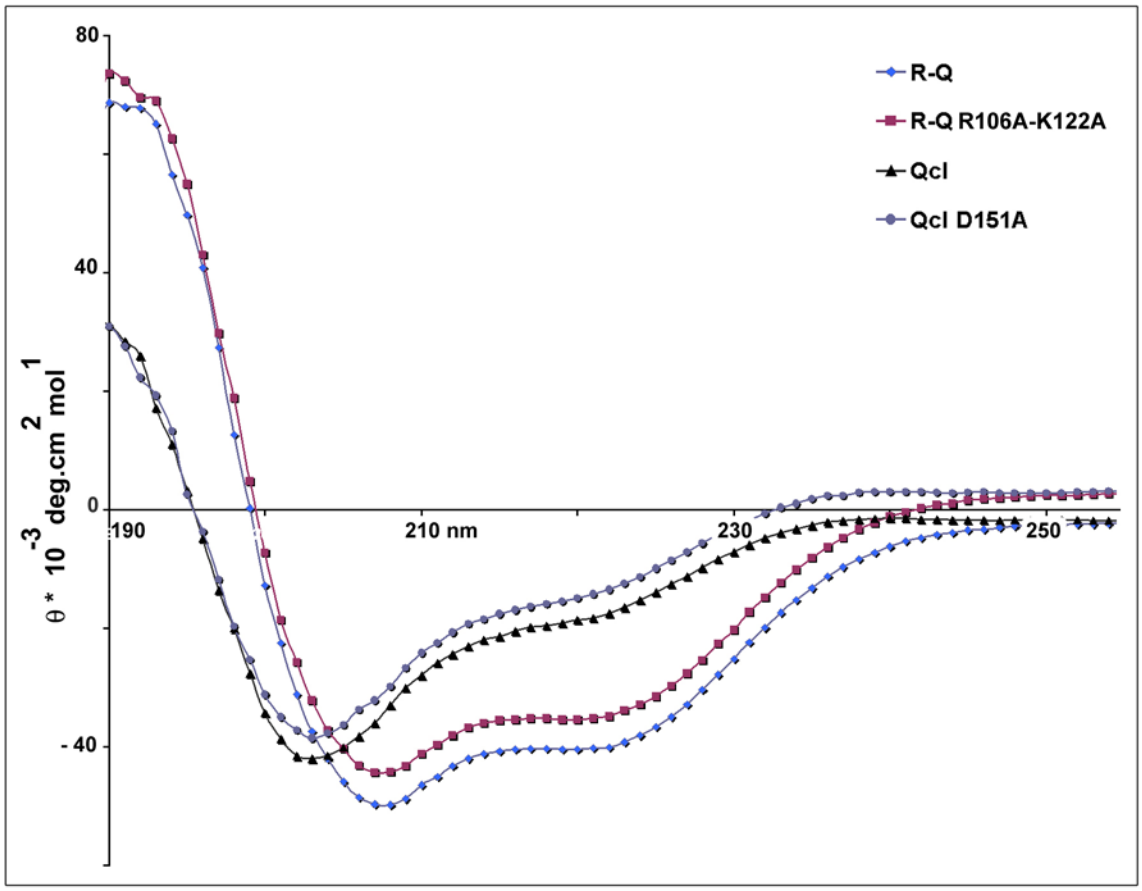

Figure S4, related to Figure 5. Circular dichroism spectra are shown for wild type and mutant Qcl and IcmR-IcmQ. Characteristics of the spectra are consistent with the determined crystal structure. Spectrum pairs have been offset vertically by 5 units to allow a clearer comparison. Spectra are color coded as indicated in the right most upper corner.

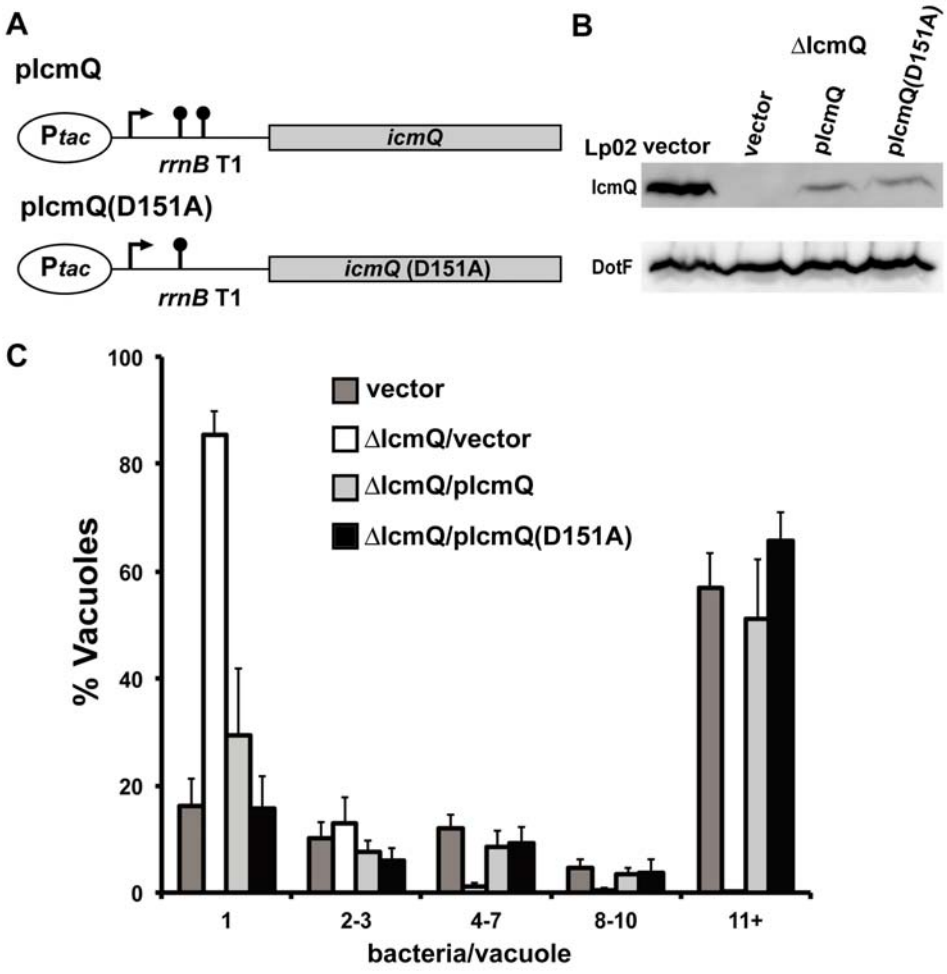

Figure S5, related to Figure 5. The *IcmQ*(D151A) mutation has no effect on intracellular replication for cells showing matched expression levels of mutant and wild type IcmQ.
A. Plasmid maps are shown of constructs used to express equivalent low levels of IcmQ and IcmQ (D151A) from the P*tac* promoter. The number of copies of the *rrnB* T1 transcriptional terminator are indicated by black stem loop structures (Brosius et al., 1981). The double terminator structure used to express wild type protein was necessary to reduce steady state levels to be equivalent to the D151A mutant. **B.** Artificial terminators allow identical levels of steady state IcmQ to be obtained in the two constructions. Western blot analysis was performed with anti-IcmQ (Dumenil and Isberg, 2001) on whole cell extracts fractionated on 12.5% gels from post-exponential cultures of *L. pneumophila.* **C.** Efficient replication vacuole formation was observed for *L. pneumophila* having low level expression of *IcmQ* and the *IcmQ*(D151A) mutation. Quantitative data for the number of bacteria/vacuole in A/J bone marrow derived macrophages is shown for cells challenged 14 hours with each Lp strain. Data are the mean ± standard deviation of three independent experiments performed in triplicate (9 samples total for each strain)

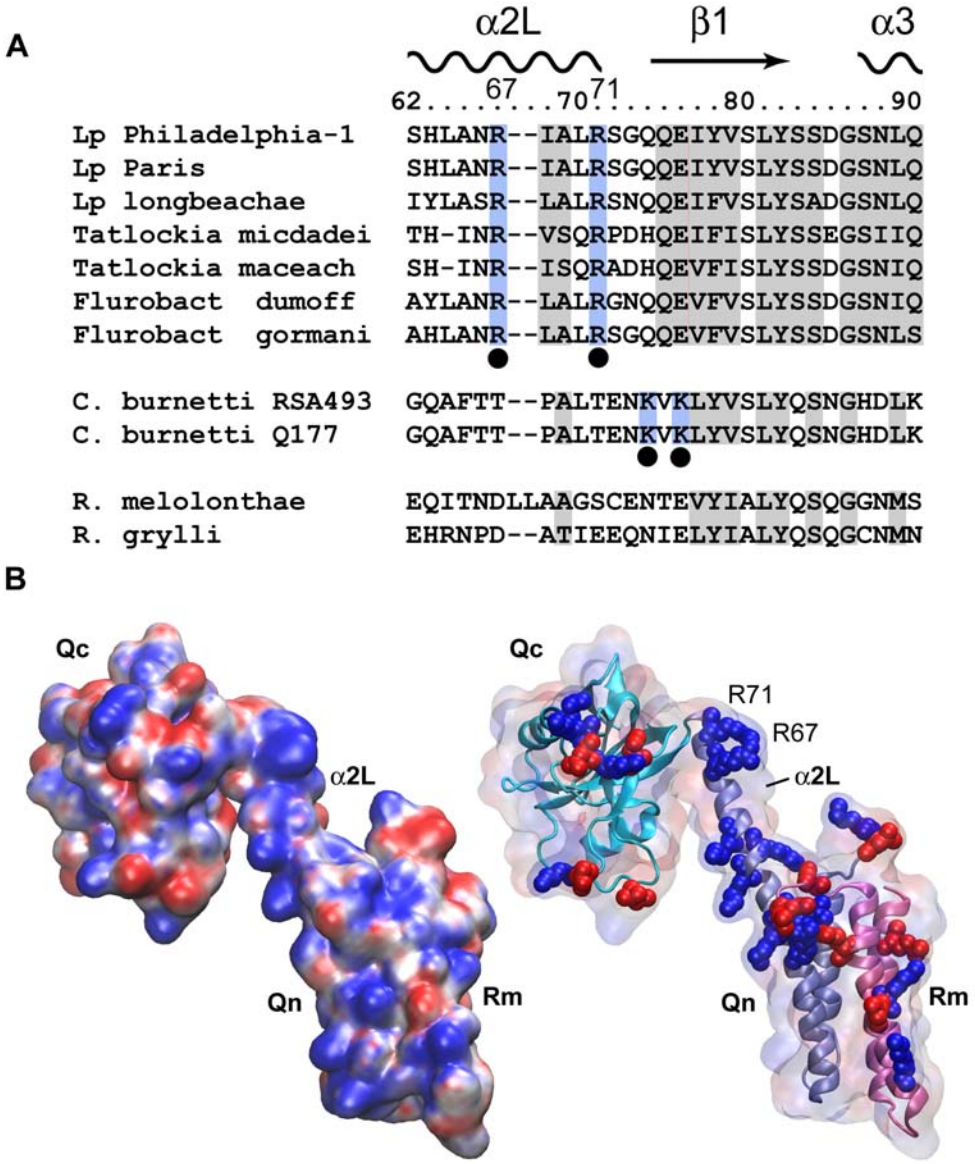

Figure S6, related to Figure 6. Positively charged residues in the linker helix of IcmQ.
A. Sequence conservation of Arg67 and Arg71 is shown. B. The charge distribution in the α2 linker helix of IcmQ is indicated. Basic residues may drive membrane association. The molecule is viewed from the membrane plane and residues within 5Å of the bilayer surface during the simulation are highlighted.

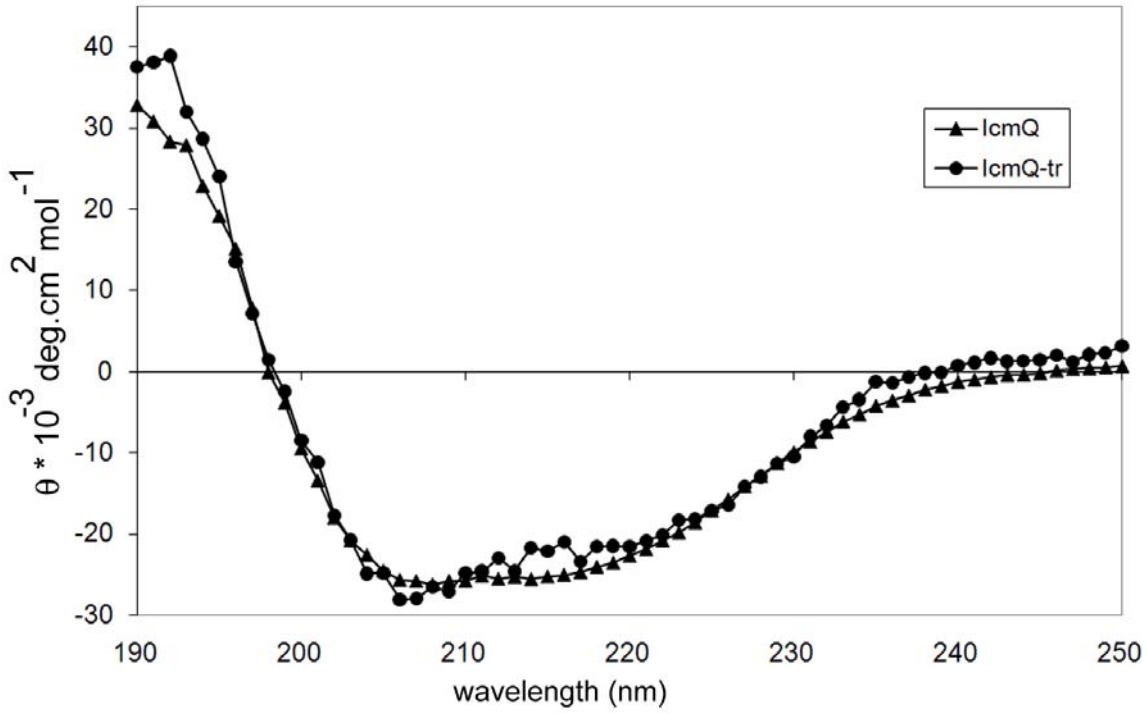

Figure S7, related to Figure 6. Circular dichroism spectra of IcmQ-tr and wild type IcmQ are super-imposable. This suggests that four glutamine mutations in the linker may not significantly alter the overall structure of IcmQ in solution.

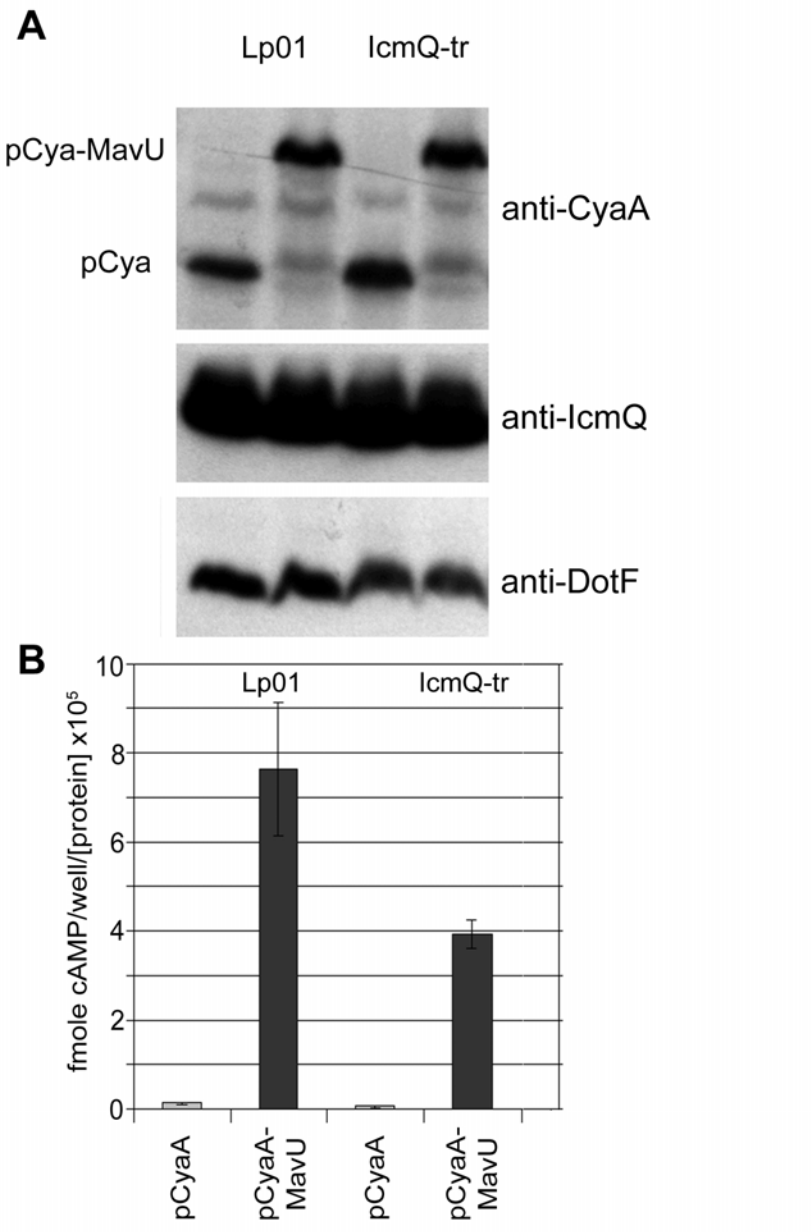

Figure S8, related to Figure 6. Translocation assays with *Legionella pneumophila* strains carrying wild type and a quadruple mutant *icmQ* (K57Q/K59Q/R67Q/R71Q; *Icm*Q-tr).
A. Protein expression levels were monitored with appropriate antibodies (as indicated) for wild type and mutant Lp strains. **B.** Results of inter-cellular translocation assays are shown for wild type and mutant Lp cells. Cyclic AMPase activity of CyaA-MavU fusion proteins was measured in macrophages relative to a CyaA control.