**lvgA, a Novel Legionella pneumophila Virulence Factor**

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Several novel *Legionella pneumophila* virulence genes were previously discovered by use of signature-tagged mutagenesis (P. H. Edelstein, M. A. Edelstein, F. Higa, and S. Falkow, Proc. Natl. Acad. Sci. 96:8190-8195, 1999). One of these mutants appeared to be defective in multiplication in guinea pig lungs and spleens, yet it multiplies normally in guinea pig alveolar macrophages. Here we report further characterization of the mutated gene and its protein and the virulence role of the gene. The complete sequence of the gene, now called *lvgA*, is 627 bp long, and its protein product is approximately 27 kDa in size. *lvgA* was present in all 50 strains of *L. pneumophila* tested. No significant nucleic acid or protein homology was found in the GenBank database for the parent. Subcellular fractionation studies localized LvgA to the outer membrane fraction, and protease digestion studies suggested that at least some of the protein is surface expressed. No change in bacterial lipopolysaccharide composition or reactivity to serogroup-specific antisera was detected in the mutant. Growth competition studies with alveolar macrophages showed that the mutant was outcompeted by its parent 3-fold in 24 h and 24-fold in 48 h, in contrast to what was observed with the null phenotype in parallel testing with alveolar macrophages or with the A549 alveolar epithelial cell line. This macrophage defect of the mutant bacterium was due to slower growth, as the mutant invaded alveolar macrophages normally. Electron microscopy showed that the mutant bacterium resided in a ribosome-studded phagosome in alveolar macrophages, with no distinction from its parent. The *lvgA* mutant was outcompeted by its parent about sixfold in guinea pig lungs and spleens; prolonged observation of infected animals showed no late-onset virulence of the mutant. Transcomplementation of the mutant restored the parental phenotype in guinea pigs. The *lvgA* mutant was twofold more susceptible to killing by human β-defensin 2 but not to killing by other cationic peptides, serum complement, or polymorphonuclear neutrophils. *lvgA* is a novel virulence gene that is responsible for pleiotropic functions involving both extracellular and intracellular bacterial resistance mechanisms.

*Legionella pneumophila*, the most common cause of Legionnaires’ disease, is a facultative gram-negative intracellular parasite. Legionnaires’ disease is a type of pneumonia that affects mainly adults, especially those who have altered local lung defenses or who have cellular immune system-suppressing diseases. A multitude of *L. pneumophila* virulence factors have been described; the majority of these affect the ability of the bacterium to grow and survive within blood monocytes and alveolar macrophages or within free-living amoebae (5). To attempt to find *L. pneumophila* virulence factors responsible for growth or survival in vivo but not necessarily in macrophages, Edelstein et al. previously performed signature-tagged mutagenesis of *L. pneumophila* by using a guinea pig pneumonia model (12). Several different new and previously discovered putative virulence factors were found, and the transposon insertion mutations in all but one mutant were found to be responsible for significant defects in bacterial growth in guinea pig alveolar macrophages. This mutant, 47:1e, apparently grew as well in macrophages as did its parent, and partial sequencing of the interrupted gene revealed no homology with other known bacterial genes. In this study, we fully sequenced the 47:1e gene, which we now call *lvgA*, performed gene complementation studies, and characterized more completely the virulence characteristics of this gene. We demonstrate that *lvgA* is a novel guinea pig and macrophage virulence gene found in *L. pneumophila*, that it encodes an outer membrane protein, and that it is responsible for enhanced growth in macrophages detectable only by competition studies of the mutant and its parent.

**MATERIALS AND METHODS**

Bacteria, plasmids, and growth conditions. *L. pneumophila* serogroup 1 strain F2310, also known as AA100jm, is a spontaneous streptomycin-resistant mutant of strain 130b (ATCC BAA-74), originally isolated from a patient with combined Legionnaires’ disease and pneumococcal and meningococcal pneumonia (12, 27). AA100jm is virulent in guinea pigs, macrophages, and amoebae (12, 28). *L. pneumophila* strain F2341 contains a transposon insertion mutation in *lvgA*. F2341 was made by transposon mutagenesis of AA100jm with Tn903H7 (Tn903 harboring a signature tag) (12). Complement-sensitive (K-12) and -resistant (K-29) *Escherichia coli* colib strains were obtained from Marcus Horwitz (20). *E. coli* strain K-29 and *Pseudomonas aeruginosa* ATCC 25619 were used as controls in neutrophil killing and defensin susceptibility assays, respectively. *L. pneumophila* strains having mutations in *dotO-icmB*, *dotB*, *icmX*, and *dotF-icmG* were made from strain F2310 by random transposon mutagenesis and used in studies of LvgA expression (12). *L. pneumophila* bacteria were grown at 35 to 37°C in ambient air on 3-(N-
morpholinio)-propanesulfonic acid (MOPS)-buffered charcoal-yeast extract agar medium supplemented with α-ketoglutarate (BCYE-α) (14), in N-(2-acetamide)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract broth supplemented with α-ketoglutarate (BCYE-α broth), or in ACES-buffered charcoal-yeast extract broth supplemented with α-ketoglutarate (BCYE-α broth). Plasmid pJS207 (29) was a gift from Howard Shuman. Plasmid pUC18 was purchased from Life Technologies, Gaithersburg, Md.

Nucleic acid manipulations. All nucleic acid manipulations were accomplished according to standard molecular biology techniques unless otherwise stated (2). PCR was performed by using Taq polymerase (Promega) unless otherwise stated. Complete sequencing of the lvgA gene. Genomic DNA from mutant clone 47:1e (AA100jm bg4:Tn903HT) was digested with restriction enzymes known not to cut the transposon insertion upstream of the kanamycin resistance cassette lvgA 47:1e (AA100jm) according to standard molecular biology techniques unless otherwise stated (2). Shuman. Plasmid pUC18 was purchased from Life Technologies, Gaithersburg, Md.

bacteria were suspended in Sorenson's balanced salt solution (pH 7.4, 10 mM) (32). Sorenson's balanced salt solution was used to suspend the bacteria and neutrophils, as the low-energy sonication as described previously (13); this process does not affect the viability of L. pneumophila.

Invasion assays. Guinea pig alveolar macrophages (2.5 × 10^5 to 5 × 10^5 cells/well) were infected with bacteria (MOI, 100) in 24-well microplates, after which the plates were centrifuged at 800 × g for 8 min at room temperature. Five wells were inoculated for each bacterium. The infected macrophages were incubated at 37°C in 5% CO_2 in air for 2 h; washed three times with warm M199; incubated with gentamicin (100 μg/ml) for 1 h; washed three times with warm M199, which was replaced with sterile distilled water; and lysed by low-energy sonication as described previously (13); this process does not affect the viability of L. pneumophila.

Neutrophil killing assays. Macrophages or alveolar epithelial cells were prepared as described above, infected with L. pneumophila (multiplicity of infection [MOI], 0.1), and incubated in 5% CO_2 in air at 37°C. Culture supernatants were harvested at various times, diluted appropriately with MHB, and plated on BCYE-α agar. Bacterial competition studies took advantage of the kanamycin resistance of L. pneumophila strain F2341 (bg4 mutant) and the kanamycin susceptibility of its parent. In these competition studies, the concentration of the parent strain was determined by subtracting the total bacterial concentration determined by plating on nonselective BCYE-α agar from the bacterial concentration determined by plating on BCYE-α agar containing kanamycin.

Intracellular growth assays. Macrophages or alveolar epithelial cells were infected at a multiplicity of 100. The infection was initiated by incubating the bacteria or exponential-phase broth-grown bacteria or exponential-phase broth-grown P. aeruginosa bacteria in suspension with ice-cold PBS. Fixed materials were processed by using standard techniques and examined by using an electron microscope, specifically seeking ribosome-studding of the L. pneumophila-containing phagosome (University of Pennsylvania Biomedical Image Core Facility).

Electron microscopic observations. To determine whether F2341 (bg4 mut) resided in a ribosome-studded phagosome, electron microscopy of F2341-infected guinea pig alveolar macrophages was performed, and the results were compared with the electron microscopic findings for F2310-infected alveolar macrophages. Guinea pig alveolar macrophages were cultured on sterile plastic dishes and infected with L. pneumophila (MOI, 0.1). The infected cells were incubated for 2 or 3 days, fixed with 2% glutaraldehyde in PBS, and washed with ice-cold PBS. Fixed materials were processed by using standard techniques and examined by using an electron microscope, specifically seeking ribosome-studding of the L. pneumophila-containing phagosome (University of Pennsylvania Biomedical Image Core Facility).

Transcomplementation of the L. pneumophila bg4 mutation. A DNA fragment containing the L. pneumophila A gene was amplified from L. pneumophila strain AA100j genomic DNA by PCR with an upstream sense primer (mu1e U79; TACCGAGTTTATCGTTGATTTGTT) and a downstream sense primer (mu1e L1003; CGGGATCCGGGGAGTTATGTGCTTTT); PstI and BamHI sites (underlined sequences) were incorporated into these primers, respectively. Amplification was performed by using Vent polymerase (New England Biolabs, Beverly, Mass.). The 958-bp PCR product of the bg4 gene was BamHI/PstI digested and then ligated into identically digested pU2719. E. coli XL-1 blue was transformed with the ligated product by electroporation. Plasmid DNA of Cm^R transformants was mapped by restriction digestion to confirm proper insertion of the desired DNA fragment into the plasmid. The cloned bg4 (Complete sequencing of the bg4 gene was sequenced by using M13 forward and reverse primers to verify that there was no erroneous incorporation of nucleotides during amplification. A single clone containing the whole bg4 gene ORF, 231 bp 5' of the ORF, and 84 bp 3' of the ORF was picked for further study, and the plasmid was designated pH292. The 731-bp Dral/SalI Klowen-treated fragment from pH292, which contains the bg4 ORF and its predicted promoter, was then cloned into Smal I cuts in pMMB207, creating the recombinant plasmid pMMB207 bg4. Complementing plasmid pMMB207 bg4 contains the entire bg4 ORF as well as 77 bp 5' and 31 bp 3' of bg4; it included the first 37 bp of the putative ORF 5' of bg4. The bg4 mutant of AA100j, clone 47:1e, than on plate-grown L. pneumophila F2310 and F2341, so that is why plate-grown bacteria were used. The assays were carried out with autoclaved siliconized microcentrifuge tubes (Fisherbrand siliconized low retention; Fisher Scientific).

Neutrophil killing assays. Human peripheral polymorphonuclear leukocytes were purified by density and size exclusion on a ficoll (BIOGEL A-50M) gradient from peripheral blood, as described previously (4). Killing assays were performed with 20% human serum as previously described, with one exception (22). M199 rather than Hanks' balanced salt solution was used to suspend the bacteria and neutrophils, as the L. pneumophila bacteria were killed by the salt solution.

Determination of flagellation. Plate-grown Legionella bacteria were suspended in sterile distilled water and stained with the presence of flagella by using Rya stain (Remel Laboratories, Lenexa, Kans.) as described previously (9, 24).

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referred to in this report as F2341, was transformed with pEG15 by electroporation as previously described (6). Plasmid DNAs from several CMR transformants were mapped by restriction digestion to confirm the presence of the desired plasmid. One of the transformants containing the desired plasmid was picked and further studied and was designated as F2462. PCR testing of strains with hsg-specific primers showed that it contained the full-length hsg4 gene, in contrast to the noncomplemented mutant. In addition, immunoblot analysis of the transcomplemented mutant with LvgA antibodies demonstrated that the transcomplemented mutant made detectable LvgA of the same size as that found in the parent strain and that LvgA production was not detectable in the hsg4 mutant. A recombinant plasmid was constructed for the mutant and parent strains by electroporating empty pMMB207 into F2341 and F2310, respectively, creating F2462 and F2463, respectively.

Detection of hsg4 in other Legionella sp. strains. The presence of hsg4 in 50 different strains of *L. pneumophila* (15 serogroups) was detected by PCR with hsg-specific primers US6 (TATAATTTCTCTTGGCGGAGT) and L711 (TGT CAATTCTTTGGGCTAAT), which gave a 643-bp product. BCYE-α agar plate-grown bacteria were harvested into 5 mM EDTA to approximate the density of a no. 1 MacFarland barium sulfate standard. The bacterial suspensions were heated at 99 to 100°C for 25 min. PCR was carried out by using a 25-µl volume with 1 µl of the heated bacterial suspensions as a template.

Animal model. The guinea pig model of *L. pneumophila* pneumonia was used as described previously (11). *L. pneumophila* was grown in BYE-α broth under the appropriate selective conditions and diluted in sterile water at a concentration of 3.3 × 10^6 CFU/ml. A total of 10^6 CFU was injected into the surgically exposed tracheae of Hartley strain male guinea pigs weighing 250 g. The animals were killed 2 days later. The right lower lung lobe and spleen were removed aseptically, weighed and ground in MHB, and diluted in diluent fluids with the same type of broth. Diluted tissue homogenates were plated on BCYE-α agar with or without kanamycin. Another experiment extended the postinfection observation time to 7 days postinfection. Analysis of the amount of lung consolidation was performed by low-power microscopic analysis of blinded histologic sections of animal lungs that had been stained with hematoxylin and eosin as described previously (11). The inflammatory pattern was characterized by using a similar method but at a higher-power magnification.

Expression of LvgA and production of anti-LvgA polyclonal antibodies. To produce LvgA antibodies for use in immunoblot studies, the protein was expressed in *E. coli*. The hsg4 ORF was amplified from a plasmid containing the gene by PCR with primers (CCGGGGATCCGAGGACCGCCGCGATATC and CGGGAATTCCTTGTGGATCGACCGCGGATCC) having BamHI and EcoRI sites (underlined sequences) to allow directional cloning into pGEX-2T (Pharmacia). PfuTurbo DNA polymerase enzyme (Stratagene) was used in the PCR. The subcloned plasmid (pBH1) was transformed into *E. coli* BL21. The sequence of the amplified gene was confirmed by sequencing in both directions with pGEX forward and reverse primers (Pharmacia). The expression of LvgA in BL21 was accomplished by growth of the bacterium in ampicillin-containing LB broth at 37°C for 5 h after previous overnight growth at 25°C. Diluted tissue homogenates were plated on BCYE-α agar with or without kanamycin. The expression of LvgA from hsg4 determined promoter site and a probable Shine-Dalgarno site were deduced from the amino acid sequence; the Tn916 transposon insertion site was removed aseptically, weighed and ground in MHB, and diluted in diluent fluids. Determination of the preservation of phase bacteria. Lipopolysaccharide (LPS) preparations of *L. pneumophila* were created by electroporating empty pMMB207 into F2341 and F2310, respectively. The expression of LvgA in BL21 was accomplished by growth of the bacterium in ampicillin-containing LB broth at 37°C for 5 h after previous overnight growth at 25°C. The expression of LvgA from hsg4 determined promoter site and a probable Shine-Dalgarno site were deduced from the amino acid sequence; the Tn916 transposon insertion site was removed aseptically, weighed and ground in MHB, and diluted in diluent fluids.

Subcellular localization of LvgA. To determine whether LvgA was secreted into culture supernatants, culture supernatants were processed as previously described (40), except that antibiotic-free BYE-α broth was used as the culture medium. The subcellular location of LvgA was determined by using the method described by Roy and Ishberg (35); the only exceptions to their protocol were the use of BYE-α broth and growth of the bacteria to log phase or stationary phase (optical density at 600 nm, 0.2 or 3.0, respectively). To determine whether LvgA was surface expressed and therefore subject to proteolytic cleavage, trypsin (bovine pancreatic trypsin type I; Sigma) digestion of whole and sonicated bacteria was performed as described previously (39) with BYE-α broth-grown log-phase bacteria. Lipopolysaccharide (LPS) preparations of *L. pneumophila* were made as described by Nolte and colleagues (30) with BYE-α broth-grown log-phase bacteria. Determination of the preservation of *L. pneumophila* serogroup 1 LPS antigens was performed as previously described with rabbit polyclonal anti-*L. pneumophila* serogroup 1 fluorescein-conjugated antibody (M Tech, Atlanta, Ga.) (9). Immunofluorescence staining of LvgA in whole bacterial cells, either unfixed or acetone treated, was attempted with rabbit polyclonal LvgA antibody as the primary antibody and goat anti-rabbit immunoglobulin G fluorescein-labeled antibody as the secondary antibody (U.S. Biochemicals).

Nucleotide sequence accession number. The hsg4 sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession number AF181867.
bacterial cells. LvgA had an apparent molecular size of 27.7 kDa and appeared as a single band under both reducing and nonreducing conditions. The apparent size of LvgA was the same for both the native \textit{L. pneumophila} protein and the recombinant protein (after GST cleavage) in \textit{E. coli}, indicating that the native protein is monomeric and is not extensively modified posttranslation. Strain F2341 (\textit{hvgA} mutant) produced no detectable LvgA, as expected (Fig. 1, even-numbered lanes). Both F2310 and F2341 produced equivalent amounts of DotA and IcmX, as judged by the density of the specific stained bands when equivalent amounts of total bacterial protein were analyzed (data not shown). \textit{L. pneumophila} strains having mutations in \textit{dotO-icmB}, \textit{dotB}, \textit{icmX}, or \textit{dotF-icmG} all produced normal amounts of LvgA, indicating that the \textit{dot-icm} complex played no role in the production or transport of LvgA (data not shown).

Subcellular localization of LvgA. Bacterial cell fractionation studies showed that LvgA was located in the non-Triton X-100-soluble fraction (Fig. 1, lane 9), (fraction O, as defined by Roy and Isberg [35]). No secretion of LvgA into the culture medium could be detected. These findings were independent of growth phase, as both early-logarithmic- and late-stationary-phase cultures of F2310 (\textit{hvgA} \textsuperscript{+}) contained LvgA in the non-secreted, non-detergent-soluble fraction. This result provided strong evidence that LvgA is an outer membrane protein. To determine whether LvgA is surface expressed, whole and sonicated bacterial cells were treated with trypsin. If LvgA is surface expressed, then trypsin exposure of both whole and sonicated bacterial cells will be expected to degrade the protein. If LvgA is not surface expressed, then trypsin will be expected to degrade the protein only in sonicated bacterial cells. LvgA was relatively trypsin resistant, but high trypsin concentrations degraded the protein in both intact and sonicated bacterial cells, indicating that it is at least partially surface expressed (Fig. 2). Coomassie blue-stained polyacrylamide gels of trypsin-treated whole bacterial cells showed that many bacterial proteins were not degraded by trypsin, excluding leaky or partially lysed bacterial cells as the explanation for the trypsin degradation of LvgA in whole bacterial cells. Since many outer membrane proteins are constituents of bacterial LPS, we determined whether there was any difference in LPS composition between F2310 (\textit{hvgA} \textsuperscript{+}) and F2341 (\textit{hvgA} mutant). LPS gels stained by silver staining showed no difference in the 15 visualized bands between the two bacteria. Also, immunofluorescence staining of the bacterial strains with polyclonal antibody to the \textit{L. pneumophila} serogroup 1 LPS antigen showed that both bacterial strains stained strongly with the antibody. Attempts to detect LvgA by immunofluorescence microscopy of F2310 with LvgA polyclonal antibody were unsuccessful, either in the presence or in the absence of acetone permeabilization of bacterial cells.

\textbf{Growth and flagellar expression of the hvgA mutant.} \textit{L. pneumophila} strain F2341 (\textit{hvgA} mutant) grew as well as its parent in \textit{BYE-\alpha} broth, as determined by monitoring of its optical density with time (data not shown). The mutant possessed single or double monopolar or bipolar flagella indistinguishable from those of its parent.

\textbf{Susceptibility to extracellular killing.} Initial studies with guinea pigs and their alveolar macrophages showed that \textit{L. pneumophila} strain F2341 (\textit{hvgA} mutant) apparently grew as well as its parent in the macrophages but that the mutant bacterium was less virulent than its parent in the guinea pig pneumonia model. To determine whether this phenotype could be attributed to the susceptibility of the mutant bacterium to non-macrophage-related host defenses, the resistance of the bacterium to killing by serum complement, neutrophils, and defensins was determined. The mutant bacterium was as resistant as its parent to killing by serum complement and human neutrophils. After 1 h of incubation in 20\% fresh non-immune guinea pig serum or heated 20\% \textit{L. pneumophila} immune guinea pig serum, there was no change in the bacterial concentration; incubation in combined 20\% fresh nonimmune serum and heated immune serum resulted in a decrease in the bacterial concentration of log\textsubscript{10} 3.8 CFU/ml. The serum-sensitive \textit{E. coli} control strain showed a decrease in concentration of log\textsubscript{10} 4.2 CFU/ml after incubation in 20\% fresh serum but showed no change in concentration after incubation in heated 20\% \textit{L. pneumophila} immune serum. Similarly, incubation of the \textit{L. pneumophila} A mutant strain with human neutrophils for 1 h resulted in a decrease in bacterial concentration of log\textsubscript{10} 0.55 CFU/ml, whereas incubation of the same strain in all reaction components except for neutrophils resulted in a decrease of log\textsubscript{10} 0.45 CFU/ml, an insignificant difference. The neutrophil-sensitive \textit{E. coli} control strain was killed by neutrophils under the same conditions, with a change in concentration of log\textsubscript{10} −2.85 CFU/ml; under the same conditions without neutrophils, there was an increase in bacterial concentration of log\textsubscript{10} 0.08 CFU/ml.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Fig1.png}
\caption{Immunoblot of various bacterial fractions with LvgA antibody. Odd-numbered lanes contain F2310 fractions (\textit{hvgA} \textsuperscript{+}); even-numbered lanes contain F2341 fractions (\textit{hvgA} mutant). Lanes: 1 and 2, culture supernatants (secreted protein); 3 and 4, total bacterial sonicate; 5 and 6, soluble subfraction; 7 and 8, Triton X-100-soluble subfraction; 9 and 10, non-Triton X-100-soluble subfraction. Identical results were obtained in two additional independent experiments.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2.png}
\caption{Immunoblot of F2310 (\textit{hvgA} \textsuperscript{+}) with LvgA antibody after trypsinization of sonicated bacterial cells or of whole bacterial cells with subsequent washing and sonication. Equivalent amounts of bacteria were added to each lane, as confirmed by Coomassie blue staining of gels before blot transfer. The trypsin concentrations (conc.) used are shown. The data are representative of findings of three independent experiments.}
\end{figure}
L. pneumophila strain F2341 (lvgA mutant) was significantly more sensitive to HβD-2 (5 μg/ml) than its parent, strain F2310 (Fig. 3). After 1 h of incubation with the peptide, the F2341 concentration was 0.43% (95% confidence interval [CI], 0.01 to 0.85%; n = 3) of its concentration under the same conditions in the absence of the peptide. In contrast, F2310 survival was about twice that of the mutant (0.91%; 95% CI, 0.09 to 1.7%); the difference between the survival concentrations of the two bacterial strains was significant (P value, 0.04, as determined by a paired t test; 95% CI for the difference, 0.05 to 0.90%; three independent experiments). Lower concentrations of HβD-2 had minimal killing effects on the L. pneumophila bacteria. The limited amount of material precluded extensive studies with HNP-1 (5 μg/ml), but one experiment showed that the F2310 concentration after 1 h of incubation with the peptide was not significantly different from that found for F2341 under the same conditions (Fig. 3). Both F2310 and F2341 grew equally well on BCYE-α medium containing polymyxin B (80 U/ml, or ~8 μg/ml).

Intracellular growth and invasion. Multiple studies of L. pneumophila strain F2341 (lvgA mutant) showed that it grew in guinea pig alveolar macrophages and A549 cells apparently as well as its parent (Fig. 4). There was a consistent difference in bacterial concentrations of ~0.5 log₁₀ unit on the second day of macrophage infection, a difference that we originally dismissed as being both biologically and statistically insignificant. However, when the mutant and parent bacteria were combined in the same tissue culture wells, the parent strain out-competed the mutant strain by 4- to 24-fold (Fig. 5). Microscopic observation of F2341-infected alveolar macrophages at later stages of infection showed almost complete lysis of all macrophages after 3 to 4 days postinfection, a pattern indistinguishable from that seen with F2310-infected macrophages. F2341 was internalized as effectively as its parent, as shown by gentamicin protection studies; 0.06% of extracellular F2341 bacteria were internalized after 1 h of incubation with alveolar macrophages, as opposed to 0.07% internalization of F2310 bacteria (P value, 0.75, as determined by an unpaired t test; one experiment performed in quadruplicate). A second gentamicin protection experiment confirmed these results.

Electron microscopy. To determine whether the lvgA mutation affected the ribosomal studding of phagosomes ordinarily observed in L. pneumophila infection of macrophages (21), electron microscopy of guinea pig alveolar macrophages infected with either the mutant or its parent was performed. This examination showed that both bacterial strains were located...
F2310 (Fig. 6B and A, respectively). Two of seven animals infected with F2310 died of pneumonia, both on day 5 postinfection, whereas all eight F2341-infected animals survived. As was found in the shorter-observation-period animal studies, the clearance of F2341 from the lungs of 7-day survivors was significantly greater than that seen in animals infected with F2310 (P value, <0.01, as determined by a t test; pooled data) (Fig. 6D). Lung weights were also significantly higher in the F2310-infected 7-day survivors (P value, <0.01, as determined by a t test; pooled data) (Fig. 6C). Animals infected with F2341 did become ill, as measured by the presence of fever and weight loss. Thus, F2341 was significantly less virulent in guinea pigs than its parent, but the mutant bacterium was not completely avirulent.

Infection of guinea pigs with either F2310 or F2341 resulted in lung inflammation and consolidation, although the histologic patterns differed qualitatively for the different infections. Infection with the parent strain resulted in more neutrophilic lung infiltration than infection with the lvgA mutant strain. An average of 45% (two independent experiments; n = 7; 95% CI, 19 to 70%) of the total lung area was consolidated in lungs taken from animals infected with the lvgA mutant strain for 2 days; this value was greater than the average 23% consolidation observed in animals infected with the parent strain (two independent experiments; n = 14; 95% CI, 14 to 32%), but this difference was not significant (P value, 0.09, as determined by an unpaired t test with Welch’s correction). The degrees of lung consolidation were almost exactly the same for animals infected with these bacteria at 7 days after infection (one experiment; n = 7 in each group; means of 64 and 66% for mutant and parent, respectively; P value, 0.8, as determined by an unpaired t test). In striking contrast, neutrophilic infiltration was significantly greater in the lungs of animals infected with the parent strain than in those of animals infected with the mutant strain at both 2 and 7 days after infection. Macrophages represented the major type of inflammatory cells observed in the lungs of animals infected with either bacterium. Neutrophils represented an average of 40% (n = 14; 95% CI, 32 to 47%) of the total inflammatory cells present in the lungs of animals infected with the parent at 2 days after infection, versus 24% (n = 7; 95% CI, 17 to 31%) for infection with the mutant (P value, 0.009, as determined by an unpaired t test). At 7 days after infection, neutrophils represented an average of 43% (n = 7; 95% CI, 25 to 60%) of lung inflammatory cells for parent infection, versus 2% (n = 7; 95% CI, 0 to 4%) for mutant infection (P value, 0.001, as determined by an unpaired t test with Welch’s correction).

Guinea pig virulence of the transcomplemented lvgA mutant. Transcomplementation of lvgA in F2341 (lvgA mutant) restored the virulence of the bacterium to the levels observed for the parent strain (Fig. 7). Animals infected with the transcomplemented mutant had body temperatures and spleen and lung clearances of L. pneumophila that were indistinguishable from those observed for animals infected with the parent strain containing an empty vector. Animals infected with the transcomplemented mutant showed slightly more weight loss than animals infected with the parent strain, although this difference was not significant.
DISCUSSION

These results show that \( lvgA \) is a novel \( L. \) \( pneumophila \) virulence factor which may act both through altering intracellular growth and through resistance to one or more pulmonary defenses. The discovery of \( lvgA \) as a virulence factor was possible only by testing bacterial mutants in guinea pigs, as the macrophage growth defect of the \( lvgA \) mutant is extremely subtle and would have been missed by the usual cell culture screening methods. These factors suggest that more such undiscovered \( L. \) \( pneumophila \) virulence factors may exist.

Since the initial macrophage testing of \( L. \) \( pneumophila \) F2341 (\( lvgA \) mutant) showed no significant defect in macrophage growth, we explored several possible reasons for the attenuated virulence of the mutant in guinea pigs, focusing on extra-alveolar macrophage lung host defense mechanisms. We speculated that either during initial deposition in the lungs or during the release of the bacterium from infected macrophages, the non-macrophage-dependent lung defenses could affect the survival or lung clearance of the mutant bacterium. Our studies demonstrated that the mutation did not confer serum complement sensitivity or neutrophil killing susceptibility, two major mechanisms for host defenses against pathogens in general. However, we did show that one lung antimicrobial peptide, \( H\beta D-2 \), was significantly more active against the mutant than against its parent. This defensin susceptibility did not appear to be the result of global susceptibility to defensins or cationic peptides, in view of the resistance of the bacterium to polymyxin B and probably its resistance to HNP-1. However, sufficient studies were not performed with HNP-1 to confirm that the mutant bacterium was truly resistant to this defensin.

The twofold increase in the sensitivity of the mutant bacterium to \( H\beta D-2 \) may be biologically significant. \( H\beta D-2 \) concentrations in normal human lungs are not known with certainty but are probably in the range of 1 \( \mu \)g/ml (15). Localized concentrations of \( H\beta D-2 \) in inflamed lungs may be considerably higher, such that resistance to extracellular or intracellular \( H\beta D-2 \) activity could be an important \( L. \) \( pneumophila \) virulence mechanism (15). \( H\beta D-2 \) is an inducible bactericidal cationic peptide produced by skin and respiratory epithelial cells, monocytes, and alveolar macrophages (8, 36, 37). A number of host inflammatory mediators increase \( H\beta D-2 \) production; these include gamma interferon and tumor necrosis factor \( \alpha \), the levels of both of which are increased in \( L. \) \( pneumophila \) infection (3, 8, 18, 36, 37). Intracellular \( H\beta D-2 \) concentrations...
and the subcellular localization are unknown, but it is possible that this defensin plays a role in killing intraphagocytic bacteria (23). HβD-2 is thought to be most important in host lung mucosal immunity against infection and in aiding in the clearance of inhaled bacteria, and it could play this role in Legionnaires’ disease (36, 37). In both normal and inflamed lungs, even a small effect on bacterial survival could tip the balance in favor of the host, especially early in the course of infection. Proof of the role of HβD-2 in host defenses against L. pneumophila will require studies with L. pneumophila-resistant animals that are defective in HβD-2 production, an animal model that does not yet exist, to our knowledge.

Cationic peptide resistance is important for the virulence of many pathogenic bacteria. Some of the known gram-negative bacterial cationic peptide virulence factors include pagP and the sap family for Salmonella enterica serovar Typhimurium, htrB for Haemophilus influenzae, and rcp for L. pneumophila (16, 17, 33, 38). S. enterica serovar Typhimurium pagP controls LPS acylation, and the sap family virulence factors act as peptide and ion transporters (16, 17, 31). Starner and colleagues described the enhanced susceptibility of nontypeable H. influenzae htrB mutants to HβD-2 but not HβD-3 and attributed the specific HβD-2 resistance to a defect in lipo-oligosaccharide acylation (38). Robey and colleagues reported that an L. pneumophila rcp mutant had reduced sensitivity to cationic peptides, including polymyxin B and C18G; neither the susceptibility of this mutant to HβD-2 nor studies of its LPS were reported, but an abnormality of LPS acylation could be expected because of the homology of rcp to pagP (33). The rcp mutant, in contrast to the hvgA mutant, showed substantially reduced growth in several different cell types. Unlike the H. influenzae htrB mutant and probably the L. pneumophila rcp mutant, the L. pneumophila hvgA mutant had no discernible abnormality in LPS structure, although it is possible that such an LPS abnormality existed but was not detected by our methods. The degree of increased sensitivity of the L. pneumophila rcp and H. influenzae htrB mutants to the peptides relative to what was observed for their parents was of the same order of magnitude as that which we observed for the increased sensitivity of F2341 relative to its parent.

We were able to demonstrate that the hvgA mutant grew less well in macrophages than its parent, but only by using bacterial competition studies and alveolar macrophages. The hvgA mutant was outcompeted by its parent starting 1 day after infection. There appeared to be a plateau in the parent/mutant ratio by day 2. Whether the mutant would eventually “catch up” to the parent with prolonged observed is impossible to know because of the entire loss of the cell monolayer by infection day 3 as a result of bacterial infection and cytotoxicity.

Virtually all studies of L. pneumophila putative virulence genes have used intracellular growth or cytotoxicity studies to determine whether a gene is a virulence factor. This study demonstrates that this cellular infection method may not detect all virulence genes, even ones that cause significant defects in macrophage multiplication, as determined by competition studies. Bacterial competition studies may be required to exclude attenuated intracellular virulence of an L. pneumophila gene mutation, despite negative cell infection or cytotoxicity studies.

Several guinea pig studies demonstrated that hvgA is an L. pneumophila virulence factor in this animal model. A competition study in which both the parent and the mutant were given to the same guinea pigs in equivalent amounts demonstrated that the mutant was outcompeted by the parent by...
about five- to sevenfold after 2 days. Several parallel noncompetition studies confirmed the greater lung clearance of the mutant than of the parent and that animals infected with the mutant appeared less ill. A clinical study with a longer observation period confirmed that infection with the mutant attenuated the clinical illness, compared to that observed with the parent, and that the lvgA mutant did not cause late-onset illness. The lvgA mutant bacterium is not avirulent in guinea pigs, however, as infection with this bacterium causes animal illness and lung inflammation. Thus, lvgA is only one of multiple *Legionella pneumophila* virulence genes responsible for causing pneumonia.

Guinea pig infection with the lvgA mutant bacterium resulted in considerable lung inflammation, as has been observed for other *L. pneumophila* bacteria with mutations in other virulence genes (28; unpublished observations). In addition, partial suppression of the acute neutrophil response in the lungs of animals infected with the lvgA mutant was seen, as was almost complete elimination of the neutrophil response to infection with the mutant bacterium in the recovery phase of lung inflammation. These results may indicate that LvgA promotes a neutrophilic inflammatory response, either directly or indirectly, or that the more efficient lung clearance of the mutant bacteria results in less of a proinflammatory stimulus, perhaps one elicited by macrophages.

Whether the lvgA mutant is attenuated in guinea pig virulence solely because of its slower growth in alveolar macrophages is unanswered by this study. The enhanced susceptibility of the lvgA mutant to HβD-2 suggests that both HβD-2-dependent extracellular and HβD-2-independent intracellular factors play a role in the pathogenesis of Legionnaires’ disease although, as discussed above, it is possible that intracellular HβD-2 plays a role in host defenses. It is possible that HβD-2 and other extracellular factors play roles during the initial entry of the bacterium into the lungs, before phagocytosis of the bacterium by lung cells, or that an extracellular factor plays a role in host defenses during the period between the release of the bacterium from one phagocyte and its entry into a new host cell. Why lvgA would have a pleiotropic virulence effect is unanswered.

The mechanism by which lvgA exerts its phenotype is unknown. We have strong evidence that LvgA is an outer membrane protein that is surface expressed and not secreted. Based on this probable location, LvgA could function in a variety of ways, such as acting as part of a bacterial sensing system or as a trigger for the host cell or immune system response. LvgA is unlikely to function as a macrophage or other immune cell binding ligand, as the lvgA mutant bacterium was phagocytosed normally by alveolar macrophages and because the bacterium was killed normally by neutrophils. A variety of *L. pneumophila* surface-expressed virulence factors have been described; these include lipid A, flagellae, pili, a porin, MIP, a 25-kDa outer membrane protein, and a heat shock protein (Hsp60) (5). LvgA is not clearly related to any of these previously described virulence factors on genetic or structural grounds. The lvgA mutant has no apparent effect on LPS composition or production of flagella, and the lvgA gene has no significant DNA homology with any other described bacterial gene. LvgA also has no apparent structural motifs or significant protein homology with previously bacterial proteins.

In conclusion, *L. pneumophila* lvgA is a novel virulence factor. This virulence factor is necessary for full virulence of the bacterium in guinea pigs and presumably humans. It is unique in that a lvgA mutant shows subtly decreased growth in alveolar macrophages, yet infection of the whole animal with the mutant results in attenuated disease seemingly out of proportion to the macrophage growth defect. The susceptibility of the mutant to a pulmonary defense and the relative to complete lack of a neutrophilic response to infection suggest that lvgA has a pleiotropic effect on macrophage growth, local host defenses, and the global host inflammatory response to infection. It is very likely that many more such *L. pneumophila* virulence factors exist that have subtle or nonexistent cell infection phenotypes but are still capable of causing disease because of synergistic pleiotropic functions.

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**REFERENCES**


17. Guo, L., K. B. Lim, C. M. Poduje, M. Daniel, J. S. Gunn, M. Hackett, and...


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