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Legionella pneumophila Mip, a Surface-Exposed Peptidylproline *cis-trans*-Isomerase, Promotes the Presence of Phospholipase C-Like Activity in Culture Supernatants

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The type II secretion system of *Legionella pneumophila* promotes pathogenesis. Among the *Legionella* type II-dependent exoenzymes is a *p*-nitrophenol phosphorylcholine (*p*-NPPC) hydrolase whose activity is only partially explained by the PlcA phospholipase C. In a screen to identify other factors that promote secreted hydrolase activity, we isolated a *mip* mutant. *L. pneumophila* Mip is a surface-exposed, FK506-binding protein that is needed for optimal infection and has peptidylproline *cis-trans*-isomerase (PPIase) activity. Since the molecular target of Mip was undefined, we investigated a possible relationship between Mip and the secreted *p*-NPPC hydrolase activity. In the *mip* mutant there was a 40 to 70% reduction in secreted activity that was successfully complemented by providing *mip* on a plasmid. A similar phenotype was observed when we examined four other independently derived *mip* mutants, and in all cases the defect was complemented by reintroduction of *mip*. Thus, *mip* promotes the presence of a *p*-NPPC hydrolase activity in culture supernatants. We also found that the C terminus of Mip is required for this effect. When supernatants were examined by anion-exchange chromatography, the *p*-NPPC hydrolase activity associated with Mip proved to be type II dependent but distinct from PlcA. This conclusion was supported by the phenotype of a newly constructed *mip plcA* double mutant. Thus, Mip promotes the elaboration of a new type II exoprotein. These data provide both the first evidence for a target for Mip and the first indication that a surface PPIase is involved in the secretion or activation of proteins beyond the outer membrane.

Legionella pneumophila, a gram-negative bacterium, is the agent of Legionnaires' disease, a potentially fatal pneumonia (27, 32). As an environmental organism, it naturally replicates in biofilms and within freshwater amoebae and ciliates (82). Infection occurs when aerosolized legionellae are inhaled. Upon reaching the human respiratory tract, the bacteria multiply in alveolar macrophages, causing host cell death and damage to lung tissue due at least in part to bacterial degradative enzymes (27, 32).

The identification of the prepilin peptidase PilD first suggested that a type II protein secretion system is present in *L. pneumophila* (51, 52). This suggestion was later confirmed by the discovery of genes encoding the components of the type II secretion apparatus (i.e., the *lsp* genes) (39, 69, 70). Analysis of *lsp* mutants revealed that the *L. pneumophila* system is required for secretion during growth in bacteriologic media at 37°C, for optimal extracellular growth at low temperatures (12 to 25°C), for typical colony morphology on standard media, for intracellular infection of both human macrophages and monocytes and freshwater protozoans, and for virulence in a murine model of pneumonia (39, 51, 61, 69, 70, 80). A process that is present in many, but not all, gram-negative bacteria (16, 59), type II protein secretion is a two-step process in which proteins are first translocated across the inner membrane by the Sec or Tat pathway and then, after what may be a very short time, are transported from the periplasm to the exterior through an outer membrane secretin (33, 74). The *L. pneumophila* system has received attention as it is the only system that has been implicated in intracellular infection (16, 75). A variety of secreted activities have been linked to Lsp; these include protease, tartrate-resistant and -sensitive acid phosphatase, mono-, di-, and triacylglycerol lipase, phospholipase A, lysophospholipase A, glycerophospholipid:cholesterol acyltransferase, RNase, and *p*-nitrophenol phosphorylcholine (*p*-NPPC) hydrolase activities (2–4, 8, 37–39, 51, 69, 70).

In many bacteria, *p*-NPPC hydrolysis is attributed to phospholipase C (PLC) enzymes, many of which promote virulence (46, 54, 83, 86). Early reports described *p*-NPPC hydrolase activities for various *Legionella* species (6, 7, 56, 57, 84). Because of the link between type II secretion and *p*-NPPC hydrolysis (3, 69), efforts have been made to understand the basis of this secreted activity. Thus, we identified an *L. pneumophila* gene (*plcA*) that encodes a protein which is highly homologous to a new type of PLC that was found in *Pseudomonas fluorescens* (4, 62). Mutation of *plcA* decreased secreted *p*-NPPC hydrolase activity, indicating that *L. pneumophila* secretes at least one PLC (4). Recent data suggest that PlcA is transported across the inner membrane by Tat prior to its export by Lsp (68). Since loss of PlcA reduces secreted *p*-NPPC activity by only 50 to 70% (4, 68), we suspect that *L. pneumophila* secretes another PLC(s). In a previous study, we mutagenized *L. pneumophila* with mini-Tn*10* and isolated mutants that had an altered appearance on egg yolk plates and reduced *p*-NPPC hydrolase activity (3). Yet the basis for this mutant phenotype remained unknown. Therefore, as a step toward identifying other factors involved in the production of *p*-NPPC hydrolase activity, these mutants were examined further. Here we report

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that Mip (macrophage infectivity potentiator), a surface protein of *L. pneumophila* long known for its role in infection, contributes to the presence of a novel *p*-NPPC hydrolase activity in culture supernatants.

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MATERIALS AND METHODS

Bacterial strains, growth media, and chemicals. *L. pneumophila* strain 130b (= ATCC BAA-74), also known as AA100, was used as our wild-type strain. Derivatives of 130b used in this study are listed in Table 1. The legionellae were routinely cultured at 37°C on buffered charcoal yeast extract (BCYE) agar or in buffered yeast extract (BYE) broth (26). Growth in broth was assessed by measuring the optical density of a culture at 660 nm using a DU250 spectrophotometer (Beckman, Fullerton, CA). *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA), used as a host for plasmids, was grown in LB media (73). Antibiotics were added to media at the following final concentrations: ampicillin, 100 μg/ml; gentamicin, 2.5 μg/ml for *L. pneumophila* and 5 μg/ml for *E. coli*; chloramphenicol, 3 μg/ml for *L. pneumophila* and 30 μg/ml for *E. coli*; and kanamycin, 25 μg/ml for *L. pneumophila* and 50 μg/ml for *E. coli*. Unless indicated otherwise, all chemicals, were obtained from Sigma (St. Louis, MO).

Analysis of enzyme activities. Cell lysates and filter-sterilized supernatants from late-log-phase broth cultures of *L. pneumophila*, as well as stationary-phase cultures of *E. coli*, were obtained as described previously (3). Samples were assayed for *p*-NPPC hydrolase activity by monitoring the release of *p*-nitrophenol (*p*-NP) from *p*-NPPC, for lipase activity by monitoring the ability to release *p*-NP from *p*-nitrophenyl palmitate and *p*-nitrophenyl caprylate, and for acid phosphatase activity by monitoring the release of *p*-NP from *p*-nitrophenyl phosphate in the presence or absence of sodium tartrate (2–4). The activities of secreted proteases were monitored using the azocasein assay (3). The effect of FK506 on secreted *p*-NPPC hydrolase was examined by addition of 1 nM, 1 μM, 10 μM and 50 μM FK506 to cultures of wild-type *L. pneumophila* at 0, 8, 9, and 10 h postinoculation, and then supernatants were collected at the late log phase and *p*-NPPC hydrolysis was analyzed. Addition of 10 μM FK506 directly to culture supernatants was also tested to determine its effect on *p*-NPPC hydrolase activity. To investigate a potential effect of membrane material on the secreted *p*-NPPC hydrolase activity, supernatants were ultracentrifuged for 1 h at 100,000 × *g* at 4°C (71) in a Beckman L8-80 M ultracentrifuge, and then the resulting membrane-free supernatant was assayed for *p*-NPPC hydrolase activity.

Immunodetection of Mip. To obtain cell lysate samples, *L. pneumophila* strains were grown to the late log phase in BYE broth supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) as indicated below. Cells were then pelleted from 1 ml of culture by centrifugation at 14,000 rpm in a Beckman Microfuge 18 for 5 min and lysed in Laemmli buffer (73). To obtain supernatant samples, filtered supernatants from late-log-phase cultures were concentrated ca. 100-fold by overnight precipitation with 2 volumes of isopropanol, and the pellet was solubilized in Laemmli buffer (73). Samples were boiled for 10 min, separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Trans-Blot SD apparatus (Bio-Rad, Hercules, CA). Western blotting was performed by using standard protocols (73) with a 1:500 dilution of anti-Mip polyclonal antiserum (17) and a 1:5,000 dilution of goat anti-rabbit peroxidase conjugate (Organon Teknika Corp., Westchester, PA). Immunodetection was done using a chemiluminescence kit (Amersham Biosciences, Piscataway, NJ), and images were obtained using a Chemi Imager 5500 (Alpha Innotech, San Leandro, CA). Supernatant proteins were also examined by silver staining (12) following SDS-PAGE.

DNA isolation, PCR, and DNA sequencing. DNA was obtained from *L. pneumophila* as described previously (28), and plasmids were routinely isolated from *E. coli* using a plasmid Mini Prep kit (Bio-Rad). All other DNA manipulations were performed using standard protocols (73). The oligonucleotide primers used for sequencing or PCR were synthesized at Integrated DNA Technology (Coralville, IA). A standard PCR was performed using HIFI polymerase (Invitrogen). An inverse PCR was performed as previously described (52). DNA samples used for sequencing were labeled with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), and sequencing was done at our biotech facility using an automated DNA sequencer (model 3100; Applied Biosystems). Sequences were analyzed with DNASTAR (DNASTAR, Inc., Madison, WI), and homology searches were done using BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

TABLE 1. *L. pneumophila* strains used in this study

Strain	Genotype	Reference
130b	Wild type; virulent, serogroup 1 strain	29
NU247	Mini-Tn*10 phoA* (Km^r) inserted into the *mip* gene of 130b	3
NU253	Mini-Tn*10 phoA* (Km^r) inserted into the *surA* gene of 130b	3
AA108	726-bp deletion in the *mip* gene of 130b	44
AA109	Intact *mip* reintroduced into the chromosome of AA108	44
NU201	Spontaneous streptomycin-resistant 130b	19
NU203	Km^r cassette inserted into the *mip* gene of NU201	20
NU268	Km^r cassette inserted into the *plcA* gene of 130b	4
NU275	Km^r cassette inserted into the *lspF* gene of 130b	70
NU317	Km^r cassette inserted into the *plcA* gene of AA108	This study

Complementation analysis. To generate a plasmid for complementing *mip* mutants, *mip*, along with its promoter, was amplified from 130b DNA with primers MIP_c-F (5′-GCGAGCTCGTTAAAATCTCTTGTTC) and MIP_c-R (5′-GCTCTAGATTAAGATGATTTTTTCACTG). The amplified fragment was ligated into pGEM-T Easy (Promega, Madison, WI). The resulting plasmid, pGEMmip_c, was digested with SacI and XbaI, and a 906-bp product was ligated to the vector pMMB2002 (70) that had been digested with SacI and XbaI, yielding p*mip*. To produce a plasmid containing a truncated *mip* (i.e., pmip_{157}), p*mip* was digested with KpnI and XbaI, treated with the Klenow fragment, and religated. To generate a plasmid for complementing a *surA* mutant, *surA* and its native promoter were amplified from 130b DNA using $SURA_c$-F (5′-GCGAATTCGATAAATGACACAATAAAAGC) and $SURA_c$-R (5′-GCGGATCCTCATGCTAAATCCTTATC). The PCR fragment was digested with EcoRI and BamHI, and a resulting 1,462-bp fragment was inserted into pMMB2002 that had been digested with EcoRI and BamHI, yielding p*surA*. Plasmids were introduced into *E. coli* and *L. pneumophila* by electroporation (20, 73).

Mutant construction. In order to construct a *mip plcA* double mutant, plasmid pVA16-3, containing a mutated, Km^r-tagged *plcA* gene cloned in pBOC20 (4), was electroporated into *mip* mutant AA108. Strains that had undergone allelic exchange were then obtained by plating bacteria on BCYE agar containing kanamycin and sucrose and were confirmed by PCR (58).

Intracellular infection of protozoans and macrophages. To examine the ability of *L. pneumophila* strains to infect protozoan and macrophage hosts, *Hartmannella vermiformis* amoebae and U937 cell macrophages were infected as previously described (19, 68).

Anion-exchange chromatography. As described previously (7), *p*-NPPC hydrolase activity in culture supernatants was fractionated by anion-exchange chromatography. Strains were grown in 50 ml of BYE broth to the late log phase and then centrifuged at 3,500 rpm in a Beckman GPR centrifuge for 30 min at 4°C. The supernatants were removed and sterilized by passage through 0.22-μm filters (Millipore, Bedford, MA). The filtered supernatants were then concentrated 100-fold using an Amicon model 202 ultrafiltration cell having a membrane with a 10-kDa cutoff (Amicon Corp., Lexington, MA). Concentrated supernatants were applied to a 10-cm A50 column (Amersham Pharmacia Biotech, Uppsala, Sweden) buffered with 50 mM HEPES (pH 7.5)–1 mM sodium azide. Chromatography was carried out at 4°C, and 3-ml fractions were collected by gravity flow in step gradients consisting of 100, 200, and 300 mM NaCl in equilibration buffer.

RESULTS

Identification of a *mip* mutant that revealed a loss in secreted *p*-NPPC hydrolase activity. NU247 is one of several previously isolated transposon mutants of strain 130b in which the levels of secreted *p*-NPPC hydrolase activity are reduced (3). Analysis of NU247 culture supernatants reproducibly revealed a 40 to 70% reduction in hydrolase activity, a reduction

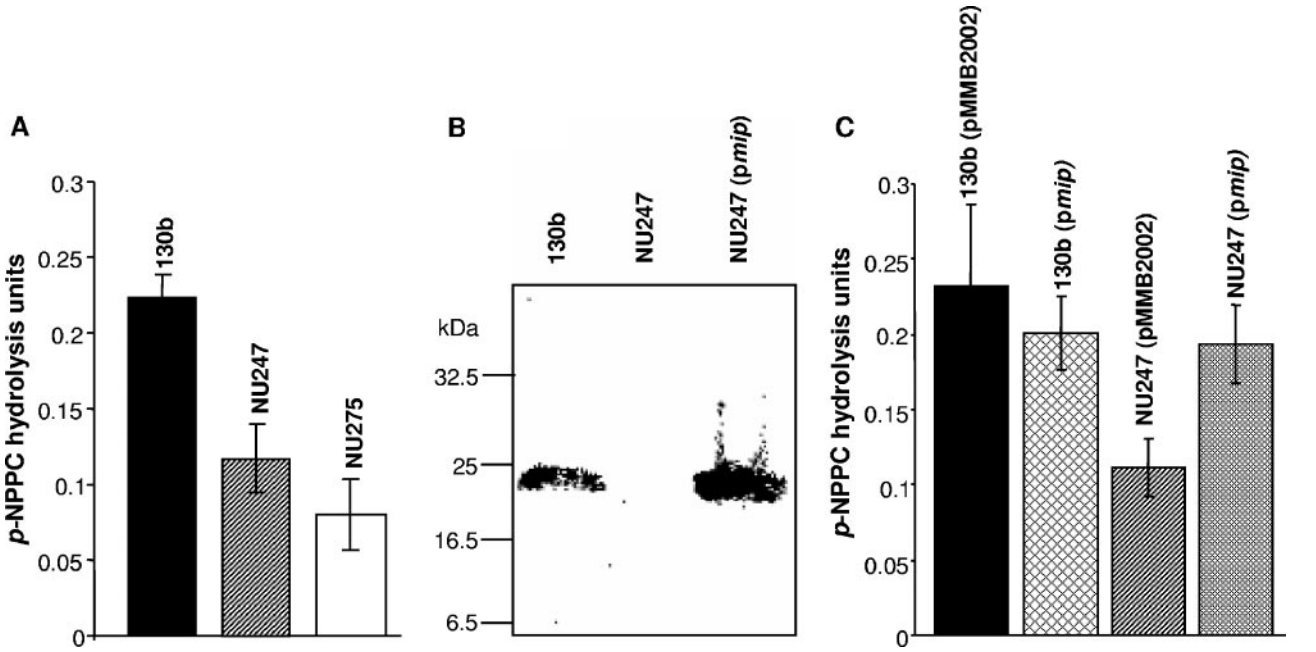

FIG. 1. *p*-NPPC hydrolase activities of and Mip expression by mutant NU247 and *mip*-complemented NU247. (A) Wild-type strain 130b, *mip* mutant NU247, and *lspF* mutant NU275 were grown in BYE broth, and then filter-sterilized culture supernatants were assayed for *p*-NPPC hydrolase activity. (B) Total cell lysates of 130b, NU247, and NU247(p*mip*) were subjected to SDS-PAGE, transferred to a membrane, and then reacted with anti-Mip antibodies. (C) *p*-NPPC hydrolase activities in BYE broth culture supernatants of 130b(pMMB2002), 130b(p*mip*), NU247(pMMB2002), and NU247(p*mip*). The data in panels A and C are the means and standard deviations for triplicate cultures for each strain. The reductions in enzymatic activity for NU247, NU275, and NU247(pMMB2002) were significant ($P < 0.05$, as determined by Student's *t* test). Similar results were obtained on at least two other occasions.

that was nearly as great as that of type II secretion mutants (Fig. 1A). Inverse PCR was used to retrieve the DNA around the transposon in NU247, and then sequence analysis showed that NU247 has a single mini-Tn*10 phoA* insertion 100 bp from the 5′ end of the monocistronic *mip* gene. *L. pneumophila mip* encodes a 24-kDa surface-exposed protein that is required for optimal intracellular infection and virulence (18–20, 28). In the *L. pneumophila* genome databases, Mip is designated lpg0791 in strain Philadelphia 1, lpp0855 in strain Paris, and lpl0829 in strain Lens (14, 15). Although Mip is a peptidylproline *cis-trans*-isomerase (PPIase) (34, 53), its molecular target(s) is unknown. To determine if the reduced *p*-NPPC hydrolase secretion of NU247 was due to the *mip* mutation, we performed *trans* complementation using *mip* cloned into pMMB2002 (i.e., p*mip*). Immunoblot analysis of total cell lysates confirmed the presence of Mip in 130b and NU247(p*mip*) and the absence of Mip in NU247 (Fig. 1B). Examination of culture supernatants then revealed that NU247(p*mip*) had *p*-NPPC hydrolase levels that were similar to those of 130b and greater than those of NU247 (Fig. 1C). Thus, *mip* alone can complement the loss of secreted *p*-NPPC hydrolase activity in NU247. Further testing of supernatants confirmed that NU247 was not generally defective for secretion, since it had normal levels of protease, lipase, and acid phosphatase activities (data not shown).

As observed previously for NU247 (3), NU247(pMMB2002) had a strong intracellular growth defect; i.e., it did not replicate in *H. vermiformis* amoebae, and replication was reduced ca. 100-fold in U937 cell macrophages (data not shown). The fact that NU247(p*mip*) and NU247(pMMB2002) behaved comparably in host cells (data not shown) suggested that the intracellular growth defect of NU247 is not simply due to the loss of *mip*. This suggestion is supported by the fact that previously constructed *mip* mutants are impaired about 100-fold and 10-fold in amoebae and U937 cells, respectively (19, 20, 44, 90). These data indicate that NU247 harbors a second-site mutation(s) that affects intracellular growth. NU247 also produced smaller colonies without opaque centers when it was grown on BCYE agar and lacked brown pigment when it was grown in BYE broth (data not shown). Neither of these phenotypes was complemented by *mip*, which increased the probability that there is another mutation in NU247. In summary, although NU247 appears to have multiple mutations, it does contain a *mip* insertion mutation that leads to reduced *p*-NPPC hydrolase activity in culture supernatants.

Multiple other *mip* mutants exhibit reduced *p*-NPPC hydrolase activity in culture supernatants. To determine whether the *mip*-associated secretion defect occurred only in NU247, we initially tested previously described *mip* mutants for *p*-NPPC hydrolase activity. Two mutants of strain 130b were examined; these mutants were AA108, which has a nonpolar deletion in *mip*, and NU203, which contains a Km^r cassette in *mip* (20, 44). For both mutants there was a 45 to 75% reduction in *p*-NPPC hydrolase activity in the supernatants (Fig. 2A). Reintroduction of *mip* into AA108, whether on a plasmid (AA108 containing p*mip*) or in the chromosome (AA109), resulted in wild-type levels of *p*-NPPC hydrolase activity (Fig. 2B). Similar complementation results were observed for NU203 (data not shown). When we examined another mutant

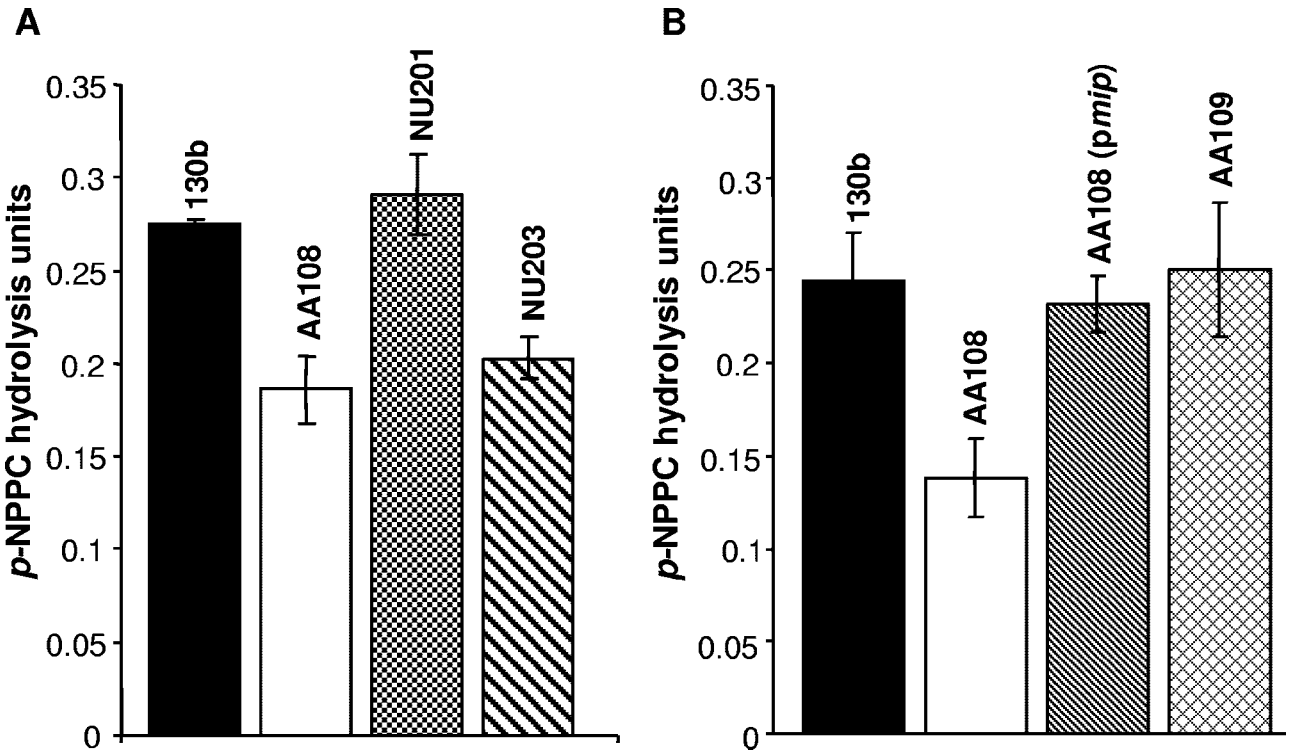

FIG. 2. Secreted *p*-NPPC hydrolase activities of previously described *mip* mutants and their complemented derivatives. (A) Enzymatic activities in culture supernatants of wild-type strain 130b, the AA108 *mip* deletion mutant of 130b, the NU201 streptomycin-resistant derivative of 130b, and the NU203 *mip* insertion mutant of NU201. (B) Enzymatic activities in culture supernatants of 130b, AA108, AA108(p*mip*), and AA109. The data are the means and standard deviations of triplicate cultures. The differences between the Mip-expressing strains and the *mip* mutants were significant ($P < 0.05$, as determined by Student's *t* test). Similar results were obtained on two other occasions.

(NU253) that had been isolated from the screen that yielded NU247, we found a transposon insertion in the *L. pneumophila surA* gene (lpg0298 in Philadelphia 1, lpp0376 in Paris, and lpl0351 in Lens). In *E. coli*, SurA is a periplasmic PPIase that belongs to the parvulin family and also functions as a chaperone that aids in the maturation of outer membrane proteins (11, 42, 47). Whereas complementation with *surA* failed to reverse the secretion defect of NU253, introduction of p*mip* did reverse it (Fig. 3). Immunoblots revealed that NU253 in fact lacks Mip (Fig. 3C). Thus, even though NU253 contains an insertion in *surA*, the reduction in secreted activity was due to a loss of Mip. In summary, since for four independently derived *mip* mutants there were similar reductions in *p*-NPPC hydrolase activity in the supernatants and since in each case the defect was reversed by providing *mip*, we concluded that Mip promotes the presence of *p*-NPPC hydrolase activity in culture supernatants.

Mip itself is not a *p*-NPPC hydrolase. Even though Mip has repeatedly been reported to be a surface-associated PPIase of *L. pneumophila* (1, 30, 31, 40, 65), it is possible that Mip itself is the *p*-NPPC hydrolase and that it can also exist in supernatants as a soluble or vesicle-bound protein. Several observations argue against this possibility. First, cell lysates of *E. coli* strains expressing *mip*, including DH5α(pGEMmip_c) and DH5α(p*mip*), had levels of *p*-NPPC hydrolase activity that were no greater than those of DH5α, with or without a vector (data not shown). Second, cell lysates of wild-type *L. pneumophila* and *mip* mutant AA108 exhibited identical (low) levels of *p*-NPPC hydrolase activity, while the type II *lspF* mutant NU275 showed the expected increase (3.5-fold) in activity due to retention of secreted enzymes in the periplasm (data not shown). Third, addition of FK506, a drug that is known to at least inhibit the PPIase activity of Mip (34, 65), to wild-type cultures and supernatants had no impact on the *p*-NPPC hydrolase activity (data not shown). Fourth, concentrated supernatants of wild-type *L. pneumophila* did not contain a protein species that reacted with anti-Mip antibodies in Western blots (data not shown). Fifth, silver staining of concentrated supernatant proteins of the wild type and Mip mutants AA108 and NU247 revealed identical profiles (data not shown), again indicating that Mip is not present in culture supernatants. Sixth, ultracentrifugation of wild-type supernatants, a procedure that should remove potential membrane vesicles, did not remove the enzymatic activity (data not shown). Taken together, these results indicate that Mip itself is not the *p*-NPPC hydrolase.

Identification of a Mip domain needed for promotion of the secreted activity. The Mip crystal structure reveals N-terminal and C-terminal domains that are connected by a long α-helix (65). The N terminus promotes dimerization, and cross-linking studies have shown that Mip homodimers form in solution and on the bacterial surface (77, 78). In contrast, the C-terminal domain of Mip contains the PPIase active site (65). To begin to determine what portion(s) of Mip is required for its role in the secretion phenotype, we generated a version of *mip* that lacked the C-terminal 76 amino acids (mip_{157}) and analyzed its ability, when cloned into pMMB2002, to complement AA108. As ex-

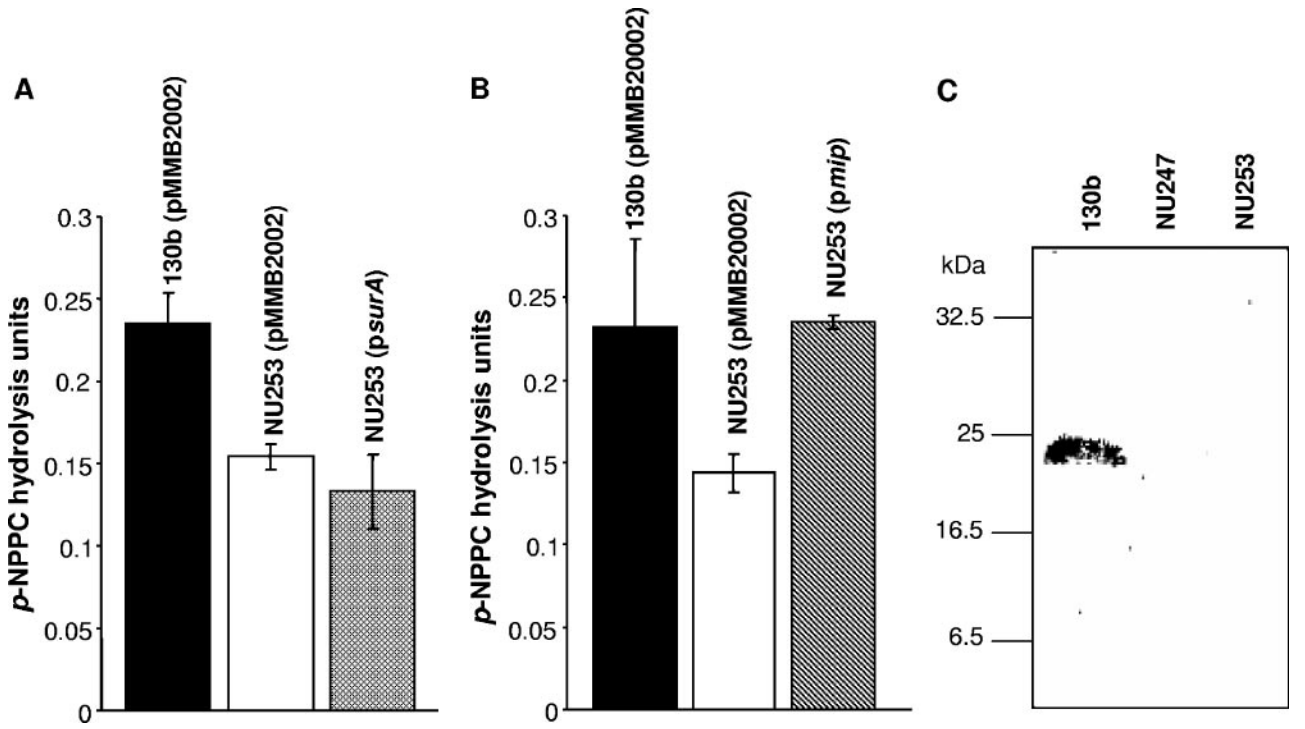

FIG. 3. Secreted *p*-NPPC hydrolase activities of and Mip expression by mutant NU253 and *surA*- and *mip*-complemented NU253. (A) Comparison of the enzymatic activities in culture supernatants of 130b(pMMB2002), NU253(pMMB2002), and NU253(p*surA*). (B) Comparison of the activities in 130b(pMMB2002), NU253(pMMB2002), and NU253(p*mip*). (C) Anti-Mip immunoblot of total cell lysates from 130b, NU247, and NU253. The data in panels A and B are means and standard deviations of duplicate and triplicate cultures, respectively, and are representative of two additional experiments. The differences in activity between 130b and NU253 containing either pMMB2002 or p*surA* were significant ($P < 0.05$, as determined by Student's *t* test).

pected, mip_{157} produced a 15-kDa protein that was detectable on immunoblots (data not shown). In contrast to AA108 carrying a plasmid with intact *mip*, the strain containing mip_{157} did not show complementation even if the gene was induced by IPTG (Fig. 4). Thus, the C terminus of Mip is required for promotion of the secreted *p*-NPPC hydrolase activity.

Mip influences the secretion of a novel PLC-like activity. To begin to identify the target of Mip action, we analyzed concentrated culture supernatants by anion-exchange chromatography in order to determine if the Mip-dependent activity was similar to or distinct from the previously described PlcA activity (4). Strain 130b supernatants obtained from late-log-phase BYE broth cultures had multiple peaks of *p*-NPPC hydrolase activity; some of these peaks were eluted by 100 mM NaCl, and another peak was eluted by 200 mM NaCl (Fig. 5). Analysis of an *lsp* mutant of strain 130b (70) showed that all of the activity peaks were dependent on *L. pneumophila* type II secretion (Fig. 5). When supernatants from a *plcA* mutant of 130b (strain NU268) were examined, the peak typically associated with elution by 200 mM NaCl was not seen, indicating that it represented PlcA (Fig. 5). Finally, when samples from the AA108 *mip* mutant were examined, they contained the PlcA peak but lacked peaks that eluted with 100 mM NaCl (Fig. 5). These data suggest that the secreted activity that is Mip dependent is not PlcA. In support of this conclusion, we observed that supernatants from a *mip plcA* double mutant exhibited a reduction in *p*-NPPC activity that was greater than that of either the *mip* or *plcA* mutant (Fig. 6), indicating that Mip and PlcA do

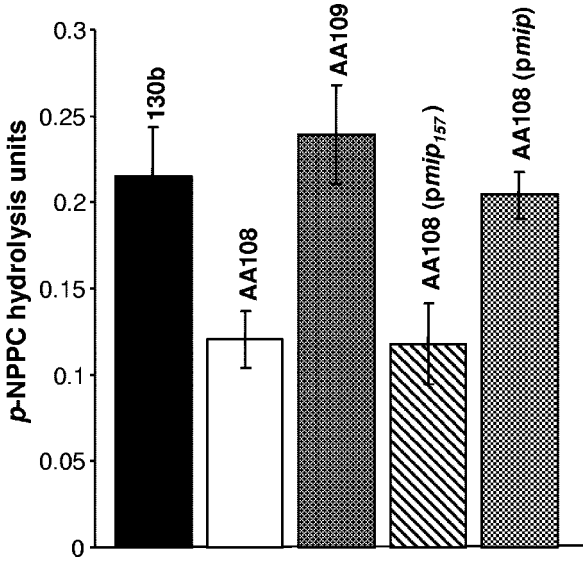

FIG. 4. Secreted activity associated with a truncated *mip* gene. Strains 130b, AA108, AA109, AA108(pmip_{157}), and AA108(p*mip*) were grown in BYE broth, and then filtered supernatants were assayed for *p*-NPPC hydrolysis. The data are means and standard deviations for triplicate cultures. Similar results were obtained on two other occasions. The differences in activity between 130b and AA108 with or without pmip_{157} were significant ($P < 0.05$, as determined by Student's *t* test).

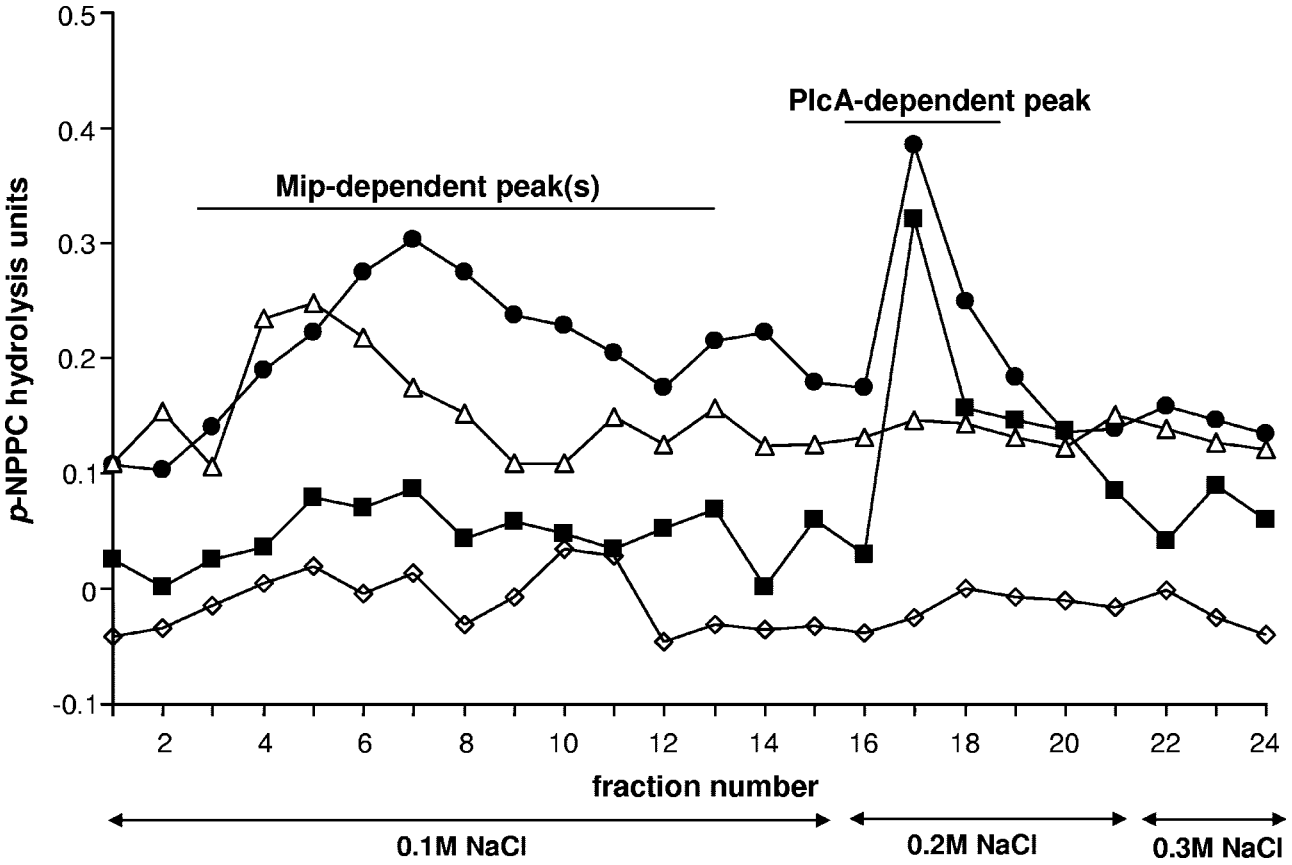

FIG. 5. Separation of secreted *L. pneumophila* *p*-NPPC hydrolase activities by anion-exchange chromatography. Concentrated supernatants of wild-type strain 130b (●), *mip* mutant AA108 (■), *plcA* mutant NU268 (△), and *lspF* mutant NU275 (◇) were applied to an A50 column, and then eluted fractions were assayed for the ability to hydrolyze *p*-NPPC. Fractions 1 to 15, 16 to 21, and 22 to 24 were eluted with 100 mM, 200 mM, and 300 mM NaCl, respectively. For each strain, a similar profile was obtained in at least two other trials.

not operate in the same pathway promoting secreted activity. Taken together, these biochemical and genetic data indicate that the Mip-dependent secreted activity involves a new type II-dependent enzyme. Although in some instances *p*-NPPC cleavage has been associated with phosphodiesterases and phosphatases (36), *L. pneumophila* does not secrete a phosphodiesterase or an alkaline phosphatase activity, and *Legionella* mutants lacking the major secreted acid phosphatase Map do not exhibit reduced secretion of *p*-NPPC hydrolase activity (2, 3). Thus, we hypothesized that the Mip-dependent *p*-NPPC hydrolase is another secreted type II-dependent PLC.

DISCUSSION

The Mip protein of *L. pneumophila* is one of the most studied *Legionella* proteins and has long been known to promote virulence (19, 28, 29). Indeed, the infectivity defects of *mip* mutants show that Mip is needed for optimal establishment and replication of *L. pneumophila* in macrophage cell lines, alveolar macrophages, blood monocytes, lung epithelial cells, amoebae, ciliates, and guinea pigs (18–21, 90). Other studies have shown that *mip* is transcribed and Mip is expressed in infected host cells (40, 48, 81, 87), and anti-Mip antibodies are present in sera from Legionnaires' disease patients (10). The *mip* gene is present and expressed in other species of *Legionella* (9, 17, 41, 63, 66), and *mip* mutants of *L. micdadei* and *L. longbeachae* are also defective for intracellular infection (25, 58). Based on immunoelectron microscopy, the absorption of anti-Mip antibodies to intact legionellae, and Mip-PhoA fusion studies, the Mip protein is expressed at least in part on the surface of *L. pneumophila* (1, 30, 31, 40). As noted above, enzymology and crystallographic studies have shown that Mip is a PPIase that exists as homodimers on the bacterial surface (65). Because the PPIase activity of Mip is inhibited by the immunosuppressant drug FK506 (34), the *Legionella* protein is considered an immunophilin and more specifically a member of the FK506-binding protein family (24). Site-directed mutagenesis has been used to identify residues in the carboxyl domain of Mip that are required for PPIase activity (90). Whereas the N-terminal domain of Mip and the dimerization of Mip are clearly required for both intracellular infection and virulence, the PPIase activity is needed for full virulence in guinea pigs but not for intracellular infection in vitro (49, 90). Finally, *mip* has been used as a target for PCR detection of legionellae in both clinical and environmental samples (35, 89) and as the basis for identification and speciation of *Legionella* strains (64). Despite the many studies by different laboratories, the substrate(s) or molecular target(s) of Mip is unknown.

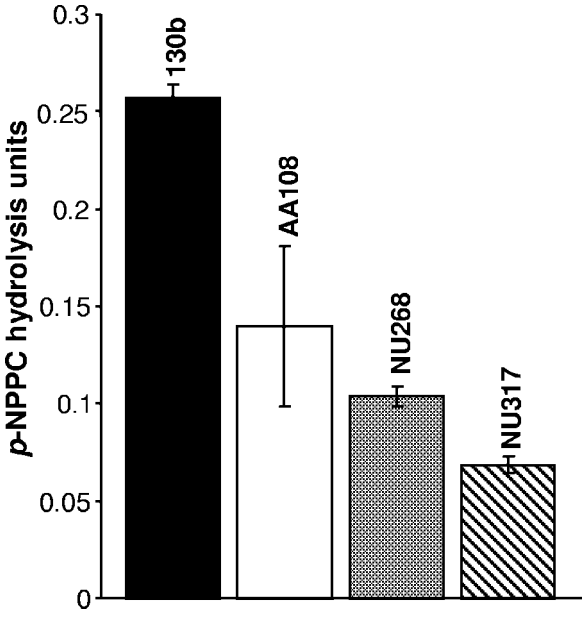

FIG. 6. Secreted *p*-NPPC hydrolase activity of a *mip plcA* double mutant. Culture supernatants of 130b, AA108, NU268, and *mip plcA* double mutant NU317 were assayed for *p*-NPPC hydrolase activity. The data are the means and standard deviations of triplicate cultures and are representative of at least two other independent experiments. The differences in activity between NU317 and the other two mutants were significant ($P < 0.05$, as determined by Student's *t* test).

The data presented here are the first evidence for a potential target of Mip. In addition to the PPIase function of Mip, it is tempting to speculate that Mip might also be a chaperone, as has been shown for other members of the FK506-binding protein family, including the periplasmic FkpA and cytoplasmic SlyD proteins of *E. coli* (5, 76, 79). We propose two basic hypotheses to account for our observations. In the first scenario, Mip, by virtue of its PPIase and/or possible chaperone activities, is involved in the extracellular release of an active enzyme that has *p*-NPPC hydrolase activity. Mip could act directly on the exoprotein. It is possible that Mip engages a subset of exoproteins (since mutant supernatants do not lack other activities); e.g., in *Pseudomonas aeruginosa* type II secretion, periplasmic LipH specifically folds a lipase (45). Alternatively, Mip might promote the maturation of proteins that form the (type II) secretion pathway. In the second scenario, Mip interacts with a newly secreted protein and, with its PPIase and/or possible chaperone action, causes changes that convert the protein from enzymatically inactive to active. More work is needed to learn how Mip facilitates secretion, what the nature of the secreted *p*-NPPC hydrolase activity is, and if this new function for Mip explains the role of Mip in pathogenesis.

To our knowledge, the data obtained in this study provide the first indication that a surface or periplasmic PPIase is involved in secretion of proteins beyond the outer membrane or in their activity. The periplasmic PPIases SurA, FkpA, and PpiD of *E. coli* have been implicated only in the maturation of outer membrane proteins (13, 23, 72). A parvulin-related, PPIase/chaperone (PrsA) that is anchored to the cytoplasmic membrane has been implicated in the secretion of proteins away from the membrane of the gram-positive bacteria *Bacillus subtilis* and *Bacillus anthracis* (85, 88). However, linkage between an extracytoplasmic PPIase/chaperone and secretion beyond the cell envelope has not been documented previously in gram-negative bacteria (13, 72). Since surface and secreted Mip-like proteins are present in other microorganisms, including *Chlamydia*, *Coxiella*, *Rickettsia*, *Neisseria*, and *Salmonella*, as well as the protozoan *Trypanosoma cruzi* (22, 43, 50, 55, 60, 67), our findings may have broad significance for a variety of diseases in which Mip-like proteins potentially promote the secretion of other important effectors.

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