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Protein Binding between PcrG-PcrV and PcrH-PopB/PopD Encoded by the *pcrGVH-popBD* Operon of the *Pseudomonas aeruginosa* Type III Secretion System

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Of the proteins encoded by the *pcrGVH-popBD* operon of the *Pseudomonas aeruginosa* type III secretion system, PcrG bound to PcrV and PcrH bound to PopB/PopD. In addition, *Yersinia* LcrG bound to PcrV, and *Yersinia* LcrH bound to PopD. The results imply a highly functional conservation of type III secretion between *P. aeruginosa* and *Yersinia* species.

Pseudomonas aeruginosa possesses a type III secretion system that is highly homologous to that of *Yersinia* species (39, 40). In type III secretion, bacteria inject their effector proteins directly into adjacent host cells (15, 20). In *P. aeruginosa* infections, exoenzyme S (ExoS) and its coregulated type III secreted toxins (ExoT, ExoU, and ExoY) are responsible for causing acute lung injury and sepsis (8, 9, 12, 21, 33, 38). In type III secretion of *Yersinia* species, translocation, a process of toxin transfer directly into the eukaryotic cytosol across the eukaryotic plasma membrane, involves LcrG, LcrV, LcrH, YopB, and YopD. These proteins are encoded by the *lcrGVH-yopBD* operon in the Yop regulon of *Yersinia* pathogenic plasmids (5, 6). In *P. aeruginosa*, a chromosomal operon, *pcrGVH-popBD*, encodes five proteins, PcrG, PcrV, PcrH, PopB, and PopD, that are homologous to *Yersinia* LcrG, LcrV, LcrH, YopB, and YopD, respectively. For *Yersinia pestis*, protective antigenic characteristics of LcrV were reported previously as a V antigen (1, 3, 4, 18, 22, 23, 25, 35). LcrV likely forms the translocation pore in eukaryotic cell membranes in conjunction with YopB and YopD (16, 17, 19, 27, 29, 30, 31, 34). LcrG, which forms a stable complex with LcrV, acts as a negative regulator that blocks secretion of Yops (7, 24, 28, 37). LcrH was reported previously as a cognate chaperone of YopD (26, 32) and was found to be necessary for YopD stabilization before secretion (10). Recently, an active role of LcrH in Yop regulation was also reported (2, 10, 11).

The importance of V antigen in cytotoxicity has been well established. Isogenic mutants of *P. aeruginosa* lacking the genes for *pcrV* or *popD* were unable to intoxicate eukaryotic cells (36). Active and passive immunization against PcrV in animal models of *P. aeruginosa*-induced lung injury greatly increased survival (36). Functional conservation from PopB and PopD of *P. aeruginosa* to YopB and YopD of *Yersinia pseudotuberculosis* was previously reported (14). However, there have been fewer studies analyzing the proteins encoded by the *pcrGVH-popBD* operon of the *P. aeruginosa* type III secretion system. In this study, we examined the interactions among the proteins encoded by the *pcrGVH-popBD* operon to investigate the functional homology between the type III secretion systems of *P. aeruginosa* and *Yersinia*.

In *Escherichia coli*, we induced the expression of glutathione *S*-transferase (GST) fusion PcrG, PcrV, PcrH, PopB, and PopD proteins whose genes were subcloned in pGEX plasmids under the *lac* promoter. We also induced the expression of the thioredoxin (Thio) fusion PcrG, PcrV, PcrH, PopB, and PopD proteins from genes subcloned into the pThio plasmid under the *lac* promoter. Induction of PopB fusion proteins appeared to decrease *E. coli* density after isopropyl-β-D-thiogalactopyranoside (IPTG) induction, suggesting bactericidal activity. We performed affinity immunoblotting to examine the interaction between PcrV and other proteins encoded by the *pcrGVH-popBD* operon. We applied *E. coli* lysate containing Thio-PcrV to a membrane blotted with the lysates of *E. coli* expressing a series of GST tag-fused proteins. From this experiment, only the GST-PcrG band was visualized (Fig. 1A). Next, we applied GST-PcrG to a membrane blotted with the lysates of *E. coli* expressing Thio tag-fused proteins. From this experiment, only the Thio-PcrV band was intensely visualized (Fig. 1B). Next, we performed affinity immunoblotting with purified recombinant nontagged PcrV and applied it to membrane-bound Thio fusion proteins to determine whether PcrV-blocking antibodies could detect the PcrV-PcrG complex. Both rabbit polyclonal anti-PcrV antibody (data not shown) and murine anti-PcrV monoclonal antibody (MAb) 166 detected PcrV bound to Thio-PcrG (13) (Fig. 1C). All affinity immunoblotting resulted in the detection of a PcrV-PcrG interaction.

Because LcrH, a *Yersinia* homolog of PcrH, was reported as a chaperone protein for *Yersinia* YopD, we purified recombinant GST-PcrH from *E. coli* transformed with pGEX-*pcrH* and examined the interaction between PcrH and other proteins in the same format as that previously used to find the PcrV-PcrG interaction. Affinity immunoblotting was performed with recombinant purified GST-PcrH to a membrane blotted with the

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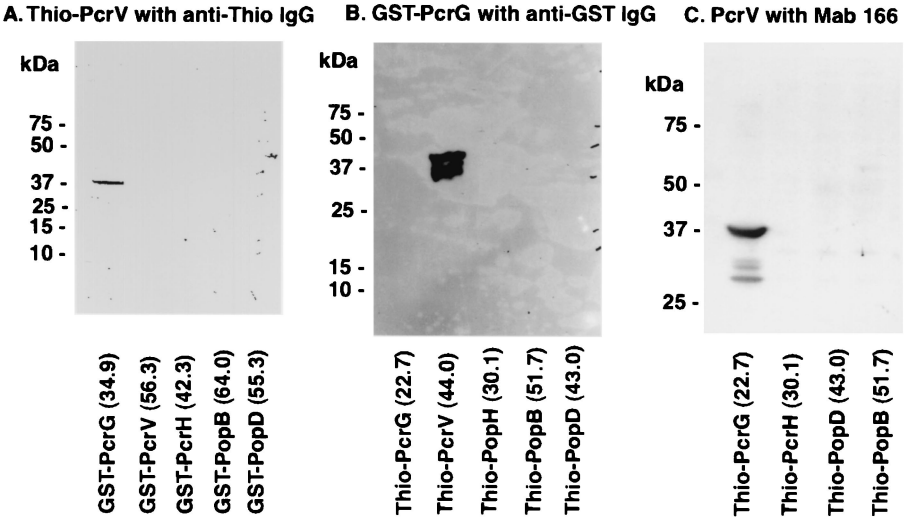

FIG. 1. Affinity immunoblot analysis. (A) Binding of Thio-PcrV to GST-PcrG. The protein samples from induced *E. coli* clones carrying pGEX plasmids were electrophoresed onto a sodium dodecyl sulfate–4 to 12% bis-Tris polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and incubated with *E. coli* lysate including expressed Thio-PcrV. The membrane was developed with anti-Thio immunoglobulin G (IgG) and secondary anti-mouse IgG conjugated with horseradish peroxidase and a chemiluminescent substrate. An intense isolated band represents binding of GST-PcrG to Thio-PcrV. (B) Binding of GST-PcrG to Thio-PcrV. The protein samples were from induced *E. coli* clones carrying pThio plasmids. The blotted membrane was incubated with recombinant GST-PcrG (10 μg/ml) and then developed with anti-GST IgG conjugated with horseradish peroxidase and a chemiluminescent substrate. An intense isolated band represents binding of GST-PcrG to Thio-PcrV. (C) Binding of PcrV to Thio-PcrG. The protein samples were from induced *E. coli* clones carrying pThio plasmids. The membrane was incubated with recombinant PcrV (10 μg/ml), then developed with murine anti-PcrV MAb 166, and developed with secondary anti-mouse antibodies conjugated with horseradish peroxidase and a chemiluminescent substrate. An intense isolated band represents binding of PcrV to Thio-PcrG.

lysates of *E. coli* expressing Thio tag-fused proteins. GST-PcrH bound to both Thio-PopB and Thio-PopD in this affinity immunoblot assay (Fig. 2). In order to verify protein interactions, a GST pull-down assay was performed on PA103 lysates with recombinant GST-PcrG and GST-PcrH. As a result, GST-PcrG coprecipitated with native PcrV, and GST-PcrH coprecipitated with PopD (data not shown).

We performed affinity immunoblotting to examine the cross-species interaction between *Yersinia* and *P. aeruginosa* type III proteins. From this experiment, we found that GST-LcrG binds to Thio-PcrV (Fig. 3A) and GST-LcrH binds to Thio-PopD (Fig. 3B). Therefore, the protein binding between LcrG and PcrV and between LcrH and PopD occurred in a cross-species manner between *Yersinia* and *P. aeruginosa*.

These findings imply high functional and structural homology among these proteins despite the fact that their amino acid sequence similarities range from 56 to 57%. Our results suggest that PcrG serves the role of a potential negative regulator of PcrV. The neutralizing epitope on PcrV appears to be different from the PcrG binding site, given that the blocking anti-PcrV MAb 166 clearly detected the PcrV-PcrG complex in our study. Since *P. aeruginosa* PcrH and PopD are homolog equivalents of *Yersinia* LcrH and YopD, respectively, our findings suggest that PcrH is a chaperone for PopD secretion. Although PcrH binds to PopB in the immunoblot that we made, a similar interaction between LcrH and PopB was not found. The experimental conditions that we tested may have been affected by the fact that the expression of recombinant PopB in *E. coli* was bactericidal. This phenomenon has been reported elsewhere for *Yersinia* YopB expression in *E. coli* without coexpression of LcrH (26). LcrV, YopB, and YopD

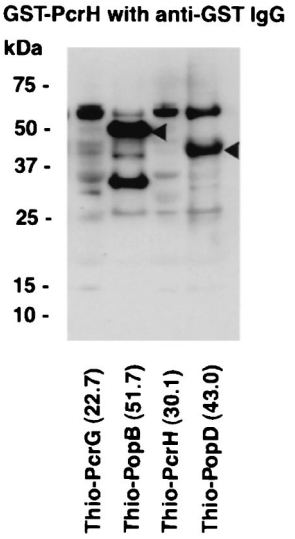

FIG. 2. Affinity immunoblot analysis demonstrates the binding of GST-PcrH to Thio-PopD and Thio-PopB. The protein samples from *E. coli* expressing Thio-tagged fusion proteins were loaded onto a sodium dodecyl sulfate–4 to 12% bis-Tris polyacrylamide gel electrophoresis gel for electrophoresis. Proteins were then blotted onto a nitrocellulose membrane for subsequent affinity immunoblot analysis. Affinity immunoblotting demonstrates the interaction between GST-PcrH and Thio-PopD. The blotted membrane was incubated in a solution with purified recombinant GST-PcrH. After the membrane was washed several times, immunostaining against the GST tag was performed with anti-GST antibody conjugated with horseradish peroxidase and chemiluminescent substrate. The intense bands demonstrating the interaction with GST-PcrH were recognized in Thio-tagged PopB and PopD lanes (arrowheads). IgG, immunoglobulin G.

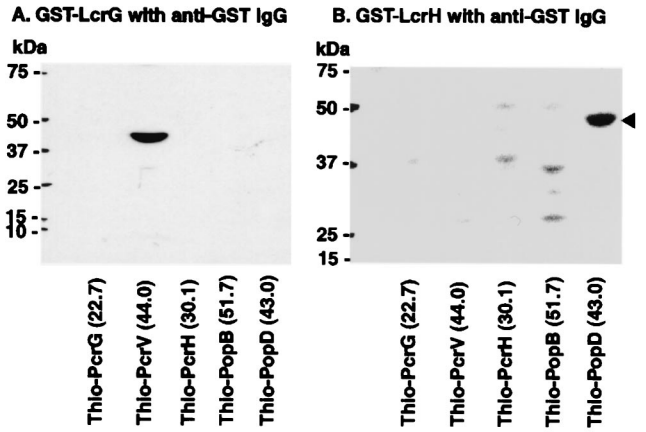

FIG. 3. Affinity immunoblot analysis demonstrates the binding of GST-LcrG to Thio-PcrV and of GST-LcrH to Thio-PopD. The protein samples from induced *E. coli* clones carrying the expression plasmids were loaded onto sodium dodecyl sulfate–4 to 12% bis-Tris polyacrylamide gels for electrophoresis. Protein was then electroblotted onto nitrocellulose membranes and incubated with each recombinant protein (8 to 10 μg/ml). The membranes were then developed with anti-GST antibodies conjugated with horseradish peroxidase and a chemiluminescent substrate. (A) An intense isolated band represents binding of GST-LcrG to Thio-PcrV. (B) An intense isolated band represents binding of GST-LcrH to Thio-PopD (arrowhead). IgG, immunoglobulin G.

are thought to form a pore complex involved in the translocation of type III secreted proteins across the eukaryotic plasma membrane, but interactions with LcrV among these proteins have not been experimentally elucidated. We tested PcrV binding to PopB and PopD, but no interaction was found.

In conclusion, PcrG binds to PcrV and PcrH binds to PopB and PopD. From interactions between Pcr and Lcr proteins, a highly functional conservation of type III system translocators was also confirmed between *P. aeruginosa* and *Yersinia*. Thus, investigations of the roles and mechanisms of PcrV secretion and anti-PcrV blockade and of LcrV secretion and anti-LcrV blockade may complement each other.

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