Legionella pneumophila proliferates within alveolar macrophages as a central property of Legionnaires’ disease. Intracellular growth involves formation of a replicative phagosome, which requires the bacterial Dot/Icm system, a multiprotein secretion apparatus that translocates proteins from the bacterium across the macrophage plasma membrane. Two components of this system, IcmR and IcmQ, are proposed to exhibit a chaperone/substrate relationship similar to that observed in other protein translocation systems. We report here that IcmQ inserts into lipid membranes and forms pores that allow the efflux of the dye calcein but not Dextran 3000. Both membrane insertion and pore formation were inhibited by IcmR. Trypsin digestion mapping demonstrated that IcmQ is subdivided into two functional domains. The N-terminal region of IcmQ was necessary and sufficient for insertion into lipid membranes and calcein efflux. The C-terminal domain was necessary for efficient association of the protein with lipid bilayers. IcmR was found to bind to the N-terminal portion of the protein thus providing a mechanism for its ability to inhibit IcmQ pore-forming activity. Localization of IcmQ on the surface of the L. pneumophila shortly after infection as well as its pore-forming capacities suggest a role for IcmQ in forming a channel that leads translocated effectors out of the bacterium.

Legionella pneumophila is a Gram-negative bacterium that multiplies inside amoebae found in fresh water ecosystems (1). It is believed that inhalation of aerosols containing either free-living L. pneumophila or amoeba laden with the bacteria leads to colonization of the lungs. Phagocytosed L. pneumophila then grow within alveolar macrophages in a fashion similar to that observed with amoeba (2, 3), eventually causing a severe form of pneumonia known as Legionnaires’ disease (4).

The ability of L. pneumophila to grow intracellularly can be recapitulated within cultured macrophages or cell lines (1). Phagosomes harboring L. pneumophila avoid fusion with the endocytic network as early as 5-min postinfection (5, 17), as endosomal markers such as Rab5 or the transferrin receptor are excluded from the phagosome (6, 7). Instead, the compart-

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bacterial envelope or on the mammalian plasma membrane, subcellular fractionation studies indicate that both IcmR and IcmQ are found in soluble fractions (25). The bulk of these two proteins is found in a complex likely to consist of one molecule of IcmQ and two or three molecules of IcmR (26). In the absence of IcmR, however, IcmQ forms large soluble homopolymers having a wide range of molecular weights, a property reminiscent of the heptamer of type III secretion substrates in the absence of their chaperones (27, 28). A chaperone-substrate interaction has also been observed in the virB secretion system of the plant pathogen Agrobacterium tumefaciens, suggesting that conjugal systems similar to Dot/Icm may use such chaperones (29, 30).

We report here that although the primary sequence of IcmQ indicates that it is a highly hydrophilic protein, purified soluble IcmQ efficiently inserts into lipid membranes allowing efflux of a fluor of defined molecular weight. This behavior is reminiscent of pore-forming toxins such as Staphylococcus aureus α-toxin (31).

MATERIALS AND METHODS

L. pneumophila Bacterial Strains and Cultures—L. pneumophila strains used in this study were derived from the wild-type strain LP02 (19). RP1095 (a derivative of LP02) was a ΔOpa mutant isogenic to LP02 (32). GD59 is isogenic to LP02, and contains a ΔEmE mutation. Strains were passaged on CYET plates and AYE broth as described previously (13).

For L. pneumophila, chloramphenicol was used at 5 μg/ml. For all experiments, L. pneumophila strains were grown to post-exponential phase (OD600 of 3.3–3.5) using bacterial motility as a post-exponential phase marker. The broad host range plasmid pMMB207 (33) was used to express the GST-IcmR fusion protein in E. coli. Production of the IcmR-IcmQ Complex—Plasmid pGD31 was used to express the GST-IcmR fusion protein in Escherichia coli (34). Plasmid pGD41 (described above) was used to express IcmQ in the absence of any tag. To allow in vivo formation of the complex, pGD31 and pGD41 were cotransformed into E. coli XL-1 blue (35), generating GD105. One-liter cultures of GD105 were grown to OD600 of 1.0 at 37°C and induced with 0.1 mM IPTG for 3 h. Bacteria were pelleted, resuspended in 40 ml of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 50 mM imidazole and the suspension was sonicated on ice for 2 min on 50% duty cycle with a Branson Sonifier 250, pelleted at 12,000 × g for 15 min, and lysates were loaded onto a 5-ml metal chelation Hi trap column (Amersham Biosciences) using a Amersham Biosciences FPLC system. After extensive washing of the column with TN buffer, His-tagged proteins were eluted using 50 m of a 0–200 m imidazole gradient. Fractions containing full-length IcmQ or IQC were pooled and dialyzed overnight against TN buffer.

IcmQ Membrane Insertion Assay—All lipids used in this study were purchased from Avanti Polar Lipids Inc. E. coli PL from this supplier represents a crude extract from bacteria with the following composition: 67% PE, 25% PG, and 10% CA. Lipids stored in chloroform at –80°C were thawed under a CO2 atmosphere and then dessicated overnight. Unless otherwise stated, 5 mg of lipids were used. Large unilamellar vesicles were prepared by two different techniques, either by detergent dilution (36) or by extrusion (37). For the detergent-based approach, lipids were resuspended in 0.5 ml of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5% N-octylglucoside in the presence or absence of protein. The mixtures were then dialyzed against TN buffer for 24 h. For the extrusion-based approach, the lipid cake was hydrated at room temperature for 30 min in 0.5 ml of 20 mM Tris-HCI, pH 7.5, 100 mM NaCl, and 1 mM EDTA (TNE). Four cycles of freeze and thaw were performed between an ethanol/dry ice mix and a water bath either at room temperature or at 60°C depending on whether the temperature of the lipids was above or below 50°C. The suspension was then extruded by several passes through a 0.1 μm pore polycarbonate membrane using a mini extruder (Avanti Polar Lipids). Certain lipid mixtures such as the PC 18:0 first required passage through a 1 μm polycarbonate membrane to prevent clogging of the filter.

To assess association of proteins with preformed lipid vesicles, 20 μg of protein was mixed with 5 mg of lipid vesicles in TNE buffer and incubated at room temperature for 30 min prior to sucrose gradient centrifugation. Sucrose in TNE buffer was added to the samples to a final concentration of 45% (w/v) and 1 ml of the mixture was placed at the tube bottom (bottom fraction). A 7 ml 30% (w/v) sucrose solution in TNE was layered on the samples (middle fraction). A 1 ml of TNE was layered on top (top fraction). The samples were then subjected to ultracentrifugation at 100,000 × g in a Beckman SW41 rotor for 15 h. The contents of the different fractions were analyzed by SDS-PAGE follow by Coomassie staining of the gel or by Western blot analysis. To generate lipid-associated IcmQ, the top fraction including the interface with the 30% (w/v) sucrose layer was aspirated and the membrane fraction was then resampled into TNE, prior to treatment with various reagents as described in Fig. 2.

Subcellular Fractionation—A 50-ml culture in AYE broth was grown until motility was observed (OD ~3.5). The bacteria were pelleted, the supernatant was precipitated with trichloroacetic acid and spheroplasts were made from the pelleted bacteria (38). Subcellular fractionation was then performed following the procedure by Coers et al. (25) except that Tris-HCl was used as a buffer instead of Heps. Briefly, sonicated spheroplasts were pelleted, resuspended in 20 mM Tris-HCl, pH 7.5, and loaded on a 3-step sucrose gradient. Membranes were collected at the 25–60% sucrose interface, washed with a

1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyrano- side; PL, phospholipid; FC, phosphatidylcholine; PE, phosphatidy- l ethanolamine; PS, phosphatidylserine; CA, cardiolipin; Ch, cholesterol; IDCH, isocitrate dehydrogenase; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)]ethyglycine; GST, glutathione S-transferase.
high salt solution containing 80 mM Tris-HCl, pH 7.5, and 0.5 M NaCl solution and then solubilized with 2% Triton X-100. Equivalent volumes of the different fractions were loaded on SDS-PAGE and analyzed by Western blot using antibodies directed against *L. pneumophila* proteins.

**Calcein Release Assay**—Lipid vesicles were generated by extrusion as described above except that lipids were resuspended at 10 mg/ml in TNE containing 80 mM calcein (pH 7.5) (39). Free calcein was then removed using a 10-ml G-75 column (Amersham Biosciences) equilibrated with TNE. The lipid vesicles were diluted in TNE to a final concentration of 60 μg/ml in cuvettes having four clear sides, and subjected to constant agitation. Fluorescence emission was monitored at 520 nm after excitation at 580 nm using an SLM Aminco spectrofluorometer. As an alternative to calcein, Dextran 3000 tetramethyl rhodamine (Molecular Probes) was resuspended at 20 mg/ml in TNE, added to the dried lipids and extruded lipid vesicles were prepared as above. Fluorescence was monitored at 580 nm after excitation at 530 nm.

**Sonication Release Assay**—PC vesicles containing IcmQ derivatives were prepared using the OG dialysis technique described above. As a control, vesicles were prepared in the absence of protein but in the presence of 80 mM calcein. Following purification, the lipid vesicles were sonicated using a Bransen Sonifier 250. To determine the efficiency of vesicle disruption, the vesicles were diluted in TNE and fluorescence emission was monitored as above. Sonication conditions were found such that there was 50% of the maximum fluorescence of the calcein-laden vesicles (30 pulses, output level 3, 50% duty cycle), indicating at least 50% of the vesicle contents had been released, relative to treatment with 0.1% Triton X-100. These conditions were then applied to lipid vesicles incubated with protein.

**SDS-PAGE and Western Blot Analysis**—Tris-Tricine gels (15% acrylamide) were performed as described (40) except that the sample buffer contained phenol red dye. For Tris glycine gels, samples were boiled for 5 min in Laemmli buffer, resolved on 15% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Immunoblotting was then performed as described (26). Membranes were processed with the Renaissance detection kit (PerkinElmer Life Science Products), and blots were either exposed to film (Kodak) or the image was directly acquired on a Kodak Image Station to allow quantitation of the signal.

**Immunofluorescence**—For IcmQ localization studies, GD132 (LP02/pGD32, PcmQ) was grown to an OD600 of 2.5 in AYET broth before adding IPTG at 0.1 mM for about 2 h until the culture reached post-exponential phase. Bacteria were then either fixed in periodate-lysine-paraformaldehyde (PLP) (41) containing 5% sucrose for 30 min or used to infect macrophages. Bacteria were deposited on bone marrow-derived macrophages at a multiplicity of infection of 20 by centrifugation at 150 × g for 5 min at room temperature and then incubated at 37 °C for various times. Cells were fixed for 30 min at 37 °C in PLP containing 5% sucrose. After removal of the fixative by washing with phosphate-buffered saline, infected cells were permeabilized with 20 °C methanol for 10 s. To analyze broth grown bacteria in the absence of detergent, IcmQ was added to the vesicles in the absence of OG prior to loading onto a sucrose gradient and collecting fractions. Quantitative Western blot analysis revealed that under these conditions, 60% of total IcmQ associated with *E. coli* PL vesicles and 100% of IcmQ was associated with liver PL vesicles (Fig. 1B, IcmQ). In contrast, little or no GST was found associated with the preformed vesicles (Fig. 1B, GST). Therefore, soluble IcmQ appears to associate with PL vesicles in the absence of detergent.

![Fig. 1. IcmQ associates with lipid vesicles.](https://example.com/fig1.png)

**RESULTS**

**IcmQ Associates with Lipid Vesicles**—Several substrates found in type III and IV secretion systems interact with lipid membranes when released from their chaperone (43). To address whether IcmQ exhibits such properties we tested whether purified recombinant His-tagged IcmQ ("Materials and Methods") could associate with and/or insert into phospholipid (PL) bilayers by incubating purified IcmQ with PL extracts in the presence of 5% n-octylglucoside (OG; "Materials and Methods"). Glutathione S-transferase (GST) was also added to the mixture as a negative control. The detergent was removed by dialysis to allow the formation of PL vesicles and the preparation was analyzed on a three-step sucrose gradient to determine the amount of PL-associated IcmQ. About 30% of the total IcmQ added in the reaction floated with *E. coli* PL to the top step of the gradient (Fig. 1A, *E. coli*). In the same sample, less than 10% of GST copurified with the PL, presumably due to the trapping of the protein within the internal volume of the vesicles. When liver PL extracts were used to make the vesicles, almost 90% of total IcmQ was found to be PL-associated (Fig. 1A, *Liver*). In the absence of PL, IcmQ was found entirely in the bottom fraction of the gradient (data not shown). IcmQ is thus able to associate with lipids and the efficiency of association depended on the composition of the PL vesicles.

We next tested whether soluble IcmQ could associate with preformed lipid vesicles (Fig. 1B). PL vesicles were first formed by incubation with OG followed by dialysis to remove the detergent. IcmQ was added to the vesicles in the absence of OG prior to loading onto a sucrose gradient and collecting fractions. Quantitative Western blot analysis revealed that under these conditions, 60% of total IcmQ associated with *E. coli* PL vesicles and 100% of IcmQ was associated with liver PL vesicles (Fig. 1B, IcmQ). In contrast, little or no GST was found associated with the preformed vesicles (Fig. 1B, GST). Therefore, soluble IcmQ appears to associate with PL vesicles in the absence of detergent.

**Phospholipid Association Is Due to Insertion**—The copurification of IcmQ with PC vesicles could be the result of peripheral association or integration into the bilayer. To determine the nature of the association of IcmQ with lipids, membrane-associated IcmQ was exposed to buffers containing 1 M NaCl, or 1% Triton X-100 and subjected to sucrose gradient fractionation (Fig. 2A). Western blot analysis allowed visualization of a major band migrating at 22 kDa corresponding to IcmQ and a minor band migrating at 50 kDa that could correspond to an SDS-resistant dimer of IcmQ. High salt did not affect the association of IcmQ with lipid vesicles indicating that IcmQ is not peripherally associated with the lipid bilayer. In contrast, IcmQ could be released by addition of Triton X-100, showing that flotation was dependent on the integrity of lipid vesicles. To further demonstrate that IcmQ was not trapped within the
IcmQ Has Pore-forming Activity—IcmQ has been reported to fractionate primarily as a soluble protein in wild-type L. pneumophila grown in AYE broth (25). As IcmQ is found associated with IcmR in a soluble fraction we hypothesized that IcmR prevents IcmQ from inserting into membranes. To test this idea, the subcellular localization of IcmQ was determined in a ΔicmR strain of L. pneumophila.

Spheroplasts from bacteria grown in AYE broth were lysed by sonication and membrane-associated proteins were separated from soluble proteins by centrifugation onto a sucrose cushion (“Materials and Methods”). The membranes were then washed with high salt and extracted with Triton X-100. In wild-type bacteria, IcmQ was localized in the soluble fraction (Fig. 3A, LP02, fraction C). In contrast, in a ΔicmR strain, about an equal amount of IcmQ was found in the soluble and the high salt resistant, Triton X-100 extractable fractions, indicating a significant fraction of IcmQ is associated with the membrane (Fig. 3A, ΔicmR, compare fractions C and E). This subcellular localization was specific for the ΔicmR strain, as it was not observed in a dotA− strain (Fig. 3A, LP03). As controls, the fractionation properties of isocitrate dehydrogenase (ICDH) and DotF proteins were analyzed. ICDH was found in the soluble fraction in all strains tested (Fig. 3A, ICDH), whereas DotF was in the Triton X-100 extractable membrane fraction in all strains (Fig. 3A, DotF).

The ability of IcmR to prevent insertion of IcmQ in liposomes was then tested. Copurified IcmR-IcmQ complex was exposed to preformed egg phosphatidylcholine (PC) vesicles and the ability of IcmQ to associate with PC on a sucrose gradient was determined. Floation of IcmQ was reduced from 25 to 5% of the total IcmQ when IcmR was present (Fig. 3B). IcmR is thus able to prevent IcmQ insertion into lipid vesicles presumably by binding to IcmQ.

Overexpression of IcmQ Allows Immunofluorescence Detection of the Protein on the Surface of L. pneumophila After Contact with Macrophages—The interference of IcmQ association with membranes by the presence of IcmR suggests that at some time during bacterial interaction with host cells IcmQ is released from IcmR to interact with a target membrane that remains to be identified. In a previous study we were not able to detect IcmQ by immunofluorescence on L. pneumophila-infected bone marrow-derived macrophages (26). To enhance the signal, IcmQ was overexpressed in L. pneumophila with an IPTG-inducible promoter and this strain was put into contact with macrophages by a short centrifugation. After 10 min of incubation at 37 °C, samples were immunolabeled using antibodies raised against IcmQ (Fig. 4A, phase contrast, Fig. 4, C and D) and against L. pneumophila (Fig. 4B; phase contrast, Fig. 4, C and D).

IcmQ could be detected on the surface of the bacteria (Fig. 4D, merged images). Cytoplasmic proteins such as IcmR or ICDH could not be detected using these probing conditions (data not shown). In addition, bacteria overexpressing IcmQ grown in AYE broth did not show any staining on their surface (Fig. 4, E and F). Immediately after centrifugation-induced contact with the macrophages, about 40% of the bacteria associated with the macrophages stained positively for IcmQ (Fig. 4G). After incubation at 37 °C, there was a slow decay in the number of positively staining bacteria to 20% at 30 min. To test whether the IcmQ staining on the surface of L. pneumophila depended on other Dot/Icm proteins, IcmQ was overexpressed in different dot/icm mutants. None of the mutations in the dot/icm genes (dotA, dotG, dotB, dotI, dotH icmR, icmT, icmXYYZ) was found to affect the ability of IcmQ to localize on the surface of the bacteria shortly after infection. With the permeabilization conditions used, detection of IcmQ on the
Fig. 3. IcmR prevents insertion of IcmQ into lipid membranes. A, subcellular localization of IcmQ, IcmR, DotF, and ICcDH, in wild-type strain (LP02; WT), ΔicmR strain (GD59), and dotA− strain (LP03, dotA−). L. pneumophila stains were grown in AYE broth and the proportion of the proteins in different subcellular fraction was determined by Western blot analysis. A, secreted proteins; B, total cellular proteins; C, soluble cellular proteins; D, membrane proteins extracted with 0.5 M NaCl; E, membrane proteins extracted with 2% Triton X-100; F, Triton X-100 insoluble membrane proteins. Levels of IcmQ present in each fraction were quantitated, and the results are presented as a fraction of the total cellular proteins (fraction B). B, IcmQ or the IcmR-IcmQ complex were incubated with preformed lipid vesicles composed of egg PC. Samples in 45% w/v sucrose were placed at the tube bottom (bottom fraction, B), a 30% sucrose solution was layered on the samples (middle fraction, M), and finally 1 ml of buffer without sucrose was layered on top (top fraction, T). The top fraction included the interface with the 30% sucrose layer. FL protein that remained lipid-associated and floated on the sucrose gradient. Results of the Western blot are presented on the bottom and its quantitation on the top.

surface of Legionella suggested that upon contact with macrophages at least a fraction of IcmQ is exported to the outer membrane of the bacteria in a Dot/Icm-independent manner. Most of this staining appeared directly associated with the bacterial surface rather than translocated across the phagosomal membrane.

Membrane-inserted IcmQ Allows Efflux of Molecules of Discrete Size—The capacity of IcmQ to exist in a soluble form associated with a chaperone and to insert into lipid bilayers in the absence of its chaperone is reminiscent of proteins such as IpaB or VirE2 that have pore-forming activity (30, 44). Therefore, we used the fluorescent dye calcein to examine whether IcmQ was able to form pores in a target membrane (39). A concentrated, self-quenched solution of this dye (80 mM) was encapsulated into liposomes prepared in the absence of detergent, and subsequently separated from the free dye by size exclusion chromatography (“Materials and Methods”). If IcmQ is able to form pores as it inserts into these vesicles, the dye should be released, resulting in fluorescence due to dilution and relief of self-quenching. Complete lysis of the liposomes with 0.1% Triton X-100 was used to determine the maximum amount of fluorescence after dilution.

Addition of IcmQ to egg PC vesicles containing calcein led to an immediate increase in fluorescence in a protein concentration-dependent manner (Fig. 5A). Protein concentrations as low as 25 nM (0.5 μg/ml) induced significant calcein release (5% of that observed with Triton X-100). At 400 nM, IcmQ induced 20% of the encapsulated store of calcein to be released. To assess the specificity of the effect, several negative controls were introduced (Fig. 5B). Trypsin-treated full-length IcmQ was not able to induce the release of calcein (Fig. 5D). Copurified IcmR-IcmQ complex at a concentration of 100 μM also was unable to induce calcein efflux. Furthermore, 20-fold molar excess of IcmR added to IcmQ was able to completely inhibit calcein efflux. Therefore, full-length IcmQ could induce calcein release in an IcmR-inhibitable fashion (Fig. 5B).

The release of calcein induced by IcmQ could be caused either by the formation of a pore or by destabilizing the membrane, perhaps by exerting phospholipase activity. To discriminate between these two possibilities, release of a molecule larger than calcein (600 Da, 13 Å diameter) (45) was analyzed. If IcmQ affects the general integrity of the vesicles, a larger dye should still be released by IcmQ. Tetramethylrhodamine-labeled Dextran 3000 (TMR Dx-3000, 26 Å in diameter) (46) was therefore tested to determine whether there was a limit to the size of molecules released by IcmQ insertion into egg PC vesicles (Fig. 5C). At 100 nM of IcmQ, no evidence of release of TMR Dx-3000 could be observed, although calcein release occurred efficiently under these conditions. TMR Dx-3000 release, however, could be induced by streptolysin O (SLO) when 50% cholesterol was introduced into the composition of the lipid vesicles, indicating that IcmQ-induced pores are limited in size and smaller than those induced by SLO.

Lipid Association Is Not Sufficient for Pore Formation—In an attempt to explore whether IcmQ exhibits specificity for lipid head groups, the pore-forming properties of IcmQ were tested on lipid extracts from different sources and synthetic phospholipids. Vesicles made out of egg PC or liver lipids exhibited comparable IcmQ-induced calcein efflux, although liver lipids were somewhat less efficient (Fig. 5D). At 100 nM of IcmQ, no evidence of release of TMR Dx-3000 could be observed, although calcein release occurred efficiently under these conditions. TMR Dx-3000 release, however, could be induced by streptolysin O (SLO) when 50% cholesterol was introduced into the composition of the lipid vesicles, indicating that IcmQ-induced pores are limited in size and smaller than those induced by SLO.

Lowered efficiency of IcmQ-induced calcein efflux observed with the E. coli lipid extract was not due to PL head groups present in the extract. IcmQ was able to induce calcein efflux from synthetic phospholipids, regardless of the headgroup composition (data not shown). In addition, a mix of 67% PE, 23% PG, and 10% CA, which mimics E. coli PL content, allowed extremely high IcmQ-induced calcein release (Fig. 5D). Therefore, membrane association is not sufficient to cause pore formation and the lipid requirements for the two events appear to be separable.
beled bacteria after 5 min of centrifugation onto macrophages, followed
by Anti antibody (mouse monoclonal) and with anti-Legionella E polyclonal antibody (staining.

The fatty acyl chain distribution of this sample.

the presence of an unidentified component of the mixture or to calcein release observed with the E. coli observed with Triton X-100. The low efficiency of IcmQ-induced Red shows anti-IcmQ B, C, and D. At 1 μg/ml of trypsin, full-length proteins were chased into four stable protein fragments (Fig. 6C, 1 μg/ml trypsin). The two larger fragments corresponded to amino acids 72–191 and 72–189 of IcmQ (Fig. 6, C and D) as seen with IcmQ alone (Fig. 6B), whereas the two bands migrating at about 9 and 8 kDa were specific to the IcmR-IcmQ complex. The 9-kDa band corresponded to amino acids 23–95 of IcmR and the 8-kDa band to amino acids 1–57 of IcmQ (Fig. 6 C and D). The presence of IcmR in the reaction thus allowed protection of the first 57 amino acids of IcmQ. Conversely, IcmQ protected a central portion of IcmR (amino acids 23–95). It is worth noting that a fragment of IcmR corresponding to amino acids 1–95 is present at the lower trypsin concentrations (0.1–0.2 μg/ml), indicating that a larger portion of IcmR may be in contact with IcmQ than the region of the protein protected against high protease concentrations.

The N-terminal Portion of IcmQ Associates with Vesicles and Promotes Calcein Efflux—As the N-terminal of IcmQ was protected by IcmR, and IcmR prevents IcmQ insertion, the ability of the N-terminal region of IcmQ to associate with preformed bilayers and promote calcein efflux was tested. A His-tagged construction corresponding to residues 1–72 of IcmQ (IQN, “Materials and Methods”) was found to be surprisingly soluble and stable. IQN was purified, incubated with preformed PL vesicles, and the mixture was subjected to sucrose gradient centrifugation to measure membrane association. At concentrations of protein that were 2-fold higher on a molar basis than was tested for full-length IcmQ (Fig. 1), no association of IQN with the vesicles could be detected (Fig. 7A, 2 μM). On the other hand, if the protein concentration were 25-fold higher than used for the full-length protein, association of IQN with the bilayer could be detected (Fig. 7A, 25 μM). At these higher protein concentrations, the lipid-protein complexes were clearly denser than in the previous experiments (compare with Fig. 3B). The protein was found in the fraction of the gradient corresponding to 30% sucrose, called the “middle” density in these experiments (Fig. 7A, M fraction), presumably because stable association of IQN with the bilayer required a high protein:lipid ratio. Perhaps association of IQN with lipid bilayers is unstable in the absence of the C-terminal, resulting in less efficient association of the N-terminal derivative with PL vesicles than observed for full-length protein.

The IQN derivative encompassing residues 1–72 was next analyzed in the calcein release assay. IQN was able to promote calcein release at levels similar to that observed for intact IcmQ using identical molar amounts of protein (Fig. 7B). The activity of IQN was blocked by either the presence of IcmR or trypsin (Fig. 7B), as had been observed for the full-length protein. Therefore, although stable association of IQN with membranes may require larger amounts of protein than observed with IcmQ (Fig. 7B), IQN retained the ability to disrupt the PL vesicles based on calcein release. The ability to detect calcein efflux at concentrations that were lower than used to get detectable quantities of PL insertion probably reflects the fact that a few monomers of inserted IQN in a single vesicle may be sufficient to allow large scale calcein efflux from that vesicle.

observed with Triton X-100. The low efficiency of IcmQ-induced calcein release observed with the E. coli extract could be due to the presence of an unidentified component of the mixture or to the fatty acyl chain distribution of this sample.

Limited Protease Digestion Indicates That IcmR Binds to the N-terminal Portion of IcmQ (amino acids 1–57)—The ability of IcmR to block association of IcmQ with lipid vesicles prompted us to speculate that interaction of IcmR with IcmQ masks a region of IcmQ involved in membrane insertion. To identify the region of IcmQ involved in binding IcmR and possibly in membrane insertion, the IcmR-IcmQ complex was exposed to trypsin and the region of IcmQ that was protected from trypsin digestion by IcmR was identified.

In the presence of trypsin at concentrations as low as 1

\[ \text{Percentage of IcmQ-stained bacteria} \]

\[ \text{Time (min)} \]

\[ 0, 5, 10, 30 \]

Fig. 4. Exposure of IcmQ on the surface of L. pneumophila after contact with macrophages. Bacteria were incubated with bone marrow-derived macrophages at 37 °C for 10 min after a 5-min centrifugation (A–D) or for the indicated times (G). After fixation, samples were labeled with a rabbit anti-IcmQ polyclonal antibody followed by an Alexa 488-labeled anti-rabbit IgG (A) and anti-Legionella mouse monoclonal antibody followed by Rhodamine-labeled anti-mouse IgG (B). C, phase contrast image of the same field. D, merged image of A, B, and C. Red shows anti-L. pneumophila staining and green shows anti-IcmQ staining. L. pneumophila grown in broth probed with the anti-IcmQ polyclonal antibody (E) and with anti-Legionella mouse monoclonal antibody (F). Quantitative assessment of the proportion of IcmQ-labeled bacteria after 5 min of centrifugation onto macrophages, followed by a 37 °C incubation for times indicated (G).
To test whether the protected N-terminal portion of IcmQ was required for promoting membrane insertion, this region was deleted and the truncated protein was purified (amino acids 68–191; designated IQC; “Materials and Methods”). Unlike the results from IQN, when IQC was incubated with preformed PL vesicles, this protein was unable to insert (Fig. 7C, post-dialysis), indicating the N terminus was necessary for this activity. Consistent with this observation, IQC was unable to promote calcein efflux when incubated with preformed vesicles (Fig. 5B). Surprisingly, however, IQC could be reconstituted into lipid vesicles by incubating the protein with lipids in the presence of OG followed by removal of the detergent by dialysis (Fig. 7C, pre-dialysis). In the same conditions, little of the GST floated on top of the gradient (data not shown). Furthermore, when purified vesicles having incorporated IQC were disrupted by sonication (“Materials and Methods”), only a small portion of the vesicle-associated protein was released (Fig. 7D), indicating that the association of IQC with PL was not due to trapping of the protein within the lumen of the vesicles. The association of IQC with the PL vesicles did not appear to be due to interaction of a mutant derivative that is poorly folded, exposing hydrophobic residues not normally revealed by this domain. IQC is extremely hydrophilic, as the protein is soluble at 80 mg/ml in neutral buffers.2 Secondly, circular dichroism revealed that the largely α-helical structure of the protein was maintained in the IQC derivative (Supplementary Materials, Fig. S1).

Taken together these data indicate that the N-terminal portion of IcmQ (the region involved in IcmR binding) is necessary for the insertion of the protein into preformed PL vesicles, can

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2 S. R. Chaudhury and C. Akey, personal communication.
insert at high protein concentrations, and is able to form pores. The C-terminal portion of IcmQ, on the other hand, cannot insert into preformed PL vesicles, but appears to stabilize protein insertion in lipid membranes. As IQC can be reconstituted into PL vesicles by the detergent dialysis strategy, this portion of the protein could have a close interaction with the bilayer in the context of the full-length protein.

**DISCUSSION**

In a previous study we showed that IcmR has biochemical properties similar to secretion chaperones found in bacterial Type III and IV secretion systems (26, 47, 48). This work prompted us to further study the behavior of IcmQ, the substrate of IcmR. We found that despite a generally hydrophilic primary sequence, IcmQ was able to insert into lipid membranes. Furthermore, membrane insertion of IcmQ allowed efflux of calcein, but not Dextran 3000, implying that there was a pore between 13 and 26 Å in diameter (45, 46).

A summary of the properties of each IcmQ derivative analyzed in this work (Table I) supports the proposition that the N-terminal of the protein is involved in insertion, with the C-terminal stabilizing this event in some fashion. That the N-terminal is involved in PL interaction is consistent with several observations. First, interaction with a PL bilayer led to protection of the N-terminal portion of IcmQ from protease digestion, suggesting that this portion of the protein is buried in the bilayer. Second, a His-tagged recombinant protein encoding the first 72 amino acids of IcmQ was able to insert into lipid vesicles and cause calcein efflux, indicating that this portion of the protein is necessary and sufficient for these processes. Third, deletion of the N-terminal portion of the protein led to a loss of the ability to insert into preformed bilayers. The first 72 amino acids of IcmQ are thus able to exist in solution at reasonably high concentration, insert into a lipid bilayer and cause calcein efflux presumably through an oligomerization step.

The C-terminal region of IcmQ is necessary for efficient association with lipid bilayers. Furthermore, a deletion derivative having only this end (IQC) could be reconstituted into lipid vesicles using a detergent dialysis approach even though this region of the protein is water soluble at remarkably high concentrations (80 mg/ml). Perhaps membrane interaction of the C-terminal is the function that stabilizes the insertion of the N-terminal of IcmQ. By this model, the insertion of IcmQ into membranes behaves as if it is a two step process, with the N-terminal portion initiating insertion, followed by the action of the C-terminus, which stabilizes this event, probably by close interaction with the lipid bilayer.
Fig. 7. Membrane association and vesicle disruption by fragments of IcmQ. A, IQN requires high protein concentrations to associate with PL vesicles. Egg PC vesicles, formed by extrusion, were incubated with IQN at concentrations of either 2 or 25 mM and subjected to sucrose step gradient centrifugation (see “Materials and Methods”). Protein from the top (T; 0% sucrose), middle (M; 30% sucrose), and bottom fractions (B; 45% sucrose) was analyzed by Western blotting with anti-polyhistidine (see “Materials and Methods”). FL, protein that floats on the sucrose gradient. B, IQN can promote calcein efflux. 100 mM IQN (IcmQ N-ter) was incubated with PC vesicles loaded with calcein, as in Fig. 5, and fluorescence as a function of time was recorded spectrophotometrically (see “Materials and Methods”). Additional to IQN were performed as in Fig. 5B. C, IQC can be incorporated into vesicles by detergent dialysis. The C-terminal region of IcmQ (IQC, amino acids 68–191) was incubated with liver extract lipids in the presence of octyl-glucoside, and the detergent was removed by dialysis (Post-dialysis). IQC was also incubated with preformed vesicles made from liver extract (Pre-dialysis). Samples were loaded on a 3-step sucrose gradient, and the contents of the 3 fractions were analyzed by Western blot. D, IQC is not released after sonic disruption of vesicles. IQC was incorporated into PC vesicles by detergent dialysis (see “Materials and Methods”), isolated on sucrose gradients, subjected to sonication, and analyzed as in Fig. 2B. To separate soluble released IcmQ from IcmQ that remained incorporated in the PC vesicles, the sonicated sample was subjected to sucrose gradient sedimentation (see “Materials and Methods”). FL, protein that remained lipid-associated and floated on the sucrose gradient. T, the top fraction of gradient, containing no sucrose. M, middle fraction of the gradient, containing 30% sucrose. B, bottom portion of gradient, containing protein released from PL that did not float.

### TABLE I

| Protein           | Incorporation by detergent dialysis | Insertion at low protein concentration | Insertion at high protein concentration | Calcein release
|-------------------|--------------------------------------|----------------------------------------|-----------------------------------------|------------------|
| IcmQ              | Yes                                  | Yes                                    | Yes                                     | Yes              |
| IcmQN-(a1–72)     | N.A.\(^b\)                           | No                                     | Yes                                     | Yes              |
| IcmQC-(72–191)    | Yes                                  | No                                     | No                                      | No               |

\(^a\) Calcein release performed on performed PL vesicles.

\(^b\) Not attempted.

Interestingly, our results indicate that membrane insertion and pore formation are separable, at least to some degree. For instance, with some types of lipids, such as the E. coli lipid extract, IcmQ is unable to induce calcein efflux but still associates efficiently with the PL. This result could reflect the fact that once inserted into the membrane a second step occurs, perhaps protein oligomerization or a conformational change. Such a two-step process, was demonstrated for perfringolysin O (49). In this case, a membrane recognition step precedes formation of the pore. One region of perfringolysin integrates into the membrane and is responsible for membrane recognition, but does not penetrate deeply into the bilayer and is not part of the actual pore. After membrane recognition, a new region of pore occurs via insertion of a second domain into the bilayer. Insertion without pore formation in E. coli lipid extracts could also explain why IcmQ overexpression in the absence of IcmR was not toxic to the bacteria.

Consistent with its role as a regulator of IcmQ activity, IcmR prevented the insertion of IcmQ into membranes both in growing cells and in artificial PL bilayers. Furthermore, our results provide a molecular mechanism for this phenomenon. The binding of IcmR to IcmQ led to the protection of trypsin-sensitive sites in the N-terminal portion of IcmQ, indicating that IcmR binds to the same region required for membrane insertion into preformed vesicles (residues 1–57). IcmR thus prevents IcmQ insertion into lipid membranes by masking the region of IcmQ involved in this process.

As an initial step toward examining the in vivo function of IcmQ, we studied its localization during infection of bone marrow-derived macrophages. IcmQ could be detected by immunofluorescence proximal to L. pneumophila shortly after infection of macrophages, suggesting that at least part of IcmQ was localized on the bacterial surface after contact with host cells. IcmQ could not be detected on the bacterial cell surface during...
growth in broth, indicating that a rapid change occurs after contact with the macrophages, leading to exposure of the protein. It should be emphasized that this was observed upon overexpression of IcmQ, and that localization of IcmQ when expressed in physiological amounts remains to be determined. In addition, the mechanism of IcmQ export to the outer membrane is unclear, as it was independent of other Dot/Icm proteins. Nevertheless these results favor a role for IcmQ either in constructing the outer portion of the translocation channel or in being part of this portion of the channel. Some known properties of IcmQ affect our understanding of this particular model. First, ΔicmQ strains are salt resistant, a property thought to be associated with a defective dot/icm translocation apparatus (50). Furthermore, mutations in the icmQ gene allow survival of L. pneumophila strains lacking the DotL protein. Mutants having lesions in dotL are not viable, but mutations in genes thought to encode components of the core type IV secretion machinery relieve the dotL lethal phenotype. Therefore, if IcmQ is part of the bacterial-associated portion of the translocation machinery, our localization data are consistent with its transit to the outer part of the complex at the time of bacterial contact with host cells. Since the pore activity of IcmQ and its cellular localization are subject to regulation, IcmR and IcmQ could be involved in the regulation of the opening and closing of the channel when in contact with host cells.

Finally, it should be pointed out that roles for IcmQ in the bacterial membrane as well as on the host cell side of the translocation channel are not mutually exclusive. In fact, the bacterial surface could represent an intermediate step prior to bacterial contact with host cells. Future research will focus on further defining the role of the pore-forming activity of IcmQ during infection and how it relates to the other Dot/Icm components.

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