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Progress in the pathogenesis of *Legionella pneumophila*

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Mutagenesis and gene cloning have been used to identify bacterial genes involved in the intracellular life cycle of selected pathogens. Examples include the detailed analysis of virulence plasmids from *Salmonella*[1] and *Shigella*,[2] the screening or selection of growth-defective mutants in *Salmonella*[3] or Listeria,[4] respectively, and the cloning of invasion-expressing genes from *Yersinia*.[5] Until recently, genetics-based studies of *Legionella* pathogenesis have been hampered by a variety of intrinsic problems: virulence-associated plasmids have not been identified, genetic manipulation of *Legionella* spp. has been difficult, and there are no obvious virulence-associated genes to clone.

There has been little difficulty in the cloning and expressing of *L. pneumophila* genes in *E. coli*. Among those genes cloned have been those expressing antigens,[6] extracellular protease,[7] a major heat-shock protein,[8] and the *recA* equivalent.[9] Introducing DNA into *L. pneumophila* has been more of an obstacle. Until the recent success with electroporation, DNA uptake had been limited to the conjugal transfer of broad host range plasmids from *E. coli*.[10]

Efficient mutagenesis of *L. pneumophila* has also been a problem. Random, chemical mutagenesis was used successfully by Mintz and co-workers to isolate a thymidine auxotroph, which was subsequently found to be unable to replicate in host cells.[11] Insertional mutagenesis by Tn*5* and its derivatives (including Tn*phoA*) is extremely inefficient.[12] In contrast, transposition of Mu derivatives occurs efficiently, and *lacZ* gene fusions have been isolated in *L. pneumophila* after conjugal transfer of a Mu*d-lac* transposon.[13] We have used a mini-Mu transposon to isolate mutants that fail to express certain lipopolysaccharide (LPS) epitopes, flagella, and bacterial phosphatase activity, and we have recently constructed a Mu*d-phoA* transposon that yields alkaline phosphatase gene fusions that are enzymatically active both in *E. coli* and in *L. pneumophila*. Because Tn*phoA* transposes so inefficiently in *L. pneumophila*, we have used a variation of the 'shuttle mutagenesis' method of Seifert and co-workers[14] to place PhoA$^+$ gene fusions on the *L. pneumophila* chromosome. Briefly, PhoA$^+$ fusions in cloned *Legionella* genes are isolated from a genomic library in *E. coli*. The cosmids carrying PhoA fusions are then transferred from *E. coli* to *L. pneumophila* and then exchanges of cloned, gene fusions for the native, chromosomal genes are selected. In the future, we anticipate that a large panel of random PhoA fusions will be used to target important secreted gene products in *L. pneumophila* in much the same way as has been done in other pathogenic bacteria.

Two *Legionella* mutants of particular relevance to our understanding of the organism's pathogenesis have been isolated by directed mutagenesis. The gene

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encoding an extracellular protease, a protein that has cytotoxic and hemolytic function as well as protective immunogenicity,[15] has been mutated.[16] Remarkably, this mutant demonstrated normal growth in a macrophage-like cell line. The second mutant was isolated in our laboratories.

Cianciotto mutated a gene encoding a 24 kDa antigenic protein that was of interest because of its specificity to *L. pneumophila* and its surface location. Compared with the parent strain, the mutant has defective infectivity for macrophages [approximately 2-log reduction in 50% infective dose (ID_{50})] and reduced virulence for guinea-pigs after intratracheal inoculation.[17,18] Both the defective cellular infectivity and reduced animal virulence were complemented by reintroduction of the wild-type gene on a transferable plasmid. Consequently, the gene was designated *mip*, for macrophage infectivity potentiator.

Preliminary studies using immunofluorescence, electron microscopy, and bacterial enumeration at early time points after infection have failed to show any qualitative or quantitative defect in the uptake of the *mip* mutant by host cells. Moreover, pre-opsonization of the bacteria with a monoclonal antibody directed at an LPS domain enhanced macrophage uptake and reduced the ID_{50} of both the parent and mutant strains to an equivalent degree. The persistence of the difference between the parent and mutant strains suggests that, although the fc-domain of the antibody enhances uptake by macrophages, it is not a substitute for the function of the *mip* gene product. That is, the Mip protein is not a simple macrophage ligand or adhesin. We also found that *mip*$^-$ and *mip*$^+$ *L. pneumophila* grow within host cells with the same doubling time, confirming that the mutant is not simply defective in intracellular replication. From these observations, we infer that the function of Mip is related to the organism's intracellular survival in macrophages, rather than to its uptake or growth.

Although the precise function of Mip is not yet known, we have several interesting clues. The amino acid sequence inferred from the DNA sequence predicts an extremely basic protein with an abundance of lysine residues, and non-equilibrium two-dimensional gel electrophoresis confirms that Mip is one of the most basic proteins in *L. pneumophila*.[19] As expected, the protein sequence begins with a consensus signal sequence, which is cleaved from the mature product. We have isolated insertions of Tn*phoA* in *mip* that yield phosphatase activity both in *E. coli* and in *L. pneumophila*, and we have recently purified Mip to homogeneity, raised monospecific antisera in rabbits, and demonstrated surface binding of the anti-Mip antibodies on the *L. pneumophila* surface, confirming our localization of this protein by less conventional methods in earlier work.[20]

Although Mip is specific to *L. pneumophila*, we have found partially homologous genes and cross-reactive proteins in virtually all members of the *Legionella* genus.[21] An initial survey of respiratory tract pathogens, normal flora, and various bacteria thought to be related to the Legionellaceae revealed no reactivity with Mip-specific antibodies nor any low stringency hybridization with a *mip* gene probe. More recently, partially homologous genes have been detected in various members of the order *Rickettsiales*, among them, *Coxiella burnetii* (N. Cianciotto, personal communication).

At the time that *mip* was sequenced, searches of the National Library of Medicine protein data bank failed to reveal significant homologies. Recently, Tropschug and co-workers reported a surprising degree of homology between *mip* and a prolyl isomerase from *Neurospora crassa*.[22] The meaning of this observation is unclear; however, we are presently engaged in studies to determine whether Mip possesses or interferes with this enzymatic foldase activity. In addition, now that purified Mip protein is available, we can determine which step in the intracellular life cycle is affected by this protein.

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