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Identification of *Legionella pneumophila* Genes Required for Growth within and Killing of Human Macrophages

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Legionella pneumophila **was mutagenized with Tn*903*dII*lacZ*, and a collection of mutants was screened for defects in macrophage killing (Mak$^-$). Of 4,564 independently derived mutants, 55 (1.2%) showed a reduced or complete lack in the ability to kill HL-60-derived human macrophages. Forty-nine of the Mak$^-$ mutants could be assigned to one of 16 DNA hybridization groups. Only one group (9 of the 10 members) could be complemented for macrophage killing by a DNA fragment containing *icm* and *dot*, two recently described *L. pneumophila* loci that are required for macrophage killing. Phenotypic analysis showed that none of the mutants were any more sensitive than the wild type to human serum, oxidants, iron chelators, or lipophilic reagents nor did they require additional nutrients for growth. The only obvious difference between the Mak$^-$ mutants and wild-type *L. pneumophila* was that almost all of the Mak$^-$ mutants were resistant to NaCl. The effects of LiCl paralleled the effects of NaCl but were less pronounced. Resistance to salt and the inability to kill human macrophages are linked since both phenotypes appeared when Tn*903*dII*lacZ* mutations from two Mak$^-$ strains were transferred to wild-type backgrounds. However, salt sensitivity is not a requisite for killing macrophages since a group of Mak$^-$ mutants containing a plasmid that restored macrophage killing remained resistant to NaCl. Mak$^-$ mutants from groups I through IX associated with HL-60 cells similarly to wild-type *L. pneumophila*. However, like the intracellular-multiplication-defective (*icm*) mutant 25D, the Mak$^-$ mutants were unable to multiply within macrophages. Thus, the ability of *L. pneumophila* to kill macrophages seems to be determined by many genetic loci, almost all of which are associated with sensitivity to NaCl.**

Legionella pneumophila is the causative agent in Legionnaires' disease, a life-threatening pneumonia in man. The bacterium has the ability to infect, multiply within, and kill human monocytes, monocyte-derived macrophages, and alveolar macrophages. The pathway for a productive *L. pneumophila* infection is a multistep process (reviewed in references 10, 15, and 38) that begins with the binding of the bacteria to a human host cell. The bacteria are engulfed by coiling phagocytosis (28) and located in a unique phagosomal vacuole that does not fuse with host secondary lysosomes (27). Smooth vesicles, mitochondria, and then ribosomes are recruited around this specialized phagosome. The bacteria multiply within the phagosome, and the host cell eventually lyses, releasing *L. pneumophila* which can initiate new rounds of infection.

This seemingly complex cycle of infection implies the existence of genetic loci that confer and/or regulate the virulence of *L. pneumophila*. However, only a few genes that are specifically required for growth within host cells have been identified. The Mip protein is needed for efficient infection of host cells, and null mutations in *mip* reduce the infectivity of *L. pneumophila* about 80-fold in both cell culture (12) and guinea pigs (11). The *icm* locus is essential for the intracellular multiplication of *L. pneumophila* within human macrophages and is also required for virulence in guinea pigs (36). Adjacent to *icm* is *dot*, a gene required for intracellular replication and the proper trafficking of cellular organelles during macrophage infection (4). Also, bacterial thymidine synthesis (42) and iron acquisition (6) are required for growth of *L. pneumophila* both in liquid culture and within macrophages.

Additional *L. pneumophila* proteins have been implicated in being important for infection. Convincing evidence suggests that the major outer membrane protein MOMP is involved in uptake of the bacteria by the host cell. MOMP is a porin (21) that binds complement components C3b and C3bi (3), which in turn mediate binding to monocyte receptors CR1 and CR3 involved in phagocytosis (43). Even though the gene encoding MOMP (*ompS*) has been isolated (25), null mutations in MOMP have not been made to demonstrate its role in infection. It is also known that *L. pneumophila* is not killed by human serum, yet the gene(s) conferring this resistance has not been identified. Finally, lesser evidence suggests that phosphatases (47), phospholipases (2), and toxins (23, 24) play a role in the pathology of *L. pneumophila* infections.

Since *L. pneumophila* strains defective in host cell killing are avirulent in guinea pigs (18, 36, 45), a subset of genes required for virulence may be identified by isolating mutants that have a reduced capacity to kill macrophages. Bacterial mutants were generated by transposition of Tn*903*dII*lacZ*, a Tn*903* derivative that transposes efficiently in *L. pneumophila* (53). We used a procedure that allowed us to collect mutants that were greater than 96% independently derived (53). These mutants, containing simple insertions of Tn*903*dII*lacZ*, were isolated and screened for their inability to kill human macrophages. Many mutants that had a reduced ability or completely lacked the ability to kill macrophages were obtained. To determine the number of *L. pneumophila* loci represented among the mutants, the mutants were categorized by their (i) site of the Tn*903*dII*lacZ* insertion, (ii) ability to be complemented by the cloned *icm* locus for macrophage killing, (iii) response to a variety of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and features	Reference or source
L. pneumophila		
25D	Icm$^-$ avirulent mutant	29
AB1156	AM511 *pig*::Tn*903*dII*lacZ*	53
AM240	25D Sm^r r$^-$	36
AM511	Philadelphia-1 Sm^r r$^-$ m$^+$	36
CS140	Philadelphia-1 *thyA*	42
JR32	Homogeneous salt-sensitive isolate of AM511	This study
LELA1–LELA4536	JR32 containing simple insertions of Tn*903*dII*lacZ*	This study
LW1066–LW1070	Mutation in LELA1650 exchanged into JR32, independent isolates	This study
LW1071–LW1076	Mutation in LELA3307 exchanged into JR32, independent isolates	This study
LW1077–LW1082	Mutation in AB1156 exchanged into JR32, independent isolates	53
Escherichia coli		
O9:K29:H$^-$	Serum resistant, wild-type isolate	30
C600K$^-$	Serum sensitive, *galK lacY1 thr-1 leu-6 thi-1 supE44 tonA21*	N. D. F. Grindley
DH5α	F$^-$ *endA1 hsdR17* (r$^-$ m$^+$) *supE44 thi-1* λ$^-$ *recA1 relA1* Δ(*argF-lacZYA*)U169 φ80d*lacZ*ΔM15 *deoR gyrA96* Nal^r	54
LE392	*hsdR514*(r$^-$ m$^+$) *supE44 supF58* Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ$^-$	5
LW211	LE392 with an integrated RP4 (ΔCb^r Tc^r::Mu) transferred by P1 transduction from SM10, Mob$^+$ Km^r	L. A. Wiater
LW413	LW211 containing pAM10	This study
SM10	*thi thr leu tonA lacY supE recA* with an integrated RP4-2-(ΔCb^r Tc^r::Mu) Km^r	49
Plasmids		
pAM10	Cm^r, *oriR*(RSF1010) *icmA*	36
pBluescript II KS(+)	*oriR*(f1) MCS *oriR*(ColE1) Cb^r	48
pLAW317	*rpsL* MCS *oriT*(RK2) Cm^r *loxP oriR*(ColEI) Cb^r *loxP*	53
pLAW330	pLAW317::Tn*903*dII*lacZ*, *tnpA* at MCS	53
pLAW334	*sacB oriT*(RK2) Cm^r *loxP oriR*(ColEI) Cb^r *loxP*	This study
pLAW344	*sacB* MCS *oriT*(RK2) Cm^r *loxP oriR*(ColEI) Cb^r *loxP*	53
pLAW339	*Eco*RI fragment containing Tn*903*dII*lacZ* from pAB18 in pLAW334	This study
pLAW340	*Eco*RI fragment containing Tn*903*dII*lacZ* from pAB22 in pLAW334	This study

reagents that probe bacterial structure and function, (iv) capacity to bind HL-60 cells, and (v) ability to survive and multiply within macrophages.

MATERIALS AND METHODS

Media and reagents. *L. pneumophila* was grown in AYE broth (31) and on ABCYE (16) or CAA plates (42). For electroporation, *L. pneumophila* was grown in AYE medium that lacked bovine serum albumin (BSA). Reagents, chemicals, and fetal calf serum were purchased from Sigma. Enzymes were supplied by New England Biolabs or Boehringer Mannheim Biochemicals. The β-galactosidase indicator 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) was added at 80 μg/ml to LB agar or at 0.6 mg/ml to 0.8% agar used in bacterial overlays. Antibiotics for *L. pneumophila* selection were used at the following concentrations: kanamycin (KM), 50 μg/ml; streptomycin (SM), 50 μg/ml; chloramphenicol (CM), 5 μg/ml. For *Escherichia coli* selection, antibiotics were used at the following concentrations: KM, 50 μg/ml; carbenicillin (CB), 100 μg/ml; CM, 25 μg/ml. Phosphate-buffered saline (PBS) was prepared as 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 (pH 7.2). Normal human serum (NHS) was obtained from healthy volunteers and stored in 5-ml aliquots at −80°C.

Bacterial strains. Bacterial strains and plasmids are described in Table 1. *L. pneumophila* strains were stored by growing the bacteria for 2 days in wells of a microtiter dish containing AYE medium plus 7% BSA and freezing at −80°C. Our stock of AM511, (Sm^r r$^-$) virulent derivative of *L. pneumophila* Philadelphia-1, contained bacteria with different levels of sensitivity to NaCl as determined by their plating efficiencies on ABCYE medium containing 100 mM NaCl. Strain JR32 was one of many (six of nine) single-colony isolates of AM511 that, after being purified twice on ABCYE medium, remained sensitive to NaCl-like wild-type *L. pneumophila* Philadelphia-1. *E. coli* DH5α was used for propagation of recombinant DNA plasmids.

Plasmids. pAM10 is a derivative of pMMB207 [chloramphenicol resistant (Cm^r) *oriT*(RSF1010)] that contains the *icm* and *dot* loci of *L. pneumophila* on a 12-kilobase pair (kb) DNA fragment. The plasmid complements the avirulent mutant 25D for intracellular multiplication within macrophages (36).

DNA containing Tn*903*dII*lacZ* insertions was isolated from various *L. pneumophila* mutants by cutting genomic DNA with *Eco*RI, which does not cut within the transposon,

and ligating the DNA fragments to the vector pBluescript II(KS)$^+$ (pBSK) (48), previously cut with *Eco*RI and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals). DH5α was transformed with the ligation mixture and plated on LB agar-KM-CB selecting for resistance to KM (conferred by Tn*903*dII*lacZ*) and CB (conferred by pBSK). The pBSK derivatives containing the *Eco*RI DNA fragments with the Tn*903*dII*lacZ* insertion are listed alongside the *L. pneumophila* mutant from which they were isolated in Table 2.

The vector used to mediate exchange of marked (KM resistant [Km^r]) mutations into a wild-type *L. pneumophila* background, pLAW334, is a pBR322 derivative that contains the counterselectable *sacB* gene of *Bacillus subtilis* and a marker for Cm^r. pLAW334 is identical to pLAW335 (53) except that the *sacB* gene is in the opposite orientation. The Tn*903*dII*lacZ* mutations from LELA1650 and LELA3307 were subcloned into pLAW344 from pAB18 and pAB22, respectively. The transposon-containing *Eco*RI DNA fragment was isolated, filled in with deoxynucleoside triphosphates by using Klenow fragment, and ligated to pLAW334, previously cut with *Eco*RV, to generate plasmids pLAW339 and pLAW340.

Transposon mutagenesis. *L. pneumophila* was mutated with the Tn*903* (22) derivative Tn*903*dII*lacZ* as described previously (53). Tn*903*dII*lacZ* confers Km^r and contains a 5′ truncated *lacZ* gene. The transposon-containing plasmid, pLAW330, was introduced into JR32 by electroporation (36). After electroporation, the bacteria were suspended in AYE medium, grown for 5 h at 37°C, and then plated on ABCYE-KM. Km^r transformants containing β-galactosidase activity, generated as a result of Tn*903*dII*lacZ* transposition, were identified as blue colonies after overlaying the plates with 0.8% agar containing 0.6 mg X-Gal per ml. Ninety-five percent Km^r blue and 5% Km^r white colonies were streaked on ABCYE medium to allow for the loss of Cm^r which marks DNA sequences outside of Tn*903*dII*lacZ* in pLAW330. Km^r Cm^s colonies were saved as simple Tn*903*dII*lacZ* insertion mutants of *L. pneumophila*.

Bacterial mating. The donor *E. coli* strain LW413 (LW211 containing pAM10) was grown to the mid-log phase in AYE medium on a roller drum at 37°C. Recipient *L. pneumophila* strains were taken from ABCYE plates after 48 h of growth and suspended in AYE medium to approximately 10^9 CFU/ml. Donors (10 μl) were mixed with recipients (100 μl), placed onto prewarmed ABCYE plates, and incubated for 6 to 18 h at 37°C before being plated on ABCYE-SM-CM. Colonies were purified twice by streaking on ABCYE-CM plates before being used to infect HL-60 cells.

Allelic exchange. Tn*903*dII*lacZ* mutations contained in pLAW339 and pLAW340 were introduced into JR32 by allelic exchange as previously described (53). For all Km^r Suc^r isolates tested, successful exchange of the wild type for a mutant allele occurred at frequencies of 84 and 94% with pLAW339 and pLAW340, respectively. The mutation introduced into each putative recombinant was verified by Southern blot analysis (data not shown).

Cell culture. The human leukemia cell line HL-60 (13) was maintained in RPMI 1640 medium (RPMI) supplemented with 2 mM L-glutamine (Gln) and 10% fetal calf serum. HL-60 cells were differentiated into macrophages by incubating them for 2 days with 10 ng of phorbol 12-myristate 13-acetate per ml in RPMI–2 mM Gln–10% NHS. Adherent cells were washed three times with RPMI–2 mM Gln and then incubated with RPMI–2 mM Gln–10% NHS prior to infection.

Identification of *L. pneumophila* mutants deficient in macrophage killing. *L. pneumophila* mutants generated by Tn*903*dII*lacZ* transposition (53) were individually grown in 0.1 ml of AYE medium for 2 days in a 96-well microtiter dish (Falcon 3072). Bacterial cultures (5 μl of approximately 10^8 bacteria per ml) were transferred to a second 96-well microtiter dish containing 4×10^5 differentiated HL-60 cells per well in 0.2 ml of RPMI–2 mM Gln–10% NHS. Microtiter dishes were incubated at 37°C under 5% CO_2–95% air for up to 5 days, during which the monolayer was visually examined to determine the extent of HL-60 cell killing. The degree of cell killing was compared with that in wells containing no bacteria, the parental strain JR32, and the avirulent *L. pneumophila* mutant 25D.

HL-60 cytotoxicity assay. The number of *L. pneumophila* bacteria required to kill 50% of an HL-60 cell monolayer (50% lethal dose [LD_{50}]) was determined as described by Marra et al. (37). Briefly, a 96-well microtiter dish containing 4×10^5 differentiated macrophages per well was infected with 10-fold serial dilutions of *L. pneumophila* in RPMI, starting with 10^8 bacteria. Data were collected only from experiments where differences between the input number of CFU and the number of bacterial particles were less than fourfold. After a 5-day incubation at 37°C under 5% CO_2–95% air, the dye MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] was added to each well at a concentration of 500 μg/ml. The microtiter dishes were incubated for 4 h at 37°C, the culture medium was aspirated, and the reduced formazan dye was suspended in 100 μl of 0.04 M HCl–1% sodium dodecyl sulfate in isopropanol. The A_{570} values of six wells containing the same multiplicity of infection (MOI) were averaged to determine the extent of macrophage killing. The number of bacteria that reduced the A_{570} by one-half as compared to the A_{570} of uninfected HL-60 cells was defined as the LD_{50} and determined by extrapolation from a graph (as shown in Fig. 1) displaying A_{570} as a function of the number of input bacteria.

Southern hybridization. Genomic DNA was prepared by the method of Smith et al. (50), digested with *Eco*RI, separated by electrophoresis in 0.7% agarose gels, and transferred to Hybond-N nylon membranes (Amersham) by the method of Southern (51). DNA probes were prepared by random primer labelling with [^{32}P]dCTP and the large Klenow fragment of DNA polymerase I (17). For some experiments, nonradioactive labelling and chemiluminescence detection were accomplished with Genius Kits 2 and 7 (Boehringer Mannheim Biochemicals).

Human serum sensitivity assay. *L. pneumophila* strains were grown in 0.1 ml of AYE medium for 2 days in a microtiter dish (Falcon 3072) under 5% CO_2–95% air at 37°C to a density of about 5×10^8 bacteria per ml. *E. coli* strains were grown overnight in 5 ml of AYE medium on a roller drum. Bacteria were diluted to approximately 2×10^6 bacteria per ml in AYE medium, mixed with equal volumes of PBS or NHS, and incubated for 1 h at 37°C. Bacteria were then diluted in PBS, plated on ABCYE plates, and incubated at 37°C. CFU were counted after 4 days.

Chemical sensitivity assays. *L. pneumophila* strains were grown as described above, and 0.1 ml of the cultures (about 5×10^7 bacteria) was mixed with 3 ml of the 0.8% agar (at 50°C) that was used to overlay ABCYE medium (25 ml). The compounds $CdCl_2$ (15 μg), 1-Cl-2,4-diaminobenzene (50 μg), hydrogen peroxide (30 μg), cumene hydroperoxide (40 μg), *t*-butylhydroperoxide (140 μg), methyl viologen (paraquat; 20 μg), deferoxamine mesylate (1 mg), 1,10-phenanthroline monohydrate (125 μg), Triton X-100 (100 μg), polymyxin B

TABLE 2. *L. pneumophila* Mak$^-$ mutants grouped by DNA hybridization

DNA hybridization group	Comp. by pAM10[a]	LELA strain	Relative LD_{50}[b]	*Eco*RI fragment (kb)[c]	Cloned *Eco*RI fragment[d]	Relative *lacZ* activity[e]
I	+	921	22	12		6
	+	1747	>500,000	12		5
	+	1802	>500,000	12		5
	+	2955	>500,000	12		5
	+	3118	>500,000	12		6
	+	3176	>500,000	12		4
	+	3993	100,000	12		3
	+	4004	>500,000	12		5
	−	4365	>500,000	12		4
	+	4510	>500,000	12		5
II	−	1205	>500,000	4.9		5
	−	1506	>500,000	4.9		5
	−	1984	13,000	4.9		4
	−	1996	22,000	4.9		3
	−	2517	40,000	4.9		5
	−	3244	>500,000	4.9		5
	−	3379	>500,000	4.9		3
	−	3393	>500,000	4.9		6
	−	3563	>500,000	4.9	pAB12*	3
	−	3896	>500,000	4.9	pAB20	4
III	−	3037	>500,000	11	pAB16	1
	−	3278	14,000	11		6
	−	3352	>500,000	11		5
	−	3451	>500,000	11	pAB17*	3
	−	3463	28,000	11		6
	−	3473	4,500	11		6
	−	4032	>500,000	11	pAB19	4
	−	4086	>500,000	11		5
	−	4378	10,000	11		5
IV	−	1223	>500,000	1.6	pAB15	6
	−	1566	>500,000	1.6	pAB14*	6
	−	3150	32,000	1.6		4
	−	3323	>500,000	1.6		5
V	−	2474	10,000	1.6		4
	−	2883	3,200	1.6	pAB11*	3
	−	4276	>500,000	1.6		5
VI	−	1012	>500,000	0.6	pAB13*	6
	−	2947	>500,000	0.6		4
VII	−	1650	>500,000	2.1	pAB18	5
	−	3307	>500,000	2.1	pAB22*	5
VIII	−	4333	>500,000	0.4	pAB21*	4
IX	−	4432	>500,000	1	pAB24*	5
X	−	1016	13,000	5.6	pAB23*	0
XI	−	4402	2,200	5.1	pAB31*	2
XII	−	2255	2,000	1.6	pAB29*	6
XIII	−	672	570	1.6	pAB32*	4
XIV	−	4210	180	2.1	pAB33*	4
XV	−	2062	50	4.1	pAB30*	6
XVI	−	877	40	5.1	pAB28*	2
Not grouped	−	1275	800			3
	−	3185	710			5
	−	1718	630			6
	−	4237	500			5
	−	3607	28			6
	−	3409	23			6

[a] Two loci known to be needed for macrophage killing, *icm* and *dot*, are present on pAM10.
[b] The relative LD_{50} is taken from Fig. 2.
[c] Represents genomic *Eco*RI fragment minus Tn*903*dII*lacZ* DNA sequences.
[d] Plasmids listed are derivatives of pBSK containing the *Eco*RI fragment with a Tn*903*dII*lacZ* insertion from the corresponding strain of *L. pneumophila*. Plasmids used as probes are indicated by an asterisk.
[e] On a scale of 0 (white) to 6 (dark blue), the Tn*903*dII*lacZ*-generated Mak$^-$ strains were rated visually after colonies were overlaid with agar containing X-Gal.

(1,000 U), and HCl (1.8 mg) were added in 10-μl aliquots to 6-mm paper discs (blank paper discs; BBL Microbiology Systems) previously placed on the bacterial overlays. NaCl (29.2 mg) and LiCl (21.2 mg) were added to 8-mm paper discs (Toyo Seisakusho Co., Ltd., Tokyo, Japan), which were then placed on top of the overlays. Cumene hydroperoxide, 1-Cl-2,4-diaminobenzene, and 1,10-phenanthroline monohydrate were dissolved in 95% ethanol, which alone had no

effect on the growth of *L. pneumophila*. The diameter of the zone of growth inhibition was measured after 2 days incubation at 37°C.

Other phenotypic traits. Calcofluor binding to *L. pneumophila* mutants was assayed by streaking bacteria on ABCYE and CAA plates containing 0.02% Calcofluor and comparing the colony fluorescence of 3-day-old colonies with that of parental strain JR32 under 254- and 365-nm UV light. Binding of Congo red was assayed by growing bacteria on ABCYE and CAA plates containing 0.01% Congo red and observing sequestration of the red dye. Temperature sensitivity was determined by patching *L. pneumophila* mutants onto ABCYE plates and observing differences in growth at 30, 37, and 42°C. Alkalinization of the growth medium was determined by growing bacterial strains overnight at 37°C on a roller drum and measuring the pH of the AYE medium with a pH probe (Orion). Pigment production was assessed by growing *L. pneumophila* on CAA plates and examining browning of the growth medium.

Bacterial binding assay. A modification of the method described by Horwitz (29) was used to quantitate the capacity of *L. pneumophila* to bind HL-60 cells. HL-60 cells were differentiated into macrophages in 24-mm Teflon containers (Savillex, Minnetonka, Mont.) as described (37). *L. pneumophila* strains were grown for 2 days at 37°C on ABCYE medium, scraped, and suspended in RPMI–2 mM Gln. Dilutions corresponding to 5×10^7 CFU of wild-type or mutant *L. pneumophila* were added to 24-well microtiter dishes (Falcon 3047) containing 10^7 differentiated HL-60 cells to obtain an MOI of approximately 5. The number of bacterial CFU per milliliter was verified by plating serial dilutions onto ABCYE medium. Immediately after mixing, the bacteria and HL-60 cells were incubated on a shaking platform at 200 rpm for 30 min at 37°C. Afterwards, 100-μl aliquots were transferred to 13-mm glass coverslips previously sterilized with 70% ethanol. The coverslips were incubated for 2 h at 37°C in 5% CO_2–95% air to allow for adherence of the infected HL-60 cells. The coverslips were washed three times with RPMI–2 mM Gln and then fixed by adding 3.7% Formalin in PBS for 30 min at room temperature. The coverslips were washed twice with PBS and then incubated with 0.2 ml PBS–3% BSA for 10 min to block nonspecific binding sites. The fixed cells were stained with rhodamine-conjugated rabbit anti-*L. pneumophila* antiserum (Monoclonal Technologies, Inc., Norcross, Ga.) for 30 min at room temperature, washed three times with PBS and once with water, dried, and examined by fluorescence microscopy. The extracellular bacteria were identified as red fluorescent rods associated with HL-60 cells. A total of 150 consecutive macrophages were examined for each infection, and both the number of associated *L. pneumophila* and the number of bacteria per HL-60 cell were determined.

Intracellular growth assay. HL-60 cells were differentiated into macrophages in Teflon containers as described above. After 2 days, the HL-60 cells were washed twice by suspending the cells in 1 ml of RPMI–2 mM Gln and pelleting the cells by centrifugation for 10 min at $200 \times g$. HL-60 cells were suspended in RPMI–2 mM Gln–10% NHS and placed on ice prior to infection. Dilutions of log-phase-growing bacteria were prepared in RPMI-2 mM Gln and kept on ice prior to infection. *L. pneumophila* bacteria were mixed with HL-60 cells at an MOI of 1 or less at 4°C and centrifuged in conical tubes for 10 min at $220 \times g$ and 10 min at $800 \times g$ as described by Horwitz (29). The tubes were transferred to 37°C and incubated for 90 min to allow synchronous phagocytosis of the bacteria. After uptake, the culture medium was aspirated and the pellet was washed twice as described previously. Infected HL-60 cells were suspended in RPMI–2 mM Gln–10% NHS, and 4×10^5 macrophages were transferred in 100-μl aliquots to 96-well microtiter dishes. The infected HL-60 cells were incubated at 37°C with 5% CO_2–95% air, and bacterial CFU were determined 0, 24, and 48 h after infection. CFU in the supernatants were determined by removing the culture medium, washing the monolayer twice with 100 μl of RPMI–2 mM Gln, combining the washes with the supernatant, and plating for CFU on ABCYE medium. CFU were determined in HL-60 cell monolayers by lysing the washed HL-60 cells with 100 μl of deionized H_2O and plating 10-fold dilutions on ABCYE medium. For a T of 0 h, 100-μl aliquots of infected HL-60 cells were centrifuged at $220 \times g$ for 10 min, and the supernatant was removed and plated for CFU. The HL-60 cell pellet was lysed with deionized H_2O and plated as described previously. Infections were performed in duplicate.

RESULTS

Identification of *L. pneumophila* mutants defective in killing human macrophages. Wild-type *L. pneumophila* JR32 was mutagenized with Tn*903*dII*lacZ* under conditions that generated independently derived (>96%) simple insertion mutants (53). The transposon carries a gene for KM resistance and a 5′ truncated *lacZ* gene that maintains an open reading frame throughout one end of the transposon. Upon transposition, Tn*903*dII*lacZ* can create *lacZ* translational fusions identified as Km^r colonies that turn blue in the presence of the β-galactosidase indicator X-Gal. Tn*903*dII*lacZ* was delivered from pLAW330, a ColE1 replicon that contains a marker for Cm^r and is maintained in *L. pneumophila* only under selection. The transposon donor replicon was introduced into JR32 in 108 separate electroporations, and 7,645 Km^r colonies with various levels of β-galactosidase activity were isolated. Of the 7,645 Km^r transformants, 95% had detectable β-galactosidase activity, while 5% remained white in the presence of X-Gal. The isolates were streaked onto nonselective medium to allow for the loss of pLAW330 as identified by their sensitivity to CM (Cm^s). Overall, 4,536 Km^r Cm^s mutants of *L. pneumophila* (LELA strains 1 through 4536) containing a simple Tn*903*dII*lacZ* insertion were collected.

The 4,536 mutants were individually screened for their ability to kill macrophage-like cells derived from the human leukemic myeloblast cell line HL-60 (37). The *L. pneumophila* mutants were grown for 2 to 3 days in microtiter dishes containing AYE medium and then transferred to a second set of microtiter dishes containing differentiated HL-60 cells so that the MOI was approximately 1. Five days after infection, the macrophage monolayers were visually examined to determine the extent of HL-60 cell killing. Wild-type *L. pneumophila* JR32 produced extensive fields of debris, leaving virtually no intact HL-60 cells. In contrast, bacteria that were attenuated in killing left few to many intact macrophages within a field of cell debris, while bacteria that were completely unable to kill macrophages left the monolayer intact (data not shown). From this initial analysis, 86 strains appeared to be defective in killing macrophages.

To quantitate these defects in macrophage killing, candidate strains were retested in a cytotoxicity assay that uses the reduction of the dye MTT by living cells as a measure of the number of macrophages surviving an *L. pneumophila* infection (37). The results from one representative cytotoxicity assay are shown in Fig. 1. By using a broad range of

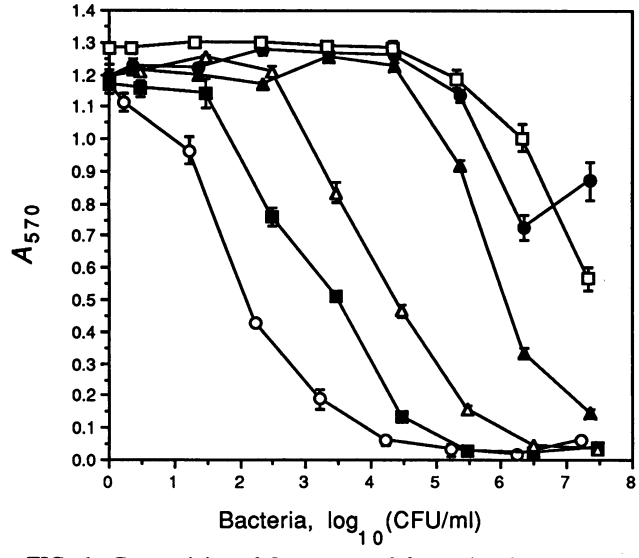

FIG. 1. Cytotoxicity of *L. pneumophila* strains for HL-60-derived human macrophages. Differentiated HL-60 cells (4×10^5 per well) were infected with 10-fold serial dilutions of bacteria to achieve final titers of 10^7 to 0 CFU/ml. After 5 days, the dye MTT was added, and the A_{570} of the blue formazan dye, an indicator of HL-60 cell survival, was measured. Wild-type *L. pneumophila* JR32 (open circles), the Tn*903*dII*lacZ*-derived Mak$^-$ mutants LELA2947 (open squares), LELA3409 (filled squares), LELA4210 (open triangles), and LELA4378 (filled triangles), and the avirulent mutant 25D (filled circles) are shown. Values are the average of six determinations. Error bars represent the standard error of the mean.

MOIs, the LD_{50} values (number of bacteria needed to kill one-half of the macrophage monolayer) of the mutants were determined and divided by the LD_{50} of the parental strain JR32 to obtain relative LD_{50} values. Because a fourfold variability existed in the determination of the LD_{50} for both mutant and parental strain JR32, any mutant displaying a 16-fold or greater relative LD_{50} value was judged to be significantly impaired in killing macrophages.

From 4,536 *L. pneumophila* mutants, 55 (1.2%) had LD_{50} values that were 16-fold greater than that of the parental strain JR32 (Fig. 2A). The mutants seemed to fall into two groups. One group (25 members) consisted of mutants that retained the ability to kill macrophages, but to a lesser extent, and had measurable LD_{50} values that were 20-fold to 10^5-fold greater than that of strain JR32. The other group (30 members) was made up of mutants that had lost the ability to kill macrophages even at MOIs of >100 and had corresponding relative LD_{50} values of $>10^5$. Both classes of mutants have been designated Mak$^-$ since their macrophage killing potential was reduced (Fig. 2C). Although the majority of the remaining candidate strains (20 out of 31) had LD_{50} values greater than that of JR32, they were still lower than the 16-fold measure of significance and were designated Mak$^+$.

Ten Mak$^-$ mutants have Tn*903*dII*lacZ* inserted within a chromosomal DNA fragment containing *icm*. Recently, the *icm* locus was shown to be required for the intracellular multiplication of *L. pneumophila* in human macrophages (36). This locus resides on a 12-kb *Eco*RI genomic DNA fragment that is contained within pAM10. Since 55 mutants had defects in HL-60 cell killing, it was anticipated that some of the Mak$^-$ mutants might contain a Tn*903*dII*lacZ* insertion within the same 12-kb *Eco*RI DNA fragment. Genomic DNA was prepared from the 55 Mak$^-$ mutants, digested with *Eco*RI, separated by gel electrophoresis, blotted onto nylon membranes, and probed with radiolabelled pAM10 sequences. Ten of the 55 Mak$^-$ mutants did not contain the

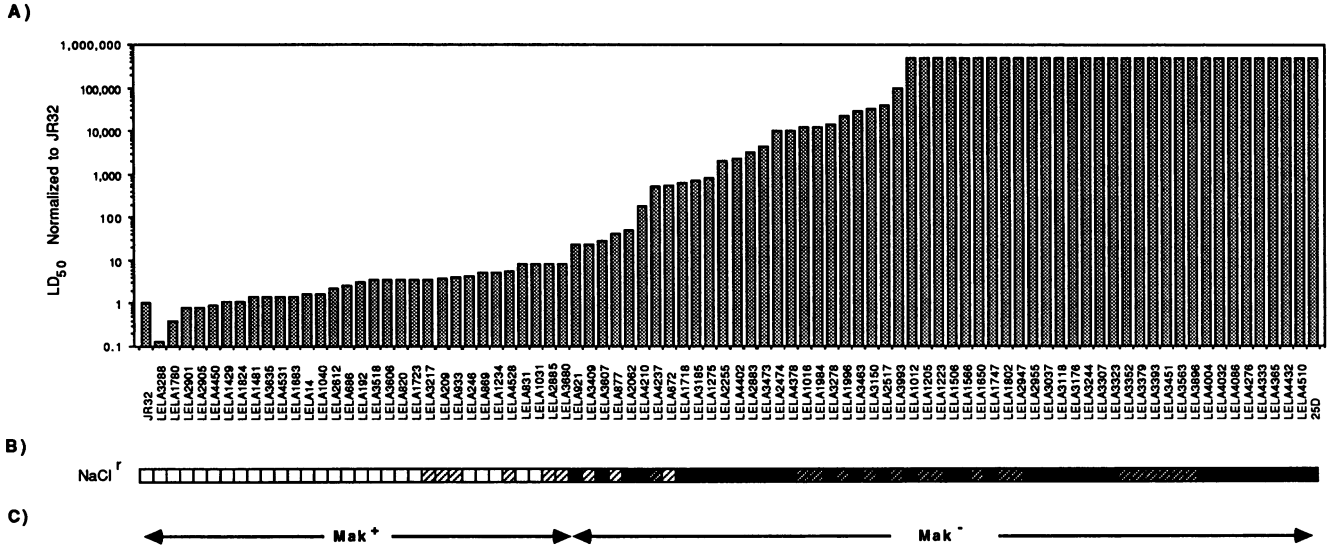

FIG. 2. Characterization of candidate Tn*903*dII*lacZ*-generated mutants of *L. pneumophila* potentially defective in macrophage killing. (A) Comparison of LD_{50} values of mutant *L. pneumophila* normalized to the parental strain JR32. LD_{50} values were extrapolated from graphs (one of which is shown in Fig. 1) displaying HL-60 cell viability as a function of the number of infecting *L. pneumophila*. The LD_{50} value for JR32 was approximately 100. (B) Sensitivity of mutants to NaCl. Sterile paper discs containing NaCl (29.2 mg) were placed on bacterial lawns, and the extent of clearing around each disc was measured after 2 days. Zones of clearing of >18 mm with no colony growth (white boxes), zones of >18 mm with dispersed colony growth (light hatched boxes), zones of <13 mm but >8 mm (dark hatched boxes), and no detectable zones of clearing (black boxes) are indicated. (C) Designation of mutants that are macrophage killing proficient (Mak$^+$) and defective (Mak$^-$).

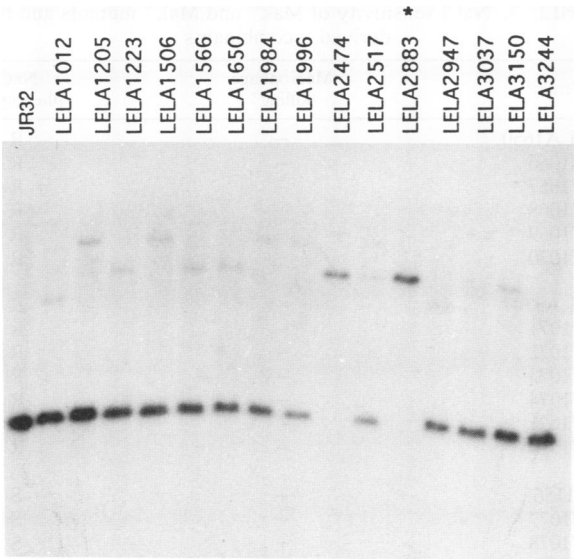

FIG. 3. Grouping of Mak⁻ mutants by DNA hybridization. An *Eco*RI genomic DNA fragment containing Tn*903*dII*lacZ* from LELA2883 (*) was isolated, and the *L. pneumophila* DNA flanking the transposon was used to probe the genomes of all 55 Mak⁻ mutants. Strain LELA2474 contains Tn*903*dII*lacZ* within the same *Eco*RI DNA fragment as LELA2883 since their lanes do not contain the 1.6-kb hybridizing genomic fragment found in the parental strain JR32. Instead, both mutants contain a 6-kb hybridizing DNA fragment that consists of the 1.6-kb *Eco*RI genomic fragment and the 4.4-kb transposon. Thus, LELA2474 and LELA2883 belong to the same DNA hybridization group (see Table 2, group V). The probe contained an additional 23 bp of Tn*903*dII*lacZ* sequences, resulting in weak hybridization with those DNA fragments containing transposon sequences.

12-kb *Eco*RI DNA fragment found in wild-type JR32 but instead contained an expected 16.4-kb hybridizing DNA species consisting of the 12-kb *Eco*RI fragment and the 4.4-kb Tn*903*dII*lacZ* (data not shown). These 10 Mak⁻ mutants were placed in DNA hybridization group I (Table 2).

Mak⁻ mutants can be divided into a minimum of 16 DNA hybridization groups. The remaining 45 Mak⁻ mutants were placed in DNA hybridization groups on the basis of Tn*903*dII*lacZ* inserting into a common DNA fragment. Initially, DNA containing the transposon was isolated as an *Eco*RI fragment from the chromosome of a Mak⁻ mutant. This fragment was cloned into the vector pBSK, and the resulting plasmid was cut with *Bam*HI and *Xba*I to separate Tn*903*dII*lacZ* and pBSK sequences from *L. pneumophila* DNA. The *L. pneumophila* DNA flanking the transposon was isolated and used to probe the genomes of all 55 Mak⁻ mutants. Mutants that had transposon insertions within the same *Eco*RI genomic DNA fragment contained a hybridizing DNA fragment that migrated 4.4 kb greater than the fragment present in the parental strain JR32.

For example, the *Eco*RI fragment containing Tn*903*dII*lacZ* was isolated from the strain LELA2883 and inserted into pBSK to form pAB11. The genomic DNA flanking the transposon in pAB11 was used to probe the genomes of the Mak⁻ mutants, a subset of which are shown in Fig. 3. Since *Eco*RI does not cut within Tn*903*dII*lacZ*, the hybridizing DNA in LELA2883 is 6 kb, 4.4 kb greater than the 1.6-kb *Eco*RI DNA fragment seen in the parental strain JR32 (Fig. 3). In fact, the pattern observed for LELA2474 was identical to that of LELA2883 (Fig. 3). Because the two mutants contained a Tn*903*dII*lacZ* insertion within the same *Eco*RI fragment, they were placed in the same DNA hybridization group. Continued probing of nylon membranes containing the rest of the Mak⁻ mutants identified one additional mutant, LELA4276, with the same hybridization profile as that of LELA2883. Thus, the strains LELA2883, LELA2474, and LELA4276 constituted one DNA hybridization group (Table 2, group V).

The transposon and flanking DNA sequences were isolated from other ungrouped mutants, and the procedure was repeated until 49 of the 55 Mak⁻ mutants were assigned to 16 DNA hybridization groups. The six remaining Mak⁻ mutants were not grouped because their transposon-containing DNA fragments were not isolated. Table 2 lists the DNA hybridization groups, their Mak⁻ members, the pBSK derivatives containing the cloned *Eco*RI fragments used as probes, the relative LD_{50} values for HL-60 cell killing, and the levels of β-galactosidase activity for each mutant. Seven of the groups (I through VII) contain multiple mutants, suggesting that the transposon insertions are responsible for the Mak⁻ phenotypes. Three groups (I, II, and III) contain 9 or 10 Mak⁻ mutants with transposon insertions contained within *Eco*RI DNA fragments of 12, 4.9, and 11 kb, respectively. The occurrence of multiple insertions suggests either that a hot spot for transposition is present or that a substantial portion of the DNA within the *Eco*RI fragment encodes information necessary for killing macrophages.

None of the Mak⁻ mutants have Tn*903*dII*lacZ* inserted within *mip*. A number of the Mak⁻ mutants (LD_{50} values, <500,000) retain some capacity to kill human macrophages, a phenotype expected for *L. pneumophila* containing null mutations in *mip*, a gene which is required for optimizing *Legionella* infections (12). To determine whether Tn*903*dII*lacZ* inserted within *mip*, genomic DNA prepared from Mak⁻ mutants representing all of the DNA hybridization groups (including ungrouped members) was probed with sequences encoding *mip* (gift of Yousef Abu Kwaik, University of Michigan). None of the Mak⁻ mutants had Tn*903*dII*lacZ* inserted within the *L. pneumophila mip* gene (data not shown), suggesting that the reduced ability of some Mak⁻ mutants to kill HL-60-derived macrophages may be the result of mutations in genes other than *mip*.

Complementation of DNA hybridization group I for macrophage killing. Marra et al. recently demonstrated that the *icm* locus contained on pAM10 complements the intracellular multiplication defect in the Mak⁻ mutant 25D (36). To determine whether pAM10 could complement any of the killing defects present in the Mak⁻ mutants, the plasmid was transferred by mating into all 55 Mak⁻ strains, the parental strain JR32, and AM240, an Sm^r r^- derivative of 25D. Since the plasmid may be lost without selection, *L. pneumophila* strains containing pAM10 were grown on ABCYE-CM plates before being used to infect macrophages at MOIs of 0.5, 10, and 200. The extent of macrophage killing was visually determined in a blind side-by-side comparison of strains containing and lacking pAM10.

Only nine of the Mak⁻ mutants showed an increased ability to kill macrophages when containing pAM10 (summarized in Table 2). The 25D derivative AM240 was also complemented, while JR32 showed no change in its ability to kill macrophages. These nine mutants are all members of DNA hybridization group I (Table 1) which contain Tn*903*dII*lacZ* insertions in the same *Eco*RI DNA fragment present in pAM10. For an unknown reason, one mutant from

this group, LELA4365, failed to be complemented by pAM10.

Phenotypic analysis of Mak$^-$ mutants. Mutants of *L. pneumophila* displaying different sensitivities to a given compound could provide insight into where in the infection cycle the Mak$^-$ mutants are blocked. For example, *L. pneumophila* requires human serum to enter macrophages by complement-mediated phagocytosis. Sensitivity to the bactericidal effects of NHS could result in a Mak$^-$ phenotype. However, none of the Mak$^-$ strains nor JR32 nor 25D showed more than a 4.3-fold reduction in CFU after incubation in 50% NHS for 1 h compared with that after incubation in PBS. In contrast, a serum-sensitive *E. coli* strain, C600K$^-$, displayed a $>10^5$ reduction in CFU when incubated with 50% NHS, while a serum-resistant *E. coli* strain, O9:K29:H$^-$, showed a 1.3-fold increase in bacterial CFU. Therefore, sensitivity to NHS does not account for the Mak$^-$ phenotype in any of the mutants.

L. pneumophila strains sensitive to oxidative damage might have reduced infectivity if they encounter toxic oxidizing compounds during macrophage infection. Sensitivity to oxidizing compounds was tested by exposing lawns of Mak$^-$ strains to paper discs containing $CdCl_2$, 1-Cl-2,4-diaminobenzene, cumene hydroperoxide, *t*-butylhydroperoxide, hydrogen peroxide, and the intracellular superoxide generating reagent paraquat. None of the Mak$^-$ mutants had zones of growth inhibition significantly larger than those of the parental strain JR32 (data not shown), suggesting that none of the Mak$^-$ mutants are any more sensitive to oxidants than the wild type.

Gamma interferon interferes with productive *L. pneumophila* infections by reducing the availability of intracellular iron within macrophages (7). A disc sensitivity assay using the iron chelators deferoxamine mesylate and 1,10-phenanthroline monohydrate was used to test the Mak$^-$ mutants for increased sensitivity to iron limitation. Again, none of the Mak$^-$ mutants appeared any more sensitive than the parental strain JR32 (data not shown), indicating that the defects in macrophage killing are not due to an increased sensitivity to iron limitation.

Additional phenotypic traits were examined in an attempt to differentiate the Mak$^-$ mutants. Calcofluor binding, used to screen *Rhizobium meliloti* strains for alterations in the exopolysaccharide needed for nodule formation (34), was similar for JR32, 25D, and the 55 Mak$^-$ strains. Likewise, binding of the dye Congo red, known to distinguish virulent from avirulent strains of *Shigella flexneri*, *Yersinia pestis*, and *Aeromonas salmonicida* (32, 39, 44, 52), was not useful in discriminating the Mak$^-$ mutants from JR32. The lipophilic reagents Triton X-100 and polymyxin B created similar zones of clearing for all of the Mak$^-$ mutants and JR32, indicating that cell membrane integrity was not grossly different. None of 55 Mak$^-$ strains was any more sensitive to growth at 42 or 30°C than JR32. In fact, all of the Mak$^-$ strains grew on minimal CAA plates, increased the pH of the growth medium from 6.9 to 8.3, and produced the brown pigment similarly to wild-type *L. pneumophila* (data not shown).

Mak$^-$ mutants are resistant to NaCl. It has long been recognized that passage of virulent *L. pneumophila* strains on suboptimal medium (supplemented Mueller-Hinton) enriches for avirulent populations (40). Indeed, an avirulent mutant, 25D, was selected by repeated passage on this medium (29). The component in supplemented Mueller-Hinton medium that suppressed growth of virulent *L. pneumophila* was shown to be NaCl (9). Not surprisingly, mutant 25D had a 93% plating efficiency on ABCYE medium containing 100 mM NaCl, while JR32 plated at an efficiency of 1.0%, similar to *L. pneumophila* Philadelphia-1 (data not shown).

TABLE 3. NaCl sensitivity of Mak$^-$ and Mak$^+$ mutants and their derived recombinants

Strain[a]	Macrophage killing[b]	NaCl phenotype[c]
LELA1650	−	R
LW1066	−	R
LW1067	−	R
LW1068	−	R
LW1069	−	R
LW1070	−	R
LELA3307	−	R
LW1071	−	R
LW1072	−	R
LW1073	−	R
LW1074	−	R
LW1075	−	R
LW1076	−	R
AB1156	+	S
LW1077	+	S
LW1078	+	S
LW1079	+	S
LW1080	+	S
LW1081	+	S
LW1082	+	S

[a] Strains containing the original Tn*903*dII*lacZ* mutation are listed first in each section. Their allelic exchange recombinants follow.

[b] The ability of *L. pneumophila* strains to kill macrophages was determined by visual inspection of the differentiated HL-60 cell monolayers 5 days after infection.

[c] NaCl sensitivity was determined by measuring the zone of growth inhibition around 8-mm paper discs containing 29.2 mg of NaCl. Sensitive (S) strains had clear zones of inhibition of >18 mm, while resistant (R) strains had zones of inhibition of <13 but >8 mm.

To determine whether resistance to NaCl is linked to the inability to kill macrophages, we tested the salt sensitivity of the Mak$^-$ and Mak$^+$ mutants. Most of the Mak$^-$ mutants were as resistant to NaCl as 25D (Fig. 2B). In contrast, most of the Mak$^+$ strains were as sensitive to salt as JR32 (Fig. 2B). This suggests that salt resistance is linked to the inability of *L. pneumophila* to kill macrophages. However, three Mak$^-$ mutants (LELA672, LELA877, and LELA3409) remained sensitive to NaCl. These Mak$^-$ mutants had relative LD_{50} values of <1,000 and do not represent the majority of the Mak$^-$ mutants which have much higher relative LD_{50} values.

To determine whether salt resistance and the inability to kill macrophages were caused by the transposon insertion, mutations from two Mak$^-$ strains (LELA1650 and LELA3307) and one Mak$^+$ strain (AB1156, a derivative of AM511 containing Tn*903*dII*lacZ*) were introduced into JR32 by allelic exchange, and the recombinants were tested for both traits. All of the recombinants derived from the Mak$^-$ strains were unable to kill macrophages and were resistant to NaCl (Table 3). In contrast, all of the recombinants derived from the Mak$^+$ strain AB1156 remained competent to kill macrophages and remained sensitive to NaCl (Table 3).

Salt resistance and the ability to kill macrophages are separate traits. The macrophage killing defect could be complemented by pAM10 in all but one member of DNA hybridization group I. To test whether pAM10 could also

restore salt sensitivity, each of these complemented mutants was examined for its sensitivity to NaCl. All of the group I mutants and AM240 (25D, Sm^r) remained salt resistant regardless of the presence or absence of pAM10 (data not shown). No changes in salt sensitivity were observed for JR32 containing pAM10. Thus, the ability of *L. pneumophila* to kill macrophages can be uncoupled from the mutation conferring salt resistance.

A subset of Mak$^-$ mutants is not defective in binding HL-60 cells. A subset of the Mak$^-$ mutants in DNA hybridization groups I through IX is unable to kill macrophages (LD_{50}, >500,000) and is resistant to salt, similar to the well characterized *L. pneumophila* mutant 25D. Mutant 25D, normal in its binding and entry into human monocytes (29), is defective only in intracellular multiplication (Icm$^-$). Unlike heat-killed *L. pneumophila*, mutant 25D persists within phagosomes of monocytes before fusing with host lysosomes (29). Like 25D, mutants in group I can be complemented for defects in macrophage killing by pAM10, a plasmid known to contain the *icm* locus (36). This suggests that mutants in group I harbor Icm defects like 25D. In contrast, Mak$^-$ mutants in groups II through IX failed to be complemented by pAM10. Since only these groups (I through IX) contained members that are similar to 25D, only representatives of these were tested for their ability to multiply within macrophages. Prior to determining their Icm phenotype, it was necessary to ascertain if this subset of mutants was defective in the ability to associate with HL-60 cells. For example, defects in macrophage binding could decrease the number of bacteria entering the host cell which would result in a decrease in the ability to kill macrophages.

The abilities of representative mutants from groups I through IX to associate with HL-60 macrophages were compared with that of JR32. Differentiated HL-60 cells were infected with *L. pneumophila* so that the average MOI was between 5 and 10. The infected HL-60 cells were then fixed, washed, and incubated with rhodamine-labelled anti-*L. pneumophila* antibodies to identify extracellular bacteria. The number of fluorescent rods found in close association with macrophages was then counted. For each strain tested, the percentage of macrophages associated with *L. pneumophila* and the average number of bacteria per macrophage containing at least one bacterium was determined for 150 consecutive HL-60 cells.

Compared with wild-type strain JR32, each of the Mak$^-$ mutants selected from groups I through IX showed a similar capacity to associate with HL-60 cells (Table 4). For HL-60 cells carrying one or more bacteria, all of the Mak$^-$ mutants and JR32 bound an average of 1.7 to 3.3 *L. pneumophila* bacteria per macrophage (Table 4). Thus, none of these Mak$^-$ mutants appeared to be defective in its ability to associate with HL-60 cells, suggesting that their defects in macrophage killing occur at a later step in the infection pathway. Because *L. pneumophila* bacteria that associate with macrophages are known to be phagocytosed efficiently, even if they are formalin- (26), glutaraldehyde-, or heat-killed (28), it is unlikely that the inability of these mutants to kill macrophages can be explained by a defect in uptake.

A subset of the Mak$^-$ mutants that bind HL-60 cells is defective in intracellular multiplication. The ability of the Mak$^-$ mutants to multiply intracellularly was determined by assaying changes in bacterial CFU during infection of HL-60-derived macrophages. The same Mak$^-$ mutants tested for macrophage association were used to infect differentiated HL-60 cells at MOIs of 1 or less. HL-60 cells and bacteria were pelleted together by centrifugation and incubated for 90 min at 37°C to allow for phagocytosis. Infected HL-60 cells were washed twice in RPMI–2 mM Gln, and 4×10^5 HL-60 cells was transferred to 96-well microtiter dishes. Bacterial CFU in culture supernatants and in washed HL-60 cell monolayers were determined separately, and CFU per whole well were determined by adding the two components.

TABLE 4. Ability of the *L. pneumophila* Mak$^-$ mutants to bind HL-60 cells

Strain	DNA hybridization group	Initial bacterial CFU/HL-60 cell[a]	% HL-60 cells associated with *L. pneumophila*[b]	Avg. no. of *L. pneumophila*/HL-60 cell with 1 or more associated bacteria
JR32		4.9	67	2.3
LELA4510	I	21	55	2.2
LELA3896	II	3.8	49	3.3
LELA4032	III	8.0	48	2.3
LELA1223	IV	8.0	50	2.1
LELA4276	V	12	54	2.4
LELA1012	VI	13	57	1.7
LELA1650	VII	11	57	2.2
LELA4333	VIII	11	45	2.1
LELA4432	IX	6.0	43	2.1

[a] Ratio of input bacteria to HL-60 cells was calculated by dividing the bacterial CFU determined on ABCYE medium by the number of HL-60 cells.
[b] The number of rhodamine-labelled *L. pneumophila* bound to HL-60 cells was determined for 150 HL-60 cells by fluorescence microscopy.

Two non-transposon-derived Mak$^-$ mutants were also included in the infection assay: *L. pneumophila* mutant 25D and the thymidine (Thy$^-$) auxotroph CS140. Mutant 25D is known to be defective in intracellular multiplication, and CS140 is unable to survive in human macrophages presumably because the intracellular environment is limiting for thymidine (42). Because both mutants are defective in macrophage killing (Mak$^-$), the assay should distinguish between defects in survival versus those in intracellular multiplication. Pilot experiments showed that differences in CFU between CS140 and 25D were not appreciable until 48 h postinfection. For this reason, bacterial CFU were determined 0, 24, and 48 h after infection.

Similarly to mutant 25D, all 10 Mak$^-$ mutants showed 2- to 100-fold decreases in CFU per whole well 48 h after infection (Table 5). The decrease in CFU per whole well paralleled the decrease in CFU per monolayer (Table 5), indicating that the diminished ability of these mutants to survive in HL-60 cells was like that of 25D. In contrast, the Thy$^-$ auxotroph (CS140) decreased about 100- to 1,000-fold in CFU per whole well 48 h after infection, compared with the 2- to 100-fold decrease seen for 25D and the selected Mak$^-$ mutants (Table 5). This approximate 10-fold difference in viability probably reflects CS140's inability to survive intracellularly as reported previously (4, 42). Wild-type JR32 was the only bacterial strain that increased in CFU in both the whole well and macrophage monolayer at both 24 and 48 h after infection (Table 5). By 48 h, the HL-60 cell monolayer was completely lysed by JR32, whereas mutant 25D, strain CS140, and the 10 Mak$^-$ mutants left the macrophage monolayers intact (data not shown).

The mutant from DNA hybridization group I, LELA4510, appeared to have the same defect as mutant 25D since it has an LD_{50} of >500,000, it was salt resistant, and its macrophage killing defect could be complemented by pAM10. The remaining mutants from groups II through IX are thought to

TABLE 5. *L. pneumophila* Mak$^-$ mutants defective in intracellular multiplication

Strain	DNA group[a]	CFU/whole well[b]			CFU/monolayer		
		T_0	T_{24}/T_0	T_{48}/T_0	T_0	T_{24}/T_0	T_{48}/T_0
JR32 (wild type)		2.5×10^3	7	412	1.7×10^3	4	288
		2.9×10^3	8	231	1.6×10^3	6	200
		3.1×10^3	2	79	2.2×10^3	2	37
		7.7×10^3	3	117	4.5×10^3	1	17
25D (*icmA1*)		1.0×10^3	0.17	0.33	3.6×10^2	0.33	0.35
		1.4×10^3	0.15	0.11	9.3×10^2	0.12	0.06
		2.7×10^4	0.06	0.06	2.3×10^4	0.05	0.09
		7.7×10^4	0.03	0.06	6.4×10^4	0.03	0.02
CS140 (*thyA*)		1.5×10^3	0.05	<0.0007	9.8×10^2	0.01	<0.001
		2.3×10^3	0.03	0.009	1.0×10^3	0.01	<0.001
		4.6×10^4	0.006	0.008	3.1×10^4	0.007	0.005
		1.1×10^5	0.002	0.0002	6.9×10^4	0.0009	0.0001
LELA4510 (*icmA2*)	I	6.0×10^2	0.39	0.31	2.8×10^2	0.61	0.46
	I	1.3×10^5	0.15	0.06	8.0×10^4	0.09	0.06
LELA3896 (*icmB*)	II	1.1×10^5	0.11	0.07	5.1×10^4	0.10	0.12
	II	8.4×10^5	0.02	0.008	2.9×10^4	0.20	0.17
LELA4032 (*icmC*)	III	5.7×10^4	0.27	0.21	2.5×10^4	0.28	0.37
	III	1.1×10^5	0.13	0.09	5.3×10^4	0.12	0.15
LELA1223 (*icmD*)	IV	1.8×10^4	0.46	0.50	1.3×10^4	0.53	0.58
	IV	7.9×10^4	0.07	0.02	2.4×10^4	0.07	0.04
LELA4276 (*icmE*)	V	1.5×10^4	0.61	0.21	1.1×10^4	0.75	0.15
	V	1.8×10^4	0.53	0.17	1.4×10^4	0.53	0.11
LELA1012 (*icmF*)	VI	1.5×10^4	0.05	0.09	1.1×10^4	0.04	0.03
	VI	1.9×10^4	0.06	0.07	1.1×10^4	0.04	0.03
LELA1650 (*icmG*)	VII	7.4×10^2	0.65	0.04	4.9×10^2	1.43	0.02
	VII	1.6×10^3	0.49	0.38	8.7×10^2	0.48	0.61
LELA4333 (*icmH*)	VIII	8.5×10^2	1.1	0.54	4.8×10^2	1.00	0.85
	VIII	9.3×10^2	0.73	0.59	5.9×10^2	0.71	0.73
LELA4432 (*icmI*)	IX	1.6×10^4	0.34	0.55	5.3×10^3	1.58	1.70
	IX	2.6×10^4	0.38	0.38	1.3×10^4	0.50	0.72

[a] DNA hybridization groups are based on the location of Tn*903*dII*lacZ* (see Table 2).
[b] This value is the sum of the CFU in the infected culture supernatant and the CFU in the HL-60 cell monolayer.

represent additional *icm* loci because they did not appear to multiply within human macrophages.

DISCUSSION

L. pneumophila is a facultative intracellular pathogen that subverts many natural host defense mechanisms, enabling it to cause disease in man. Like *Mycobacterium tuberculosis* (1), *Mycobacterium microti* (35), *Chlamydia psittaci* (20), and *Toxoplasma gondii* (33), *L. pneumophila* infects human cells, surviving and multiplying within specialized vacuoles that do not fuse with host secondary lysosomes (26). To identify the genes that allow *L. pneumophila* to effectively parasitize human macrophages and monocytes, the bacterial chromosome was mutated with Tn*903*dII*lacZ*, a derivative of Tn*903* shown to transpose efficiently and randomly in *L. pneumophila* (53). Tn*903*dII*lacZ* encodes a selectable Km^r gene and can generate *lacZ* translational fusions which can be used to identify transposition events in *L. pneumophila*.

From 4,536 independently derived Tn*903*dII*lacZ* mutants of *L. pneumophila*, 55 had defects in their ability to kill macrophages (Mak$^-$). These defects were quantitated in a cytotoxicity assay and found to range from a slight reduction to a total lack in the ability of the bacteria to kill macrophages. The mutants were grouped by the chromosomal site at which the transposon inserted. Forty-nine of the mutants could be assigned into 16 DNA hybridization groups on the basis of which common *Eco*RI genomic DNA fragment contained Tn*903*dII*lacZ*. Six mutants were not grouped, but may constitute six additional DNA groups since their transposon-containing *Eco*RI fragments migrate with different mobilities in agarose gels (data not shown). Each of the 55 Mak$^-$ mutants most likely contains unique Tn*903*dII*lacZ* mutations since they were all obtained from independent electroporations.

How many genes are needed for *L. pneumophila* to kill macrophages? If the number of DNA hybridization groups reflects the number of genes needed to kill macrophages, then it appears that many determinants are needed for *L. pneumophila* to parasitize its host. Additionally, it is possi-

ble that multiple genes needed to kill macrophages are present on a single *Eco*RI DNA fragment (>2 kb) that defines a DNA hybridization group, like *dot* and *icm* in group I. Moreover, the 55 Mak$^-$ mutants may not represent the total number of loci required to kill macrophages but probably do represent a significant fraction of the genes specifically required for intracellular growth rather than growth on bacteriological media. Nevertheless, some genes may not be equally represented among the Mak$^-$ mutants. First, *L. pneumophila* genes that are "cold spots" for Tn*903* transposition might have been missed. Second, mutations that inactivate genes which encode functions required for growth on ABCYE agar would not have been recovered. Third, genes which encode secreted or membrane proteins may be underrepresented because some of these hybrid proteins may confer a growth disadvantage to the bacteria (46). This, however, should not be a serious concern because only a subset of these *lacZ* hybrids would result in this phenotype. Finally, since few (5%) LacZ$^-$ Km^r Cm^s transposon insertion mutants were collected, genes which are expressed only during macrophage infection may have been overlooked. This is also unlikely because X-Gal is sensitive enough to detect basal levels of expression for many genes (41).

The actual number of genes needed to kill macrophages may be overestimated by the number of DNA hybridization groups. First, some groups (VI and VIII) have mutants in which the transposon inserted into small (600 bp or less) *Eco*RI DNA fragments. These DNA fragments may be contiguous with genes already represented by other groups. Second, many groups (IX through XVI and the ungrouped members) consist of one mutant that maintains some capacity to kill macrophages. For these mutants, it is not yet known if the Mak$^-$ mutation is linked to the site of the transposon insertion.

Salt resistance has been correlated with the conversion of *L. pneumophila* to avirulence. Salt-resistant avirulent variants can arise by repeated passage of *L. pneumophila* on presumably nonselective ABCYE medium (8, 40). To determine the relationship between the inability to kill macrophages and the acquisition of salt resistance, it was important to start with a virulent strain of *L. pneumophila* that was amenable to genetic manipulation and yet retained its sensitivity to salt. The *L. pneumophila* strain mutagenized in this study was JR32, a salt-sensitive isolate of the Sm^r r$^-$ virulent strain AM511 (36).

Almost all of the Mak$^-$ mutants identified were resistant to salt, similar to mutant 25D. Resistance to salt was caused by the transposon insertion and not by the repeated passage of *L. pneumophila* mutants on ABCYE medium which was necessary to obtain pure independent isolates. This was supported by two observations. First, Tn*903*dII*lacZ* insertion mutants that were able to kill macrophages (Mak$^+$) remained salt sensitive. Second, introduction of Tn*903*dII*lacZ* mutations from two Mak$^-$ strains into JR32 reestablished salt resistance. Transfer of a Tn*903*dII*lacZ* mutation from a salt-sensitive Mak$^+$ strain into JR32 did not alter its sensitivity to salt. This suggests that the many loci in *L. pneumophila* needed to kill macrophages are either closely linked to genes conferring salt sensitivity or they are one and the same.

While salt resistance and macrophage killing appear to be linked, they seem to be phenotypically distinct. Mak$^-$ mutants from DNA hybridization group I could be complemented to Mak$^+$ by the introduction of a plasmid (pAM10) containing the virulence locus *icm*. These complemented mutants remained resistant to salt, demonstrating that salt resistance cannot be used as an absolute indicator of avirulence or the inability to kill macrophages. The data also suggest that genes conferring both salt sensitivity and macrophage killing are distinct traits but are coordinately expressed. Alternatively, a formal possibility exists that salt sensitivity and macrophage killing are conferred by one gene and that group I mutants harboring Tn*903*dII*lacZ* mutations are dominant for salt resistance but recessive for macrophage killing when pAM10 is present. This seems unlikely, however, since transposon insertions in the *Eco*RI fragment that defines group I mutants are dispersed (data not shown), and the probability of obtaining such unique dominant mutations at nine different locations would seem rare.

That traits for salt sensitivity and macrophage killing are tightly linked and conferred by many loci may explain two characteristics of *L. pneumophila*. First, avirulent derivatives of *L. pneumophila* are easily obtained after being passed on suboptimal medium containing NaCl. Salt-resistant mutants of *L. pneumophila* that were able to grow on this medium would inevitably contain macrophage killing defects. Second, virulence conversion of *L. pneumophila* is thought to be a one-way phenomenon (8). Catrenich and Johnson found that avirulent populations of *L. pneumophila* isolated from suboptimal medium containing salt never regained their ability to cause disease in guinea pigs (8). These mutants may have accumulated multiple salt resistance mutations at dispersed genomic sites, making reversion of the macrophage killing defects very unlikely.

All of the Mak$^-$ mutants that have relative LD_{50} values of >500,000 are resistant to salt, similar to the avirulent mutant 25D. This mutant was shown to be taken up by monocytes and placed in a phagosomal compartment where it failed to multiply, yet persisted for hours, up to 24 h postinfection (29). Because of the similarity between 25D and these Mak$^-$ mutants, representative mutants (one from each DNA hybridization groups I through IX) were assayed for their ability to associate with and multiply within human macrophages. Compared with the parental strain JR32, this subset of Mak$^-$ mutants showed no overall difference in its capacity to bind to or associate with HL-60 cells. In addition, these same Mak$^-$ mutants displayed no demonstrable increase in CFU during infection of HL-60 cells. This contrasts with *act* mutants of *Listeria monocytogenes*, which replicate intracellularly but fail to form plaques on L929 fibroblast cells (14). Thus, the Mak$^-$ mutants in the 10 DNA hybridization groups seem to possess an intracellular multiplication (Icm) defect like mutant 25D, indicating that there are many *L. pneumophila* genes involved in intracellular multiplication.

Mutants that display reduced levels of macrophage killing (LD_{50}, <500,000) may affect steps in the infection cycle other than intracellular multiplication. For example, mutations in *mip* impair the ability of *L. pneumophila* to infect macrophages and yet retain the ability to kill (12). Interestingly, none of the 55 Mak$^-$ mutants contained Tn*903*dII*lacZ* insertions within *mip*. However, a few Mak$^-$ mutants that retain some ability to kill macrophages belong to DNA hybridization groups (I through V) that contain mutants that are not only unable to kill but are also unable to multiply within macrophages. These mutants may have partial Mak$^-$ or Icm$^-$ defects and may be useful in assigning function to the wild-type genes.

Mutants of *Salmonella typhimurium* that exhibited decreased survival in mouse macrophages had easily detectable physiological defects such as auxotrophies and increased sensitivity to oxidizing compounds and serum (19). In contrast, none of the Mak$^-$ mutants exhibited any of

these characteristics. Their defects, however, may be in the prevention of phagosome-lysosome fusion, the prevention of phagosome acidification, or, as has been suggested for the *dot* gene (4), organelle recruitment. Alternatively, the mutants may be defective in a metabolic pathway that is important for intracellular multiplication. Further characterization of macrophages infected with the Mak$^-$ mutants should address these points.

In addition to identifying *L. pneumophila* genes required for killing macrophages, the Mak$^-$ mutants may be useful for identifying analogous genes in genetically recalcitrant intracellular pathogens like *M. tuberculosis*, *Mycobacterium leprae*, *C. psittaci*, and *T. gondii*. Furthermore, it is known that *L. pneumophila* infections are acquired from the reservoir of bacteria living in association with protozoa. Since the parasitism of these freshwater organisms by *L. pneumophila* parallels the infection of human macrophages in many respects (18), the Mak$^-$ loci may also be needed to perpetuate this infectious reservoir.

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