短　報

Legionella pneumophila の *Acanthamoeba* 内増殖を調べる定性検査法（アメーバ寒天法）の開発

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（平成14年12月6日受付）
（平成15年1月28日受理）

Key words：　*Legionella pneumophila*, *Acanthamoeba culbertsoni*, virulence

〔感染症誌　77：343～345，2003〕

序　文

Legionella pneumophila が病原性を発揮するうえで最も重要な性質は細胞内増殖能と考えられている[1]．近年，この細胞内増殖を支配する *L. pneumophila* の遺伝子群が明らかにされ *Icm/Dot* と名付けられた[2][3]．これらの遺伝子群は本菌の *Acanthamoeba*（以下アメーバと略す）内増殖にも必須な遺伝子であることも明らかにされた[4]．この知見は菌株のアメーバ内増殖能を評価すれば，その菌株の病原性を評価することが可能であることを示唆している．菌株のアメーバ内増殖能を簡便に評価出来る定性法を考案すれば，多数の環境分離株の病原性を簡単に調べることができるので有益と考えられる．

材料と方法

供試菌；強毒株として臨床分離株である *L. pneumophila* Philadelphia-1（血清群［SG］1，ATCC 33152），Nagasaki-80045（SG1），Togus-1（SG2，ATCC 33154），EY 3702（SG3），GIFU 11491（SG 4），GIFU 11395（SG5）を用いた．これらの菌株は全てモルモットMφ内で増殖できる[5][6]．一方，弱毒株としてモルモットMφ内で増殖できない *L. pneumophila* 25D（SG1）[7]，LELA 4086（SG1）[4]を使用した．Gil and Shuman の方法[4]に準じこれらの菌株のアメーバ内増殖を経時的に測定した．また，ヒメネツ染色による形態学的観察も経時的に行った．アメーバ寒天培地の作製；*Acanthamoeba culbertsoni* GS89-1001（鶴原　喬博士より供与）をAC buffer[4]に懸濁し，約 1×10^6 cells/ml の濃度に調整後，その3mlをBCYE培地に重層した．シャーレを適度に揺らしてアメーバ懸濁液が培地表面全体を覆うようにしたのち，3時間，30℃で培地を静置した．その後，培地表面に付着しなかったアメーバと培地に吸収されなかったAC bufferを吸引除去した．アメーバがBCYE培地の表面全体に付着した培地を「アメーバ寒天培地」と名付けた．培養；供試菌株の単一集落を滅菌爪楊枝で穿刺し釣菌した後，まずBCYE培地に接種し，次にアメーバ寒天に接種した．その後，30℃で6日間静置培養した後，BCYE培地とアメーバ寒天培地上での集落形成を比較・観察した．また，アメーバ寒天培地上でアメーバに捕食されなかった非被食菌が集落を形成できるかを調べるため，アメーバ内増殖能を欠く *L. pneumophila* 25Dの濃厚菌液（MacFarland No4）を作成し，その5μlをBCYE培地とアメーバ寒天培地にそれぞれスポットし

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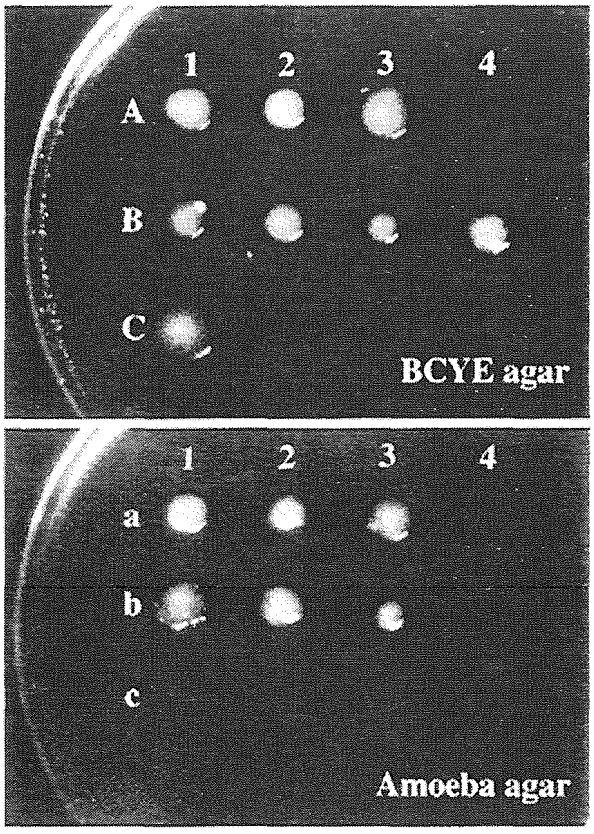
平成15年5月20日

Fig. 1 Colony formation of *L. pneumophila* on BCYE (upper) and amoeba (below) agar plates. A1 and a1 ; strain Philadelphia-1, A2 and a2 ; strain Nagasaki 80045, A3 and a3 ; strain Togus-1, A4 and a4 ; no bacteria inoculated, B1 and b1 ; strain EY 3702, B2 and b2 ; strain GIFU 11491, B3 and b3 ; strain GIFU 11395, B4 and b4 ; strain 25D, C1 and c 1 ; strain LELA4086.



た. 30℃ で 6 日間培養した後, 対象の BCYE 培地とアメーバ寒天培地上での集落形成を比較した.

結　果

6 株の強毒株はアメーバ内で増殖し, 感染 2 日後には 100～500 倍に増殖した. ヒメネツ染色でも増殖像が観察された. これらの強毒株は BCYE 寒天培地, アメーバ寒天培地の両者で集落形成が観察された(Fig. 1). 一方弱毒株 2 株はいずれもアメーバ内で増殖できなかった. これらの弱毒株では BCYE 寒天培地のみで集落が観察され, アメーバ寒天培地では集落を形成することができなかった(Fig. 1 ; B4 と b4, C1 と c1). また, アメーバ寒天培地に弱毒株を多量に接種した場合, 培養 2 日後に非被食菌による微小な集落が形成された. しかし, 培養 6 日後にはこの集落は完全に消失し, 消失した微小集落の跡にはアメーバの増殖による培地表面の円状混濁が観察された.

考　察

L. pneumophila の病原性の解析にはヒトや動物由来の培養細胞などが利用されてきた[1]. *Acanthamoeba* はこれらの細胞に比べ乾燥に強く, また固相に付着する性質があることに着目し, 本研究を行った. その結果, BCYE 寒天培地のみで集落が観察され, アメーバ寒天培地で集落が観察されない場合は調べた菌株はアメーバ内での増殖能を失った菌株と判断できること, アメーバ内増殖能を欠く菌株は, たとえ非被食菌が多くてもアメーバ寒天上で集落を形成できないこと, それゆえ BCYE 寒天培地, アメーバ寒天培地の両者で集落が観察された場合は, その菌株はアメーバ内で増殖能を持つと判断できること, が明らかになった. 以上の結果はアメーバ寒天法が分離株のアメーバ内増殖能を調べる定性法として有効であり, 多数の *L. pneumophila* 環境分離株の病原性を評価する際のスクリーニング方法として有益であることを示していると考えられる.

謝辞 : 本研究は平成 14 年度厚生労働科学研究費補助金(研究課題 : 生活環境におけるレジオネラ感染予防に関する研究)の支援を受けて行われた. *Acanthamoeba culbertsoni* GS89-1001 を分与頂きました鶴原　喬博士に感謝申し上げます.

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A Simple Qualitative Assay for Intracellular Growth of *Legionella pneumophila* within *Acanthamoeba culbertsoni*

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平成15年 5 月20日

JOURNAL OF BACTERIOLOGY, Nov. 2003, p. 6712–6718 Vol. 185, No. 22
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.22.6712–6718.2003

Virulence Conversion of *Legionella pneumophila* by Conjugal Transfer of Chromosomal DNA

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Received 22 January 2003/Accepted 19 August 2003

In this study, we examined whether virulence conversion occurs in *Legionella pneumophila* by conjugal transfer of chromosomal DNA. A virulent strain, K6, which has the genes for Km^r and LacZ$^+$ transposed in the chromosome of strain Philadelphia-1, which belongs to serogroup 1, was used as one parent, and an avirulent strain, Chicago-2S, which is a spontaneous streptomycin-resistant derivative of strain Chicago-2 belonging to serogroup 6, was used as the other parent. Experiments in which K6 (approximately 2.6 × 10^9 CFU) and Chicago-2S (approximately 8.9 × 10^9 CFU) were mated typically yielded 10^3 Km^r Sm^r LacZ$^+$ transconjugants. Thirty-two (about 2.8%) of 1,152 transconjugants belonging to serogroup 6 acquired the ability to grow intracellularly in *Acanthamoeba castellanii* and guinea pig macrophages. When guinea pigs were infected with sublethal doses of *Legionella* aerosols generated from one of these transconjugants (HM1011), they developed a severe pneumonia similar to that caused by donor strain K6. These results show that avirulent strain Chicago-2S changed into virulent strain HM1011 through conjugation with virulent strain K6. Furthermore, we showed that *Legionella* chromosomal virulence genes (*icm-dot* locus) were horizontally transferred by the conjugation system. The chromosomal conjugation system may play a role(s) in the evolution of *L. pneumophila*.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular bacterium that can grow in human monocytes (8) and alveolar macrophages (17) and in the macrophages of guinea pigs (10). Two unlinked 20-kb regions of genes required for human macrophage killing and intracellular multiplication have been identified on the *L. pneumophila* chromosome; they have been designated *icm* (intracellular multiplication) (11, 21) and *dot* (defect in organelle trafficking) (2). Region I contains seven genes (*icmV*, *-W*, and *-X* and *dotA*, *-B*, *-C*, and *-D*) (1, 5, 27, 28), and region II contains 16 genes (*icmT*, *-S*, *-R*, *-Q*, *-P*, *-O*, *-N*, *-M*, *-L*, *-E*, *-G*, *-C*, *-D*, *-J*, *-B*, and *-F*) (1, 18, 22, 23, 24). All of these genes have also been shown to be required for intracellular growth in *Acanthamoeba castellanii* (25).

We previously reported on chromosomal conjugation as a novel DNA transfer system in *L. pneumophila* (14). In brief, virulent strain K6, which has the genes for Km^r and LacZ$^+$ transposed in the chromosome of strain Philadelphia-1 and belongs to serogroup 1, was used as one parent, and avirulent strain Chicago-2S, which is a spontaneous streptomycin-resistant derivative of strain Chicago-2 and belongs to serogroup 6, was used as the other parent. Experiments in which K6 (approximately 2.6 × 10^9 CFU/ml) and Chicago-2S (approximately 8.9 × 10^9 CFU/ml) were mated typically yielded 10^3 CFU of Km^r Sm^r LacZ$^+$ recombinants per ml, corresponding to a transfer frequency of 10^{-6} per parent. All of the recombinants tested (100 of 100) belonged to serogroup 6, which is the same serogroup as strain Chicago-2S. Genotyping of both parents and their recombinants by repetitive-element PCR, arbitrarily primed PCR, and pulsed-field gel electrophoresis (PFGE) revealed an asymmetric role for each parent; that is, strain K6 is a donor, and strain Chicago-2S is a recipient. In addition, Southern hybridization analysis made clear that chromosomal genes transferred from K6 were integrated into the chromosome of Chicago-2S by homologous recombination. We speculated that some of the genes responsible for the growth of strain K6 within amoebae or macrophages might be transferred to strain Chicago-2S and that they might be integrated into the Chicago-2S chromosome by recombination. If this were so, some transconjugants would be able to grow within amoebae or macrophages. In this study, we examined whether virulence conversion occurs in *L. pneumophila* by conjugal transfer of chromosomal DNA.

The *L. pneumophila* strains and plasmids used in this study are listed in Table 1. *L. pneumophila* was grown in BYE broth (19) and on BCYE agar plates (6). Antibiotics for *L. pneumophila* selection were used at the following concentrations: kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; rifampin, 100 μg/ml; chloramphenicol, 5 μg/ml; gentamicin, 50 μg/ml. *L. pneumophila* strain Chicago-2 or its derivative, Chicago-2S, was avirulent, although strain Chicago-2 was first isolated from a patient's lungs (12). Chicago-2 has lacked the ability to grow intracellularly in macrophages, but we do not know why this is so. There was no large deletion in the *icm-dot* genes of strain Chicago-2S (unpublished observation), and there was no severe defect in its type II secretion system (unpublished observation). Even when it was inoculated (8.4 × 10^8 CFU) into guinea pigs intraperitoneally, the bacterium could not be isolated from the guinea pig spleens on day 3 or 4 after infection.

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

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
25D	Avirulent mutant of Philadelphia-1; Icm$^-$	9
AM511	Philadelphia-1 Sm^r r$^-$ m$^+$	12
Chicago-2	Avirulent, serogroup 6; Icm$^-$	ATCC 33215
Chicago-2S	Streptomycin-resistant derivative of Chicago-2	14
Chicago-2SR	Rifampin-resistant derivative of Chicago-2S	This study
GS3001	JR32 *icmS3001*::Km^r (*icm-dot* region II)	22
GS3008	JR32 *icmM3008*::Km^r (*icm-dot* region II)	24
GS3010	JR32 *icmK3010*::Km^r (*icm-dot* region II)	24
HM1011–HM1013	Recombinants between K6 and Chicago-2S; Icm$^+$ serogroup 6	14
HM1014	Recombinant between K6 and Chicago-2S; Icm$^-$ serogroup 6	14
HM1042–HM1044	Recombinants between K6 and Chicago-2S; Icm$^-$ serogroup 6	14
HM1134	LELA3473 with pGS-Lc32	This study
HM1138	LELA3393 with pMW100	This study
HM1156–HM1164	Recombinants between LELA2883 and Chicago-2SR; Icm$^-$ serogroup 6	This study
HM1175	Chicago-2SR with pMMB207C	This study
JR32	Salt-sensitive isolate of AM511	21
K6	Philadelphia-1 chromosome::Tn*903*dII*lacZ*	14
LELA2883	JR32 *dot* B2883::Tn*903*dII*lacZ* (*icm-dot* region I)	21
LELA2883–28	LELA2883 with Gm insertion instead of *lvh* region	26
LELA3118	JR32 *dotA3118*::Tn*903*dII*lacZ* (*icm-dot* region I)	21
LELA1747	JR32 *icmV1747*::Tn*903*dII*lacZ* (*icm-dot* region I)	21
LELA4004	JR32 *icmX4004*::Tn*903*dII*lacZ* (*icm-dot* region I)	21
LELA3473	JR32 *icmR3473*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA3463	JR32 *icmO3463*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA3352	JR32 *icmP3352*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA4032	JR32 *icmO4032*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA4432	JR32 *icmE4432*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA4432–28	LELA4432 with Gm insertion instead of *lvh* region	26
LELA3244	JR32 *icmD3244*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA3393	JR32 *icmB3393*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
LELA1718	JR32 *icmF1718*::Tn*903*dII*lacZ* (*icm-dot* region II)	21
MW635	JR32 *icmG635*::Km^r (*icm-dot* region II)	18
MW656	JR32 *icmJ656*::Km^r (*icm-dot* region II)	18
Philadelphia-1	Virulent, serogroup 1	ATCC 33152
Plasmids		
pGS-Lc-32	*icmTSRQPO* in pMMB207	22
pGS-Lc-47	*icmNMLKEG* in pMMB207αb	24
pLAW317	*rpsL MCS*[a]*oriT*(*RK2*) Cm^r *loxP oriR*(ColE1) Ap^r *loxP*	30
pLAW330	pLAW317::Tn*903*dII*lacZ tnpA*(Tn*903*) *oriR*(F1)	30
pMMB207	RSF1010 derivative; IncQ *lacI*^{q} Cm^r P_{tac} *oriT*	16
pMMB207αb	pMMB207 containing MCS (α complementation)	22
pMMB207C	pMMB207 with 8-bp insertion in *mobA*; Mob$^-$	G. Segal and H. A. Shuman, unpublished data
pMW100	*icmGCDJBF* and *tphA* in pMMB207	18

[a] MCS, multiple cloning site.

Growth and maintenance of *A. castellanii* ATCC 30324 in Proteose Peptone-yeast extract-glucose medium (4, 15) in 75-cm^2 tissue culture flasks were performed as previously described (4, 15).

Intracellular growth of transconjugants in *A. castellanii*. The ability of transconjugants to grow within *A. castellanii* was screened by a spot assay in which 10^6 amoebae were spread on a BCYE plate for screening of the intracellular growth of transconjugants in amoebae. Individual colonies of *L. pneumophila* were spotted with a toothpick onto a BCYE plate with nothing added and onto a second BCYE plate on which amoebae had been spread. The plates were incubated at 28°C for 4 to 5 days and then visually inspected for the growth of each spot of *L. pneumophila*. Strains Philadelphia-1 and JR32 grew equally well on both plates. Strains Chicago-2 and 25D, which are unable to grow within *A. castellanii*, did not form visible growth on the BCYE plate spread with amoebae. Thirty-two (about 2.8%) of 1,152 transconjugants belonging to serogroup 6, that is, the same serogroup as Chicago-2S, formed visible growth on the BCYE plate spread with amoebae. Serogroups of transconjugants were determined by slide agglutination tests with monoclonal (Monoclonal Technologies, Inc., Atlanta, Ga.) or polyclonal (Denka Seiken Co., Ltd., Tokyo, Japan) antibodies against serogroups 1 and 6. All of these 32 strains belonged to serogroup 6. On the basis of these results, to evaluate the intracellular growth in the amoebae quantitatively, seven transconjugants originating from three separate conjugation experiments were selected from the 1,152 transconjugants and used for experiments. Strains HM1011, HM1012, and HM1013 formed visible colonies on amoeba-

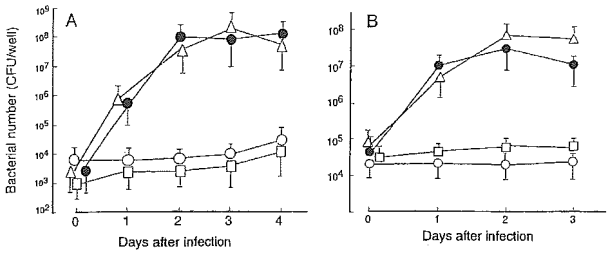

FIG. 1. Time course of intracellular growth of *L. pneumophila* strains in *A. castellanii* (A) and in peritoneal macrophages of guinea pigs (B). The experiments were done at least three times, and the data are means ± standard errors. Symbols: ●, K6; ○, Chicago-2S; △, HM1011; □, HM1014.

containing agars as well as strain K6 did, and the other strains (HM1014, HM1042, HM1043, and HM1044) did not grow on the agar plates as well as Chicago-2S did. Intracellular growth assays were performed as previously described (15), with some modifications. *L. pneumophila* was added at a multiplicity of infection of 10 to an adherent monolayer of 1.5×10^5 amoebae. After incubation for 30 min at 37°C to allow for infection, the wells were washed three times with 0.5 ml of Ac buffer (15). A sample of the infection supernatant was removed once every 24 h for 4 days. Numbers of CFU of extracellular bacteria on BCYE plates were quantified. As the Ac buffer does not support the growth of *L. pneumophila*, the CFU represent bacteria that have grown within the amoebae. As shown in Fig. 1A, strain HM1011 grew in the amoebae approximately 10^5-fold in 2 days, i.e., as well as K6 did, while strain HM1014 did not grow in the amoebae as well as Chicago-2S did. Although the data are not included here, we observed that strains HM1012 and HM1013 grew in the amoebae as well as strain K6 did, while strains HM1042, HM1043, and HM1044 did not grow in the amoebae as well as strain Chicago-2S did.

Intracellular growth of transconjugants within guinea pig macrophages. It was confirmed by light microscopic observation of Gimenez-stained macrophages infected with the 32 transconjugants that all of the transconjugants could grow in guinea pig macrophages as well. Quantitative assay of intracellular growth in guinea pig macrophages (Fig. 1B) was performed with the same strains as described in Fig. 1A. The intracellular growth of *L. pneumophila* in peritoneal macrophages from guinea pigs was examined after in vitro phagocytosis. Peritoneal exudate cells were collected by lavage of the peritoneal cavities of guinea pigs 4 days after intraperitoneal injection of 10 ml of 10% Proteose Peptone (Difco). The preparation of peritoneal macrophage monolayers from the peritoneal exudate cells, in vitro phagocytosis, and the microscopic observation of Gimenez-stained macrophage monolayers were carried out as described previously (13). *L. pneumophila* was added at a multiplicity of infection of 10 to monolayers of 10^6 macrophages. After incubation for 1.5 h at 37°C to allow for in vitro phagocytosis, the wells were washed three times with sterile phosphate-buffered saline (PBS; 136.8 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_3PO_4, 1.5 mM KH_3PO_4) to remove nonphagocytosed bacteria. The infected macrophages were incubated at 37°C in a CO_2 incubator, and bacterial CFU were determined 0, 24, 48, and 72 h after infection. The number of CFU in a whole well was determined by removing the culture medium, adding 0.1 ml of sterile water to the cell monolayer, combining the two fractions, and plating samples on BCYE plates. Strain HM1011 grew approximately 10^3-fold in the macrophages in 2 days, i.e., as well as strain K6 did, while strain HM1014 did not grow in the macrophages as well as Chicago-2S did (Fig. 1B). Although the data are not included here, strains HM1012 and HM1013 grew in the macrophages as well as strain K6 did, while strains HM1042, HM1043, and HM1044 did not grow in the cells as well as strain Chicago-2S did. These results show that some of the genes on the strain K6 chromosome that are responsible for its growth within amoebae and macrophages are transferred to Chicago-2S, and they are integrated into the Chicago-2S chromosome. These results strongly suggest that avirulent strain Chicago-2S may change into a virulent strain.

Infection of guinea pigs by *Legionella* aerosols. To assess the virulence of *L. pneumophila* strains, guinea pigs were infected with sublethal doses (approximately 10^5 CFU) of *Legionella* aerosols generated from a suspension of strain K6, Chicago-2S, or HM1011. Female guinea pigs of the outbred Hartley strain, weighing 250 to 350 g, were purchased from Shizuoka Experimental Animals (Hamamatsu, Japan). The protocols of the animal experiments were approved by the institutional animal care committee of the University of Occupational and Environmental Health. *L. pneumophila* strains were harvested from BCYE plates after 48 h of growth and suspended in BYE medium. The cultures were grown at 37°C in a shaking incubator to late log phase. The bacteria were recovered by centrifugation, washed twice with sterile PBS, and then suspended at approximately 5×10^8 CFU/ml in sterile PBS, which corresponds to a sublethal dose. *Legionella* aerosols were generated from the bacterial suspensions by an ultrasonic nebulizer (NE-U12; OMRON Co., Ltd., Tokyo, Japan). We confirmed that about 70% of the aerosols generated by the nebulizer resulted in less than 6 μm reaching alveoli and that ultrasonication by the nebulizer for 30 min did not affect the numbers of CFU in the bacterial suspension set in the machine (data not shown). The aerosols generated were transported at 7 liters/min for 15 min by a vacuum pump into an all-glass metabolic chamber (volume, 7 liters; Shibata Scientific Technology, Ltd., Tokyo, Japan) into which animals were put. The vacuum speed (7 liters of air per min) was chosen to keep constant concentrations of *Legionella* aerosols in the chamber during exposure. After exposure to *Legionella* aerosols, the body weight and rectal temperature of each animal were measured daily. The left lungs were harvested aseptically on days 0 (2 h after infection), 2, 4, and 7 postinfection, and their homogenates were cultured quantitatively on BCYE agar. The right lungs were fixed in 10% formalin for more than 2 weeks before dissection. Three or four of these sections were stained with hematoxylin and eosin and examined microscopically. Animals infected with strain K6 or HM1011 became febrile 2 days after infection. Their body temperatures rose to a maximum at 4 or 5 days after infection and then fell to normalcy by day 8. Animals infected with strain Chicago-2S did not become febrile. Animals infected with K6 or HM1011 had an average weight loss of 15% by day 5 after infection, in contrast to the 7% weight gain of animals infected with strain Chicago-2. Approximately 5×10^4 CFU of bacteria were recovered from the left lungs of

animals infected with strain K6 or HM1011 at 2 h after infection. Numbers of CFU in the left lungs increased about 10^4-fold in 4 days after infection, reached a maximum of approximately 9×10^8 CFU, and decreased thereafter. Approximately 2×10^4 CFU of bacteria were recovered from the left lungs of animals infected with strain Chicago-2S at 2 h after infection, but the bacteria were not detected ($<10^2$ CFU) 2 days after infection. Histological examination of their right lungs revealed a greater degree of exudation in animals infected with strain K6 (Fig. 2A) or HM1011 (Fig. 2C) than in those infected with strain Chicago-2S (Fig. 2B) on day 4 or 7 postinfection. These results show that strain Chicago-2S changed to virulent strain HM1011.

Conjugal transfer of chromosomal virulence genes (*icm-dot* genes). To learn about transfer of chromosomal virulence genes more precisely, we examined whether the *icm-dot* genes can be transferred. Mating experiments were performed with donor strains with the genes for Km^r or Km^r $LacZ^+$ at different sites within the *icm-dot* genes of the strain JR32 chromosome (Table 1). As a recipient strain, a spontaneous Rif^r derivative of strain Chicago-2S (strain Chicago-2SR) was used because donor strains were Sm^r. Mating experiments were performed as previously described (14). Selection for recombinants was performed on agar plates containing both kanamycin and rifampin, and top agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was also used when LELA strains were used as donors in the experiments. Table 2 shows the results obtained with LELA2883 (*dotB*) as the donor. The transfer frequency corresponded to about 10^{-4} per donor (Table 2, line 1). No Km^r Rif^r $LacZ^+$ colonies were obtained when only Chicago-2SR was plated (Table 2, line 7), and spontaneous Rif^r mutants were obtained when LELA2888 was plated on selective media (Table 2, line 6). No recombinants were obtained when the mating experiments were carried out with BYE broth (Table 2, line 3), implying that extended cell-to-cell contact or a high cell density is required for efficient transfer. Recombinant formation was not affected by DNase I (10 μg/ml) (Table 2, line 2), and no transfer was seen with one viable parent and one heat-killed parent before mating (Table 2, lines 3 and 4). No recombinants were obtained by mixing and incubating cell-free filtrates of one parent with the cells of the other parents, and no plaques were detected if filtrate from one parent was spotted onto a lawn of the other parent. These results suggested that transformation or transduction was not involved in the recombinant formation observed. The mechanism of the DNA transfer seen was most consistent with conjugal transfer.

To genetically confirm the DNA transferred from LELA2883 to Chicago-2SR, genotyping of strains LELA2883 and Chicago-2SR and their transconjugants was performed by PFGE as described previously (14). As shown in Fig. 3A, all of the transconjugants (HM1156 to HM1164) exhibited identical restriction fragment length polymorphism after PFGE of *Sfi*I-digested genomic DNA. The restriction fragment patterns of the transconjugants were distinct from the pattern exhibited by LELA2883 and were similar to that exhibited by Chicago-2SR (Fig. 3A, lanes 2 to 11). These findings show that strain LELA2883 is the donor and strain Chicago-2SR is the recipient and also suggest that chromosomal genes transferred from LELA2883 are integrated into the Chicago-2SR chromosome by recombination. To examine the presence of the LELA2883 sequences in the Chicago-2SR chromosome, we performed Southern blot experiments with pLAW330 (30) as a probe. Prehybridization, hybridization (at 68°C overnight), and

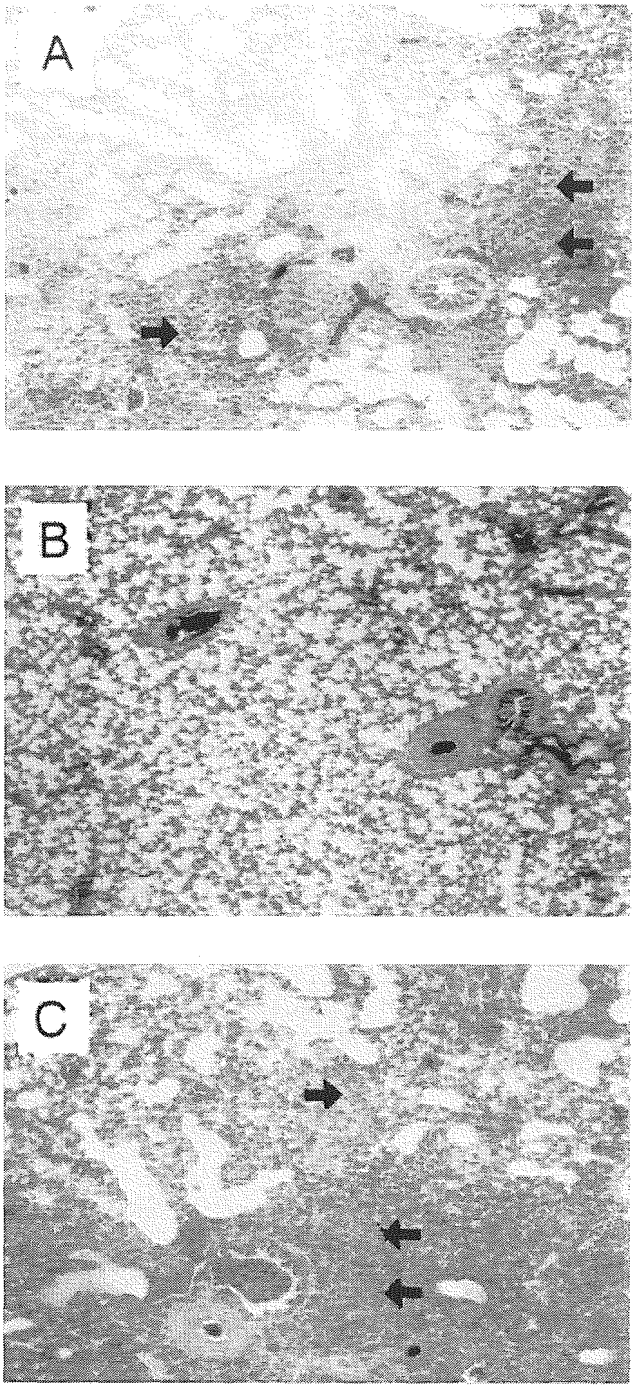

FIG. 2. Light micrographs of hematoxylin-and-eosin staining of the right lungs of guinea pigs sublethally infected with *Legionella* aerosols. The right lungs were harvested at 4 days after infection. After fixing and staining, light microscopic observations were performed (magnification, ×40). Panels: A, strain K6; B, strain Chicago-2S; C, strain HM1011. In contrast to those in panel B, alveolar spaces in panels A and C are occupied by many inflammatory cells (arrows).

TABLE 2. Transfer of chromosomal antibiotic resistance markers from *L. pneumophila* strain LELA2883 to Chicago-2SR[a]

Serogroup 1, Km^r $LacZ^-$ donor	Serogroup 6, Rif^r recipient	Mating condition(s)	No. of Km^r Rif^r $LacZ^+$ recombinants/ml	Transfer frequency (no. of recombinants/donor)
LELA2883	Chicago-2SR	BCYE agar	$(5.1 \pm 2.3) \times 10^5$	$(1.7 \pm 0.4) \times 10^{-4}$
LELA2883	Chicago-2SR	BCYE agar, DNase I	$(2.7 \pm 0.6) \times 10^5$	$(2.2 \pm 1.5) \times 10^{-4}$
LELA2883	Chicago-2SR	BYE broth	0	
LELA2883	Chicago-2SR	BCYE agar, heat-killed LELA2883	0	
LELA2883	Chicago-2SR	BCYE agar, heat-killed Chicago-2SR	$(2.0 \pm 1.7) \times 10^{2b}$	$(1.4 \pm 0.7) \times 10^{-7c}$
LELA2883		BCYE agar	$(2.1 \pm 1.0) \times 10^{2b}$	$(1.3 \pm 1.4) \times 10^{-7c}$
	Chicago-2SR	BCYE agar	0	

[a] The numbers of recombinants and transfer frequencies are based on at least three experiments and were calculated as means ± standard deviations. The numbers of LELA2883 and Chicago-2SR CFU were $(2.6 \pm 1.1) \times 10^9$/ml and $(5.0 \pm 1.8) \times 10^9$/ml at the end of the experiments, respectively.
[b] Number of spontaneous mutations of LELA2883 to Rif^r.
[c] Frequency of spontaneous mutation of LELA2883 to Rif^r.

chemiluminescent detection of the nylon membrane (Hybond-N+; Amersham Japan Co., Ltd., Tokyo, Japan) blots were performed with a DIG DNA labeling and detection kit (Boehringer GmbH, Mannheim, Germany) in accordance with the instructions of the manufacturer. As shown in Fig. 3B, the genes for Km^r and $LacZ^+$ were located on an about 582-kb *Sfi*I-digested DNA fragment of strain LELA2883 (Fig. 3B, lane 1). In all of the transconjugants tested (HM1156 to HM1164), the genes were located on an about 582-kb *Sfi*I-digested DNA fragment (Fig. 3B, lanes 3 to 11). This is direct evidence that chromosomal genes transferred from LELA2883 are integrated into the chromosome of Chicago-2SR. Some bands of more than 582 kb are probably due to partial digestions, as well as bands observed at their sample plugs. In addition, divergences in signal intensity between these samples are due to differences in the amount of DNA electrophoresed in the gel.

Figure 4 shows the results obtained in these conjugation experiments. Recombinant formations were not affected by DNase I (10 μg/ml). Frequencies (mean ± standard deviation) of spontaneous mutation to Rif^r for donor strains of *icmX* and *-V*; *dotA* and *-B*; *icmF*, *-B*, *-E*, *-P*, and *-R*; and *icmS* were $(4.9 \pm 3.9) \times 10^{-7}$ and $(1.0 \pm 0.8) \times 10^{-7}$; $(0.8 \pm 1.4) \times 10^{-7}$ and $(1.3 \pm 1.4) \times 10^{-7}$; $(1.7 \pm 0.6) \times 10^{-7}$, $(3.2 \pm 4.1) \times 10^{-7}$, $(4.5 \pm 2.3) \times 10^{-7}$, $(3.4 \pm 2.6) \times 10^{-7}$, and $(3.0 \pm 3.2) \times 10^{-7}$; and $(1.3 \pm 1.2) \times 10^{-7}$, respectively. Although the data are not included in Fig. 4, strains with insertions in seven *icm* genes (*icmQ*, *-O*, *-M*, *-K*, *-G*, *-D*, and *-J*) had transfer frequencies of 10^{-7} to 10^{-6}. When it was found that the spontaneous mutation of donor strains to Rif^r was not far off the conjugal frequency, it was confirmed that all (50 of 50) of the transconjugants tested belonged to serogroup 6, which is the same serogroup as Chicago-2SR. To distinguish transconjugants from Rif^r mutants of donor strains more clearly, we introduced a nonconjugative Cm^r plasmid (pMMB207C) as a cytoplasmic marker into Chicago-2SR. Km^r Rif^r ($LacZ^+$) recombinants were selected after mating of Chicago-2SR containing pMMB207C (HM1175, Rif^r Cm^r) with Km^r ($LacZ^+$) donor strains. Transconjugants were screened for the presence of the Cm^r plasmid by patching onto plates containing chloramphenicol. All of the Km^r Rif^r ($LacZ^+$) recombinants tested (100 of 100) were Cm^r and belonged to serogroup 6. Their conjugal frequencies were similar to the results shown in Fig. 4 (data not

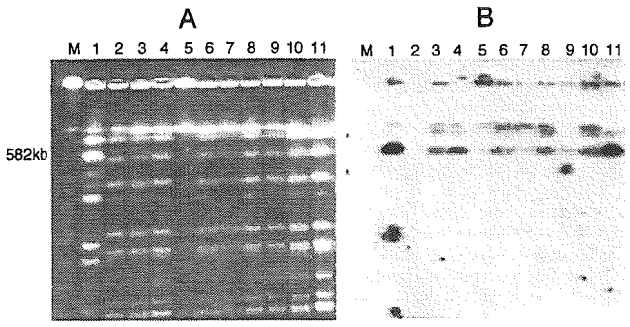

FIG. 3. Cleavage patterns (A) and Southern blot analysis (B) of *Sfi*I-digested genomic DNAs of *L. pneumophila* strains by PFGE. Plasmid pLAW330 digested with *Hin*dIII, which does not cut within Tn*903*dII*lacZ*, was labeled with digoxigenin and used as a probe. Lanes: M, lambda ladder (Bio-Rad), used as a DNA size standard; 1, LELA2883 (*dotB*); 2, Chicago-2SR; 3, HM1156; 4, HM1157; 5, HM1158; 6, HM11159; 7, HM1160; 8, HM1161; 9, HM1162; 10, HM1163; 11, HM1164.

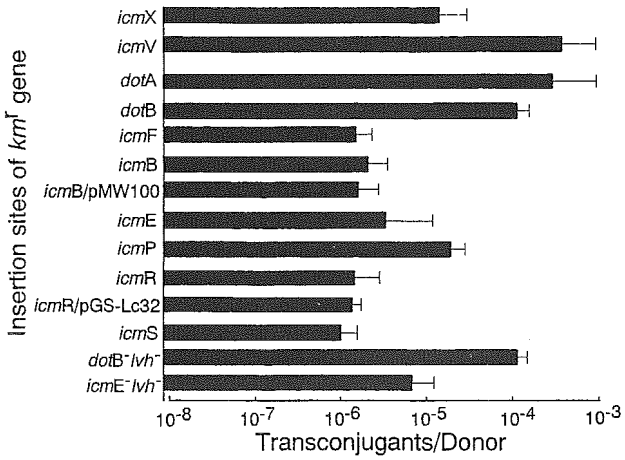

FIG. 4. Frequencies of conjugal transfer of the *icm-dot* locus. Chicago-2SR was used as a recipient in all of the conjugation experiments. The donor strains tested were LELA4004 for *icmX*, LELA1747 for *icmV*, LELA3118 for *dotA*, LELA2883 for *dotB*, LELA1718 for *icmF*, LELA3393 for *icmB*, HM1138 for *icmB*/pMW100, LELA4432 for *icmE*, LELA3352 for *icmP*, LELA3473 for *icmR*, HM1134 for *icmR*/pGS-Lc32, GS3001 for *icmS*, LELA2883-28 for *dotB lvh*, and LELA4432-28 for *icmE lvh*. The other strains tested containing insertions in *icmQ*, *-O*, *-M*, *-K*, *-G*, *-D*, and *icmJ* are listed in Table 1. The experiments were done at least three times. The values are means ± standard deviations.

shown). These results suggest that all of the genes for Km^r or Km^r $LacZ^+$ within the *icm-dot* genes were horizontally transferred into strain Chicago-2SR, indicating that the *icm-dot* locus can be transferred by the chromosomal conjugation system. Differences in transfer efficiency between *icm-dot* genes observed in Fig. 4 may reflect distances from chromosomal *oriT* to the insertion sites of the genes for Km^r ($LacZ^+$) in the strain LELA, GS, and MW chromosomes. The chromosomal *oriT* locus may be closer to the region around *dotB-dotA* than to the rest of the *icm* genes.

In addition, this result suggests that the *icm-dot* apparatus may not be involved in the chromosomal DNA transfer observed because all of the donor strains used here have defects in the *icm-dot* apparatus. When plasmid pGS-LC-32 was introduced into strains GS3001 (*icmS*), LELA3473 (*icmR*), and LELA3463 (*icmQ*), this plasmid complemented them for intracellular growth in guinea pig macrophages (data not shown), indicating that the *icmS*, *icmR*, or *icmQ* gene product is expressed from this plasmid and the intact *icm-dot* apparatus is reconstructed. However, no change in the transfer frequency of the chromosomal DNA was observed when these complemented strains were used as donors for the conjugation experiments (*icmR* and *icmR*/pGS-Lc32 in Fig. 4). When plasmid pGS-Lc-47 or pMW100 was introduced into LELA4432 (*icmE*) or LELA3393 (*icmB*), respectively, the transfer frequencies of the complemented strains were the same as those of the original mutants (*icmB* and *icmB*/pMW100 in Fig. 4). These results may support the notion that the *icm-dot* apparatus is not directly involved in the transfer of chromosomal DNA.

In addition to the *icm-dot* system, *L. pneumophila* has another type IV secretion system designated the *lvh* (*Legionella vir* homolog) system (26). The *lvh* system is dispensable for intracellular growth in human macrophages and *A. castellanii* but is able to transfer a mobilizable IncQ plasmid (26). To examine whether the *lvh* system is involved in the conjugal transfer of chromosomal DNA, conjugation experiments were performed with *lvh* deletion strains as donors, that is, LELA2883-28 (*dotB lvh*) and LELA4432-28 (*icmE lvh*) (Table 1). As shown in Fig. 4, both strains transferred their chromosomes as well as the original mutant strains did. The frequencies of spontaneous mutation of LELA2883-28 and LELA4432-28 to Rif^r were $(1.0 \pm 1.2) \times 10^{-7}$ and $(4.9 \pm 2.9) \times 10^{-7}$, respectively. This finding shows that the *lvh* system is dispensable for the transfer of chromosomal DNA. Therefore, there must be additional conjugal transfer systems in *L. pneumophila*. Recently, we found the possibility of a third conjugation system separate from *icm-dot* and from *lvh* on the *L. pneumophila* chromosome (the right end of contig 619 [CTG. WG. 013. 49. WK1. 091001]). The cluster consists of hits to TraD, -G, -H, -F, -N, -U, -W, -C, and -B, which are involved in sex pilus assembly (http://genome3.cpmc.columbia.edu/~legion/index.html). Further studies are required to reveal mechanisms or apparatuses of chromosomal transfer.

In the present study, we showed that *L. pneumophila* virulence conversion occurs by the conjugal transfer of chromosomal DNA. The chromosome conjugation system is not specific for the serogroups or the strains used in this study because some transconjugants were obtained with *L. pneumophila* strains Bloomington-2 (serogroup 3, ATCC 33155) and AM240 (serogroup 1) (11; unpublished observation). As far as we know, this is the first report on virulence conversion by conjugal transfer of chromosomal DNA. The results of this study indicate that an avirulent strain could change to a virulent one through conjugal transfer from another virulent strain coinhabiting the environment. It is known that *L. pneumophila* is recovered from biofilms on the surfaces of water systems (3, 20, 29). The relative spatial stability of bacteria in biofilms may be a good niche for conjugation. It was reported that a high frequency of plasmid conjugation from *Escherichia coli* to *Alcaligenes eutrophus* occurs in biofilms (7). We are currently investigating whether *L. pneumophila* chromosomal conjugation occurs in biofilms. That study may provide new insights into the ecology of *L. pneumophila* and reveal the consequences of chromosomal conjugation in *L. pneumophila*.

This work was supported in part by National Institutes of Health grant AI-23549 to H.A.S.

We thank Laura Hales, Gil Segal, John Chen, and Yasuo Mizuguchi for helpful discussions and technical advice. We also thank Carmen Rodriguez and Kazuki Yamauchi for laboratory maintenance.

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INFECTION AND IMMUNITY, Oct. 2005, p. 6272–6282 Vol. 73, No. 10
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.10.6272–6282.2005

Characterization of *Legionella pneumophila pmiA*, a Gene Essential for Infectivity of Protozoa and Macrophages

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Received 19 January 2005/Returned for modification 15 April 2005/Accepted 1 June 2005

The ability of *Legionella pneumophila* to cause pneumonia is dependent on intracellular replication within alveolar macrophages. The Icm/Dot secretion apparatus is essential for the ability of *L. pneumophila* to evade endocytic fusion, to remodel the phagosome by the endoplasmic reticulum (ER), and to replicate intracellularly. Protozoan and macrophage infectivity (*pmi*) mutants of *L. pneumophila*, which include 11 *dot/icm* mutants, exhibit defects in intracellular growth and replication within both protozoa and macrophages. In this study we characterized one of the *pmi* loci, *pmiA*. In contrast to the parental strain, the *pmiA* mutant is defective in cytopathogenicity for protozoa and macrophages. This is a novel mutant that exhibits a partial defect in survival within U937 human macrophage-like cells but exhibits a severe growth defect within *Acanthamoeba polyphaga*, which results in elimination from this host. The intracellular defects of this mutant are complemented by the wild-type *pmiA* gene on a plasmid. In contrast to phagosomes harboring the wild-type strain, which exclude endosomal-lysosomal markers, the *pmiA* mutant-containing phagosomes acquire the late endosomal-lysosomal markers LAMP-1 and LAMP-2. In contrast to the parental strain-containing phagosomes that are remodeled by the ER, there was a decrease in the number of ER-remodeled phagosomes harboring the *pmiA* mutant. Among several *Legionella* species examined, the *pmiA* gene is specific for *L. pneumophila*. The predicted amino acid sequence of the PmiA protein suggests that it is a transmembrane protein with three membrane-spanning regions. PmiA is similar to several hypothetical proteins produced by bacteria with a type IV secretion apparatus. Importantly, the defect in *pmiA* abolishes the pore-forming activity, which has been attributed to the Icm/Dot type IV secretion system. However, the mutant is sensitive to NaCl, and this sensitivity is abrogated in the *icm/dot* mutants. These results suggest that PmiA is a novel virulence factor that is involved in intracellular survival and replication of *L. pneumophila* in macrophages and protozoan cells.

Legionella pneumophila is the causative agent of Legionnaires' disease (29, 50). The ability of *L. pneumophila* to grow within mammalian cells, such as monocytes and alveolar macrophages, is essential for pathogenicity in humans (1, 42). Protozoa are reservoirs of *L. pneumophila* in natural environments, which play a crucial role in bacterial ecology and transmission to humans (38, 54, 63). Growth of *L. pneumophila* within amoebae enhances invasion of epithelial cells and macrophages (20). Amoeba-grown *L. pneumophila* becomes resistant to various stress conditions, such as acid, oxidative, osmotic, and heat stresses (4), and becomes more resistant to antibiotics (11). These phenotypic modulations of *L. pneumophila* may contribute to bacterial survival in harsh environmental conditions and to invasion and replication within human phagocytic cells.

The Icm/Dot type IV secretion apparatus is a major virulence system in *L. pneumophila*, since it is essential for evasion of endocytic fusion and remodeling of the phagosome in a suitable replicative niche (69, 80). The Icm/Dot system is also essential for early activation of caspase-3 in the host cell, which does not result in apoptosis until late stages of the infection (6, 83). Activation of caspase-3 results in cleavage of the Rab5 effector rabaptin-5, and this cleavage may be involved in the ability of *L. pneumophila*-containing phagosome to evade endocytic fusion and to be remodeled by the endoplasmic reticulum (ER) (55). *L. pneumophila* utilizes the Icm/Dot type IV secretion apparatus for injection of bacterial effector proteins into the host cell, leading to the creation of unique vacuoles suitable for intracellular growth (22, 58). Several proteins that are substrates of the Icm/Dot secretion apparatus, such as LepAB, LidA, RalF, and SidC, have been identified (18, 23, 47, 57). Although the functions of these substrates are unclear, it is thought that they modulate host signal transductions to establish the replicative vacuoles, allowing the organism to survive and replicate within phagocytic cells. At 15 min postinfection, *L. pneumophila* is contained in a unique phagosome with a membrane thinner than the plasma membrane, and it is surrounded by mitochondria and the ER (1, 2, 5, 41, 75–77). The *L. pneumophila*-containing phagosome excludes endosomal and lysosomal markers. In contrast, phagosomes containing *icm/dot* mutants acquire endosomal and lysosomal markers, and the mutants are unable to replicate intracellularly (64, 67, 80). Interestingly, calnexin, Rab1, and Sec22b are acquired by phagosomes containing the wild-type strain shortly after

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bacterial uptake (24, 44). Upon formation of this unique replicative niche, the organism replicates in the phagosome. During late stages of the infection, the phagosomal membrane becomes disrupted, and the bacteria escape into the cytoplasm, where the last rounds of replication are completed prior to lysis of the host cell membrane and bacterial egress (7, 51–53). Interestingly, within gamma interferon-activated macrophages, the *L. pneumophila*-containing phagosome fuses to lysosomes, and it is not remodeled by the ER (66).

Among the components of the Icm/Dot secretion apparatus, IcmT is essential for pore formation-mediated egress from the host cell upon termination of intracellular replication, in addition to a function required for phagosome biogenesis and evasion of lysosomal fusion (7, 51, 52). IcmQ has pore-forming capacities involving insertion into host cell membranes to form a channel for translocation of effectors from the bacterium into the host cell (25).

Loci other than the *icm/dot* genes are required for intracellular replication of *L. pneumophila*. The stationary-phase sigma factor RpoS and the stress-induced protease/chaperone HtrA are essential for multiplication within *Acanthamoeba polyphaga* (34, 59). The Rep helicase is required for replication within human macrophages and epithelial cells (31, 37). The type II secretion system of *L. pneumophila* is required for intracellular infection (33, 61, 62). The *pilD* gene, which encodes the prepilin leader peptidase, is required for assembly of both a type IV pilus (72) and a type II secretory apparatus, and it is also required for bacterial replication within amoebae and human macrophages (46). The LetA/LetS two-component regulatory system is required for infection of macrophages (30, 35, 48). The *csrA* gene is involved in regulation of the bacterial switch from the replicative form to the transmissible form and is essential for intracellular growth within both macrophages and amoebae (28, 56). Iron acquisition and assimilation are key factors for *L. pneumophila* virulence and intracellular growth (39, 79). The *lvgA* and *ptsP* genes of *L. pneumophila* affect colonization of the lungs and spleens of guinea pigs (26, 40). Therefore, numerous loci other than the *icm/dot* genes play major roles in the ability of *L. pneumophila* to replicate intracellularly and cause disease.

We previously isolated 89 insertion mutants of *L. pneumophila* that exhibit defects in cytotoxicity, intracellular survival, and replication within both U937 macrophage-like cells and *A. polyphaga*. These mutants were collectively designated *pmi* (protozoan and macrophage infectivity) mutants (32). Although 11 of the mutants have insertions within the *icm/dot* genes, most of the mutated genes of these strains have not been identified.

In this study, we characterized one of the *pmi* mutant strains, GB112, and identified the mutated gene, *pmiA*. The *pmiA* gene is localized outside the two genomic regions that include the *icm/dot* genes. The *pmiA* gene likely encodes an *L. pneumophila*-specific virulence factor required for intracellular survival and growth within macrophages and protozoan cells. The defect in intracellular survival is associated with trafficking of the *pmiA* mutant-containing phagosomes through the default endosomal-lysosomal degradation pathway. The predicted amino acid sequence suggests that the gene product is a transmembrane protein. Functional analyses using mutants and gene complementation indicated that the mutation does not eliminate sodium sensitivity, which is one of the phenotypes dependent on the Icm/Dot secretion apparatus, but abrogates the pore-forming activity, which is dependent on a functional Icm/Dot secretion apparatus.

MATERIALS AND METHODS

Bacterial strains and media. The *Legionella* strains used in this study were *L. pneumophila* serogroup 1 strain AA100 (3), *L. pneumophila* Philadelphia 1 JR32 (65), *L. pneumophila* Philadelphia 1 Lp02 (12), *L. pneumophila dotA* mutant LELA3118 (65), *L. pneumophila icmT* mutant GS3011 (70), *L. pneumophila icmT* mutant AA100kmT (52), *L. pneumophila* serogroup 5 strain GTC297, *L. pneumophila* serogroup 6 strain GTC748, *L. pneumophila* serogroup 7 strain GTC750, *Legionella bozemanii* GTC298, *Legionella micdadei* GTC299, *Legionella dumoffii* GTC303, *Legionella brunesis* GTC515, and *Legionella gratiana* GTC699. The GTC strains were obtained from the Gifu Type Culture Collection at Gifu University Graduate School of Medicine, Japan. The *Legionella* strains used in this study were grown on buffered charcoal-yeast extract (BCYE) plates or in buffered yeast extract (BYE) broth.

Cell culture. Macrophage-like U937 cells were maintained in RPMI 1640 (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (ICN Biomedicals, Aurora, Ohio), as described previously (32). Axenic *A. polyphaga* was cultured as adherent cells in PYG medium. All cells were cultured under a humidified atmosphere containing 5% CO_2 and 95% air at 37°C, as described previously (32).

Intracellular bacterial growth assay. U937 cells (1×10^5 cells per well in 96-well flat-bottom plates [Falcon, Becton Dickinson, Franklin Lakes, NJ]) were allowed to differentiate into macrophages by incubation for 48 h with 50 ng ml^{-1} of phorbol 12-myristate 13-acetate (Sigma, Tokyo, Japan) in RPMI 1640 containing 10% fetal calf serum. *A. polyphaga* cells were grown in a 96-well plate at a density of 1×10^5 cells per well in PYG medium. The bacterial strains were grown in BYE medium to an optical density at 550 nm (OD_{550}) of 2.0 to 2.2 (post-exponential phase). The differentiated U937 cells or *A. polyphaga* cells were infected with the bacteria at a multiplicity of infection (MOI) of 10 in triplicate cultures. The plates were spun at 250 × *g* for 20 min to bring the bacteria into contact with the host cell monolayer. The time at the end of this centrifugation was designated zero time. Then the monolayer was incubated for 1 h at 37°C to allow bacterial infection. At the end of the infection period, the cells were washed three times with an appropriate culture medium, and then they were incubated for 1 h at 37°C in culture medium containing 50 μg ml^{-1} gentamicin to kill the extracellular bacteria. The cells were washed again, and then incubation was continued in the culture medium. After several different times, cell lysis was performed by hypotonic treatment for U937 cells or by treatment with 0.04% Triton X-100 for *A. polyphaga*. Aliquots of the cell lysates were immediately diluted and plated on BCYE plates for enumeration of the intracellular bacteria. The number of intracellular bacteria was expressed as the number of CFU/ml (36, 52).

Cytopathogenicity of *L. pneumophila* for U937 cells and *A. polyphaga*. Infection of U937 cells with *L. pneumophila* strains in the post-exponential phase was performed in triplicate in 96-well tissue culture plates with 1×10^5 cells/well at an MOI of 10 for 1 h, and infection of *A. polyphaga* was performed in triplicate in 24-well tissue culture plates with 5×10^5 cells/well at an MOI of 10 for 1 h. After 23 h, the numbers of viable cells were determined by using the Alamar Blue (TREK Diagnostic Systems, Westlake, Ohio) assay for U937 cells and the trypan blue dye assay for *A. polyphaga*, and the degrees of cytopathogenicity for the two types of cells were expressed as described previously (32).

Cloning and sequencing the chromosomal junction of Kan insertions in the GB112 mutant. Genomic DNA from the GB112 mutant was digested with EcoRI, and the fragment containing the Kan cassette was ligated to EcoRI-digested pBC-SK^+ (Stratagene, La Jolla, Calif.). The cloned plasmid was digested with EcoRI and probed with pUC-4K (Pharmacia, Piscataway, N.J.) by Southern blotting to confirm the fidelity of the cloning. This plasmid was designated pGB112. pGB112 was digested with XhoI to obtain chromosomal fragment GB112 flanking half-Kan cassette. This fragment was ligated to pBC-SK^+, and the resulting plasmid was designated pGB112-XHOI. pGB112-XHOI was used for sequencing analysis with a T7 primer of pBC-SK^+. The sequence of approximately 360 nucleotides at the junction of the insertion was determined and used in database searches of the *Legionella* Genome Project at the Columbia Genome Center.

Construction of a GB112 complemented strain. Amplification of lpg1728 (*pmiA*) was performed with primer GB112-F3, complementary to a region 136 nucleotides upstream of the lpg1728 start codon (5′-GTTGATGATATGGGG

GCTG-3'), and GB112-R3, complementary to a region 138 nucleotides downstream of the lpg1728 stop codon (5'-CTCCGACAGGATAATCAGGA-3'). A 1,189-bp fragment was amplified by PCR with a Gene Amp PCR 9600 system (Perkin-Elmer, Yokohama, Japan). This fragment was cloned into the pGEM-T Easy system (Promega, Madison, Wis.). A NotI-digested fragment containing this region was subcloned into pBC-SK$^+$. The recombinant clone was designated pGB112C. pGB112C was introduced into the GB112 strain by using a Gene Pulser II (Bio-Rad, Hercules, Calif.) as recommended by the manufacturer to generate complemented strain GB112C-5.

Sequencing of *pmiA* gene. Sequencing of the whole *pmiA* region of the AA100 strain, using pGB112C, was carried by Hokkaido System Science Co., Ltd. Sequence alignments and comparisons were performed with GENETYX-MAC 8.0 (Software Development Co., Ltd.) and National Center for Biotechnology Information (blastp) programs, respectively.

Confocal laser scanning microscopy. Samples used for immunofluorescence analysis were prepared by the method described previously (36). In brief, U937 cells were cultured and then allowed to differentiate on coverslips in the wells of a six-well culture plate (Falcon, Becton Dickinson). The differentiated U937 cells were infected with *L. pneumophila* for 1 h. The cells were continuously incubated in culture medium containing gentamicin for 1 h to kill the extracellular bacteria. At different times after the initiation of infection (see below), the coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature, and then nonspecific binding sites were blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min. The cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 min. The nuclei of both the bacteria and the U937 cells were stained with TO-PRO-3 iodide (Molecular Probes, Eugene, Oreg.) for 1 h. For colocalization experiments with endosomal markers and ER markers, 1 h of infection and 1 h of gentamicin treatment, followed by 2 h and 4 h of incubation, respectively, in culture medium without gentamicin were performed. Thus, 4-h or 6-h postinfection samples were examined. To label lysosomal and late endosomal compartments, serial 1-h incubations were performed with mouse anti-human LAMP-1 (H3B3) or LAMP-2 (H4B4) monoclonal antibodies (1:10 dilution; Developmental Studies Hybridoma Bank, University of Iowa) and Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) (1:100 dilution; Molecular Probes). To label ER proteins, serial 1-h incubations were performed with mouse anti-KDEL monoclonal antibodies (StressGen Biotechnologies, Victoria, Canada) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes). In this experiment, extracellular bacteria were labeled by incubation with rabbit anti-*L. pneumophila* serum (1:10 dilution; Denka Seiken, Tokyo, Japan) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:100 dilution; Molecular Probes) before permeabilization. Between the incubations, the coverslips were washed three times with PBS for 5 min. The coverslips were then mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, Calif.). Samples were observed with a Carl Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Germany).

Transmission electron microscopy. For examination of infected amoebae by transmission electron microscopy, monolayers were infected by *L. pneumophila* strains in six-well plates at an MOI of 10 for 1 h, followed by three washes. At 6 h postinfection, the infected monolayers were washed with 0.1 M Sorenson's phosphate buffer and then incubated for 45 min in 0.1 M Sorenson's phosphate buffer containing 3.5% glutaraldehyde, pH 7.4, at 4°C. Fusion between lysosomes of the *L. pneumophila* phagosomes was determined by examination for the presence of the lysosomal enzyme acid phosphatase, as described previously (13, 51). The monolayers were washed three times with 0.1 M Sorenson's phosphate buffer and three times with 0.1 M acetate buffer (pH 5) and incubated with an acid phosphatase-specific substrate, β-glycerolphosphate (0.1 M acetate buffer, 2 mM β-glycerolphosphate as the substrate, and 1.2% lead nitrate as the capture metal), for 1 h at 37°C. After three washes in 0.1 M acetate buffer (pH 5) and Sorenson's phosphate buffer (5 min each), infected cells were postfixed with 1% OsO_4 in the same buffer for 45 min. Samples were dehydrated and processed as described previously (31). Sections were stained with uranyl acetate and lead citrate and examined with an Hitachi H-7000/STEM electron microscope (Hitachi, Inc., Japan) at 80 kV, as described previously (31).

Southern blotting. Genomic DNA was isolated from *L. pneumophila* strains using an AquaPure genomic DNA isolation kit (Bio-Rad). Samples of genomic DNA were digested with EcoRI, separated by electrophoresis in 0.8% agarose gels, and transferred to Hybond-N nylon membranes (Amersham Biosciences, Buckinghamshire, England). pGB112C was used as a DNA probe. Labeling of DNA probes and detection of signals were performed by using the ECL direct nucleic acid and detection system (Amersham Biosciences).

Contact-dependent pore formation assay. Contact-dependent pore formation in membranes was determined by examining hemolysis of sheep red blood cells (sRBCs) by *L. pneumophila* at an MOI of 10 after 2 h of bacterial contact, as described previously (45). The release of hemoglobin from the lysed red blood cells was measured by spectrophotometry at 415 nm.

Sodium sensitivity assay. Bacteria were grown in BYE broth until the post-exponential phase (OD_{550}, 2.0 to 2.2). The OD_{550} of bacterial cultures were adjusted to 1.0 to equalize the number of bacteria. Dilutions were plated on BCYE plates in the presence or absence of 0.6% NaCl. The ratio of the plating efficiency with 0.6% NaCl to the plating efficiency without 0.6% NaCl was calculated for each strain.

Nucleotide sequence accession number. The sequence of the whole *pmiA* region of the AA100 strain has been deposited in the DDBJ database under accession number AB193439.

RESULTS

Characteristics of intracellular survival, replication, and cytopathogenicity of the GB112 mutant in U937 cells and *A. polyphaga*. We showed previously that the GB112 *pmi* mutant exhibits a defect in intracellular growth within both macrophages and protozoa (32). To ensure that the GB112 mutant had no other mutations, we decided to reconstruct the Kan insertion in the GB112 mutant in the wild-type strain. A DNA fragment containing the insertion and the flanking chromosomal regions was cloned from GB112 genomic DNA and introduced into wild-type strain AA100 by homologous recombination, as described previously (73). The fidelity of the allelic exchanges was confirmed by Southern hybridization (data not shown). For all the studies described in this paper, we show data only for the reconstructed mutant, which for simplicity was designated GB112.

Since many virulence-associated phenotypes have been shown to be induced at the postexponential phase (15), we examined the phenotype of the reconstructed GB112 mutant for intracellular growth and cytopathogenicity for host cells using bacteria grown to the postexponential phase. We first examined the cytopathogenicity for U937 human macrophage-like cells using Alamar Blue assays and the cytopathogenicity for *A. polyphaga* using trypan blue. The data showed that the cytopathogenicity of the GB112 mutant for both U937 macrophages and *A. polyphaga* was diminished compared to that of parental strain AA100 (Fig. 1A and B).

The intracellular growth of the GB112 mutant was less than that of wild-type strain AA100 in U937 cells. The number of GB112 mutant bacteria within U937 cells was approximately 1/10 the number of AA100 bacteria from 24 h to 48 h postinfection (Fig. 1C). In contrast, the GB112 mutant was severely defective in survival and replication within *A. polyphaga*. At 24 h postinfection, there were no detectable viable GB112 bacteria in *A. polyphaga*, while AA100 showed robust replication by 24 h (Fig. 1D). This result indicated that the GB112 mutant bacteria were killed in *A. polyphaga*.

Intracellular trafficking of the GB112 mutant within U937 macrophages and *A. polyphaga*. Survival and replication of *L. pneumophila* within mammalian and protozoan cells are totally dependent on evasion of fusion of the bacterial phagosome to lysosomes (51, 64, 74, 82). In addition, bacterial replication has also been shown to occur in a phagosome that is remodeled by the rough endoplasmic reticulum within macrophages and protozoa (1, 2, 5, 41, 75–77). Since the GB112 mutant was defective in survival in macrophages and amoebae, we hypothesized that intracellular trafficking of the mutant was likely altered in both types of host cells. To test this hypothesis, we examined colocalization of the bacterium-containing phagosomes with

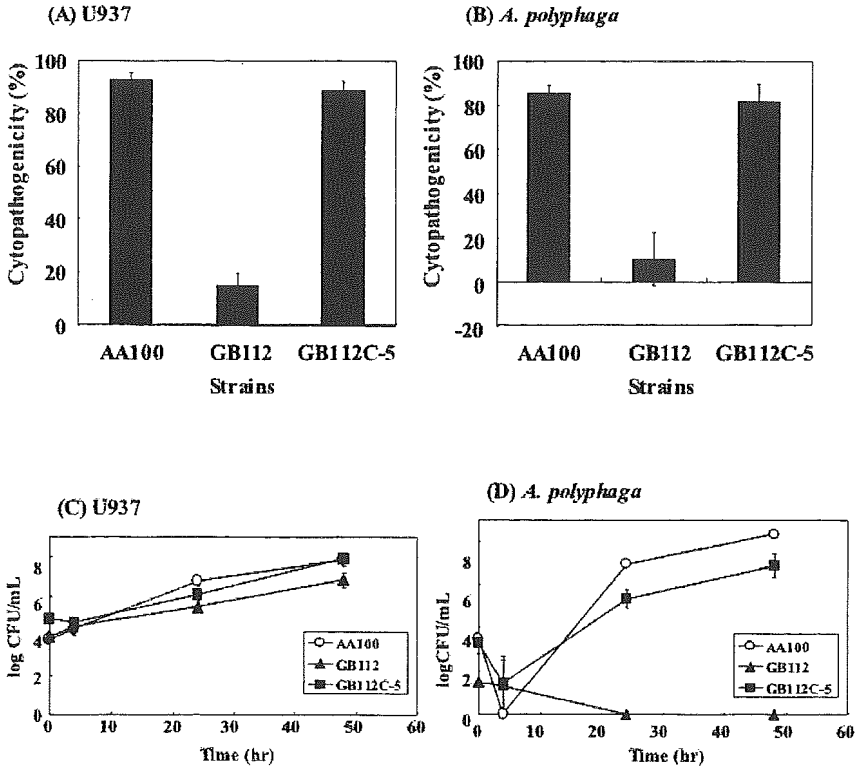

FIG. 1. Cytopathogenicity and intracellular replication of *L. pneumophila* strains in U937 macrophage-like cells and *A. polyphaga*. U937 macrophages or *A. polyphaga* cells were infected with bacteria at an MOI of 10 for 1 h, followed by gentamicin treatment to kill extracellular bacteria, and the cells were then incubated for 24 h. The viability of host cells was determined by the Alamar Blue (for U937 cells) (A) or trypan blue (for *A. polyphaga*) (B) method. The following *L. pneumophila* strains were used: AA100 (wild type), GB112 (*pmiA* deficient), and GB112C-5 (*pmiA*-complemented GB112). The percentage of killed cells after bacterial infection was normalized to the percentage of uninfected cells, which was considered 100% viable cells. (C and D) Intracellular growth kinetics of *L. pneumophila* GB112 strains within U937 macrophage-like cells and *A. polyphaga*, respectively. Infection of the monolayers was performed exactly as described above for the cytopathogenicity assay, except that at the end of the 1-h infection period the monolayers were treated with gentamicin for 1 h to kill extracellular bacteria. The intracellular bacteria were recovered at several times postinfection, and the number of viable cells was determined by enumeration of the CFU. All experiments were done three times in triplicate; the data are the data from one representative experiment, and the error bars indicate standard deviations.

the late endosomal-lysosomal markers LAMP-1 and LAMP-2 in U937 cells by confocal laser scanning microscopy showing only LAMP-2 staining (Fig. 2A). Approximately 70% of the phagosomes containing the GB112 mutant colocalized with LAMP-1 and LAMP-2 (Fig. 3). In contrast, only 10 to 25% of the phagosomes containing parental strain AA100 colocalized with LAMP-1 and LAMP-2 (Fig. 2A and 3). The phagosomes harboring heat-killed *L. pneumophila* as a positive control colocalized with both LAMP-1 and LAMP-2, as expected (Fig. 2A and 3).

We examined the presence of ER-associated proteins in the phagosomes at 6 h postinfection, using an antibody that recognizes the KDEL amino acid sequence, which is the signal for ER retention (Fig. 2B). Approximately 60% of the GB112 mutant-containing phagosomes did not acquire the KDEL marker. In contrast, more than 70% of the AA100-containing phagosomes acquired the KDEL marker (Fig. 2B and 3). Phagosomes harboring heat-killed AA100, as a control, did not acquire the KDEL marker, as expected. Thus, the defect in survival and replication of the GB112 mutant within U937 macrophages was associated with acquisition of the late endosomal-lysosomal markers LAMP-1 and LAMP-2 and with a reduction in the frequency of colocalization with the ER.

Since the GB112 mutant was severely defective in replication within *A. polyphaga*, we examined whether this defect was associated with fusion of the GB112-containing phagosomes to lysosomes. We examined the presence of the lysosomal enzyme acid phosphatase in the bacterium-containing phagosomes by electron microscopy, as described previously (51). The data showed that only 7% of the phagosomes containing AA100 contained acid phosphatase at 6 h postinfection (Fig. 4A and D). In contrast, 82% of the phagosomes harboring the GB112 mutant contained acid phosphatase (Fig. 4B and D). For the *icmT* null mutant, which was used as a positive control, ~60% of the phagosomes acquired acid phosphatase (Fig. 4C and D), which is consistent with previous observations (51, 52). On the basis of these findings, we concluded that the severe defect of the GB112 mutant in survival and replication in *A. polyphaga* was associated with the fusion of the GB112 mutant-containing phagosomes to lysosomes.

Identification of the mutated gene in the GB112 mutant. We cloned the DNA fragment containing the Kan insert and flank-

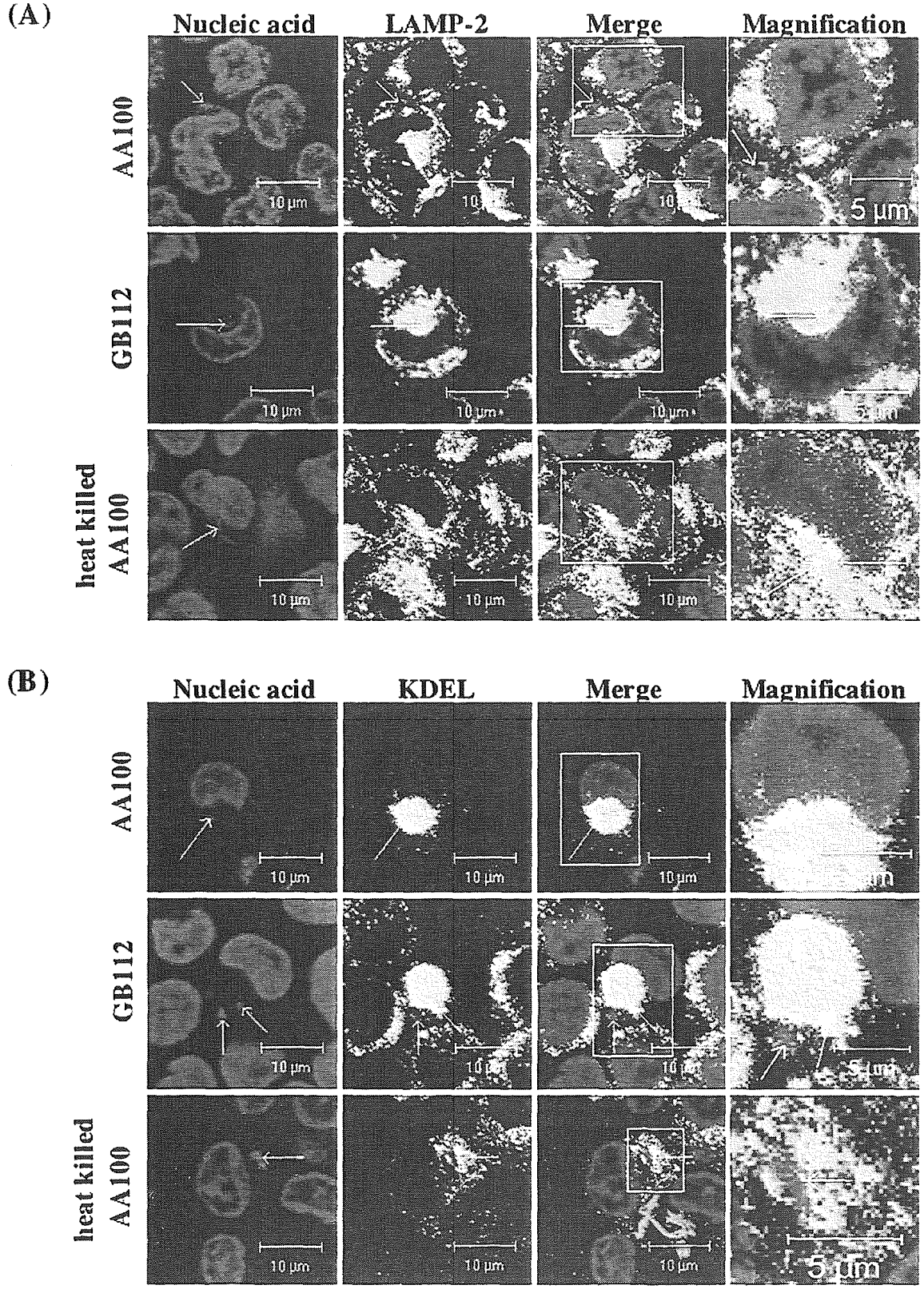

FIG. 2. Representative confocal microscopy images of colocalization of the bacterial phagosomes with LAMP-2 (A) and ER proteins (B) within U937 cells infected by *L. pneumophila* AA100 (wild type) or GB112 (*pmiA* deficient). The DNA of intracellular and extracellular bacteria, as well as the U937 nucleus, were stained with TO-PRO-3 (blue). LAMP-2 and the KDEL marker were visualized with secondary antibodies conjugated to Alexa 488 (green). Extracellular bacteria were visualized with secondary antibodies conjugated to Alexa Fluor 546 (red). The arrows indicate intracellular *L. pneumophila*. The magnified portions are indicated by the squares in the merge panels.

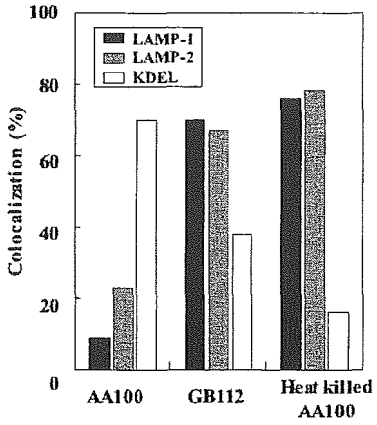

FIG. 3. Quantitation of colocalization of LAMP-1, LAMP-2, and ER proteins with the phagosomes containing *L. pneumophila* strains. Phagosomes containing AA100 (wild type), GB112 (*pmiA* deficient), or heat-killed AA100 (control) were examined for the presence of LAMP-1 and LAMP-2 at 4 h postinfection and for proteins with the ER retention signal (KDEL motif) at 6 h postinfection using confocal laser scanning microscopy.

ing sequences from the chromosome of the GB112 mutant. The sequence of the DNA fragments at the junction of the chromosomal DNA and the insert was analyzed. Using the BLAST program at the website of the *Legionella* Genome Project for the Philadelphia 1 strain (http://genome3.cpmc.columbia.edu/~legion/index.html) (19), we identified an open reading frame (ORF) that was 915 bases long (lpg1728) in the genome sequence that was interrupted by the insertion in the GB112 mutant. This ORF was also present in the complete genome sequences of two other *L. pneumophila* strains, Paris and Lens (17). We designated the lpg1728 ORF *pmiA* (accession no. AB193439) (Fig. 5A). The *pmiA* gene was located outside the *icm/dot* regions and was 1.1×10^6 bp downstream from *icm/dot* region I (*icmVWX-dotABCD*) and 1.45×10^6 bp upstream from *icm/dot* region II (*icmTSRQPO-lphA-icmMLKEGCDJB-tphA-icmF* or *dotMLKJIHGFENO*).

There are five ORFs, three upstream and two downstream of lpg1728 (Fig. 5A). Since the predicted directions of transcription of all six ORFs were the same, it is possible that the Kan insertion has a polar effect on expression of the two ORFs downstream of *pmiA*. However, a 1.19-kb PCR-generated fragment containing only *pmiA* (lpg1728) was sufficient to complement the GB112 mutation (strain GB112C-5) for the defects in intracellular growth and cytopathogenicity for U937 macrophages and *A. polyphaga* (Fig. 1). Thus, the defect of the GB112 mutant in intracellular survival and trafficking is due to the defect in *pmiA*.

Topology analysis using the TopPred program (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html; Institut Pasteur, France) suggested that the predicted PmiA protein has three transmembrane domains (Fig. 5B). Following the putative transmembrane domain near the N terminus, there is a predicted long hydrophilic region (SOSUI program [http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html]) (Fig. 5C). These results suggested that PmiA might be a transmembrane protein.

We examined the gene distribution of *pmiA* in several species of the genus *Legionella* by genomic Southern hybridization, using low-stringency conditions for hybridizations (see Materials and Methods). The *pmiA* gene was present in all *L. pneumophila* strains regardless of the serogroup, but it was not detected in any other *Legionella* species examined (Fig. 6). This result suggested that *pmiA* might be specific for *L. pneumophila*.

PmiA is essential for contact-dependent pore formation. The pore-forming activity has been shown to be dependent on a functional Icm/Dot type IV secretion system (45). Many Icm/Dot proteins that are predicted to be structural components of the Icm/Dot secretion apparatus are essential for the pore-forming activity (21, 45, 51, 52). We examined the pore-forming activity of the GB112 mutant, using contact-dependent hemolysis of sRBCs. The *dotA* and *icmT* mutants were used as controls, since both of these mutants are defective in pore-forming activity. The GB112 mutant was completely defective in pore-forming activity, similar to the *dotA* and *icmT* mutants, as well as heat-killed bacteria (Fig. 7A). Importantly, the defect of the GB112 mutant was fully complemented by the wild-type *pmiA* gene on a plasmid (Fig. 7A). These results indicated that the PmiA protein is essential for the contact-dependent pore-forming activity.

***pmiA* mutation does not affect NaCl sensitivity.** *L. pneumophila* virulent strains have been shown to be sensitive to sodium chloride (16, 65). It has been proposed that the sodium sensitivity is likely to be dependent on the function of an intact Icm/Dot secretion system because *icm/dot* mutants are resistant to high concentrations of sodium ions. It has been speculated that a functional Icm/Dot secretion apparatus is leaky to NaCl, which may explain why mutants defective in the secretion apparatus are resistant to salt (81). We examined the sodium sensitivity of the GB112 mutant and compared it to that of other *icm/dot* mutants. The GB112 mutant was similar to the wild-type strain in terms of sensitivity to 0.6% sodium chloride, whereas the *dotA* mutant was resistant to NaCl (Fig. 7B).

DISCUSSION

The hallmark of Legionnaires' disease is the intracellular survival and growth of *L. pneumophila* within alveolar macrophages. It is thought that in natural aquatic environments, *L. pneumophila* is a parasite of protozoa (38, 54, 63), and the infected protozoa are important as a source of infection of humans by *L. pneumophila* (10, 27).

The major virulence *icm/dot* gene clusters of *L. pneumophila*, which encode components of type IV secretion systems, are required for intracellular growth and killing of human macrophages (14, 49, 60, 65, 67–69, 78, 80). The *icm/dot* genes of *L. pneumophila* are also required for intracellular growth within amoebae, as well as within human macrophages (71). However, many genes other than the *icm/dot* genes are also required for intracellular replication (26, 28, 30–40, 46, 48, 56, 59, 61, 62, 79). It is plausible that different sets of genes are utilized in a coordinated manner with the *icm/dot* genes. It is also possible that different pathways of pathogen-host interaction are host cell specific.

In this study, we characterized the GB112 mutant. This mutant has been identified as one of the *pmi* mutants (32). Our confocal laser scanning and transmission electron microscopic

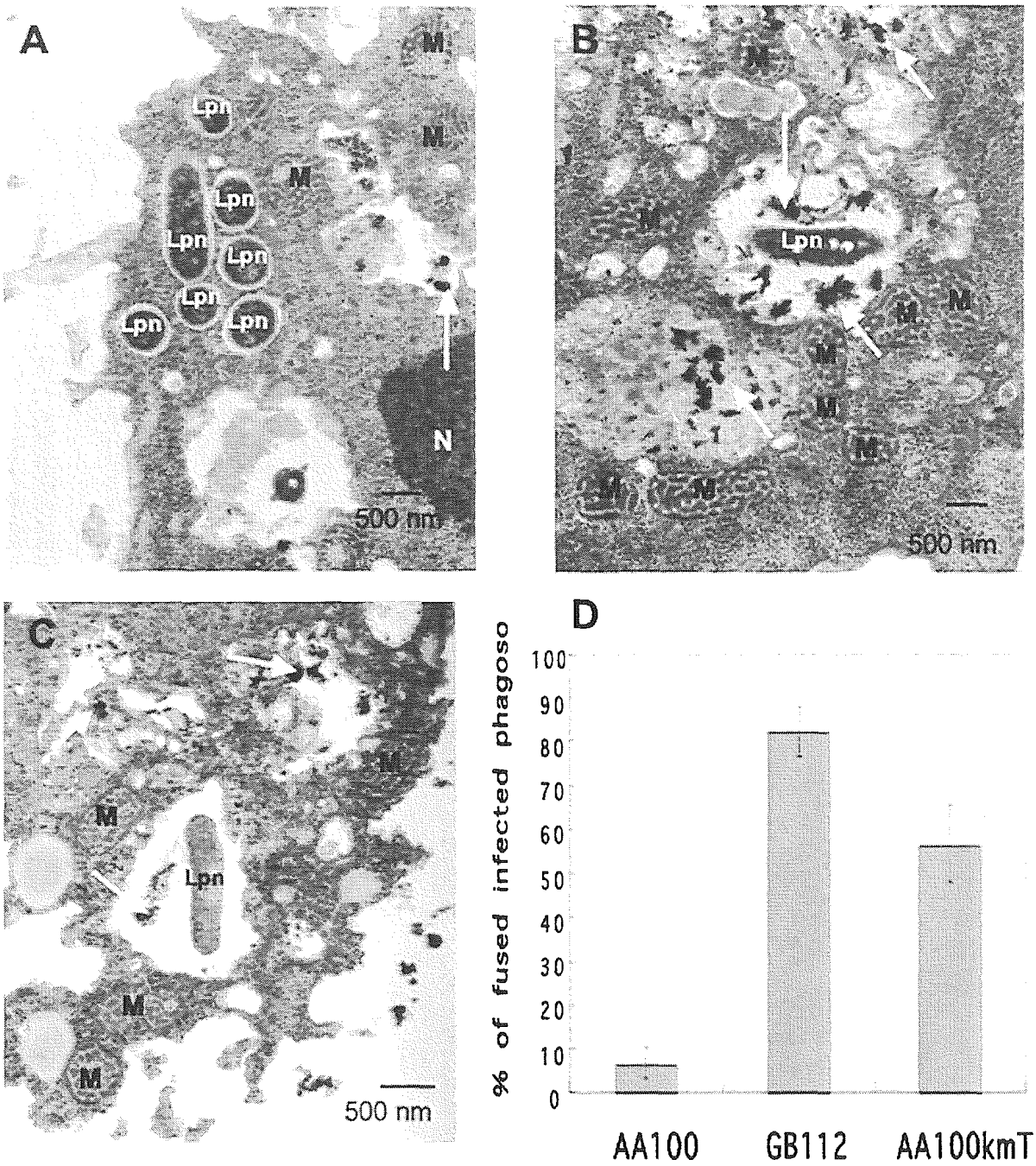

FIG. 4. Fusion of phagosomes containing the GB112 mutant in *A. polyphaga* to lysosomes. Localization of lysosomal acid phosphatase in phagosomes within *A. polyphaga* was determined at 6 h postinfection. (A to C) Representative electron micrographs of *L. pneumophila*-infected *A. polyphaga* at 6 h postinfection. Signals representing acid phosphatase were detected as electron-dense lead nitrate, as indicated by the arrows. Tight phagosomes containing wild-type strain AA100 (A) were negative for acid phosphatase. Phagosomes containing GB112 (B) or AA100KmT (C) (*icmT* deficient) exhibited positive signals for acid phosphatase. (D) Quantitative results obtained by examining 150 *Legionella*-containing phagosomes for the presence of acid phosphatase. Lpn, *L. pneumophila*; M, mitochondrion; N, nucleus; phagoso, phagosomes. The experiment was done three times in triplicate; the data are the data from one representative experiment, and the error bars indicate standard deviations.

studies showed that phagosomes containing the GB112 mutant are defective in evasion of acquisition of late endosomal and lysosomal markers at early stages of infection of both U937 macrophages and *A. polyphaga*. Thus, *pmiA* is involved in inhibition of fusion of the bacterium-containing phagosomes to lysosomes in both types of host cells. Interestingly, the *pmiA* mutant is severely defective in protozoa but exhibits a less severe defect in intracellular growth in the U937 human mac-

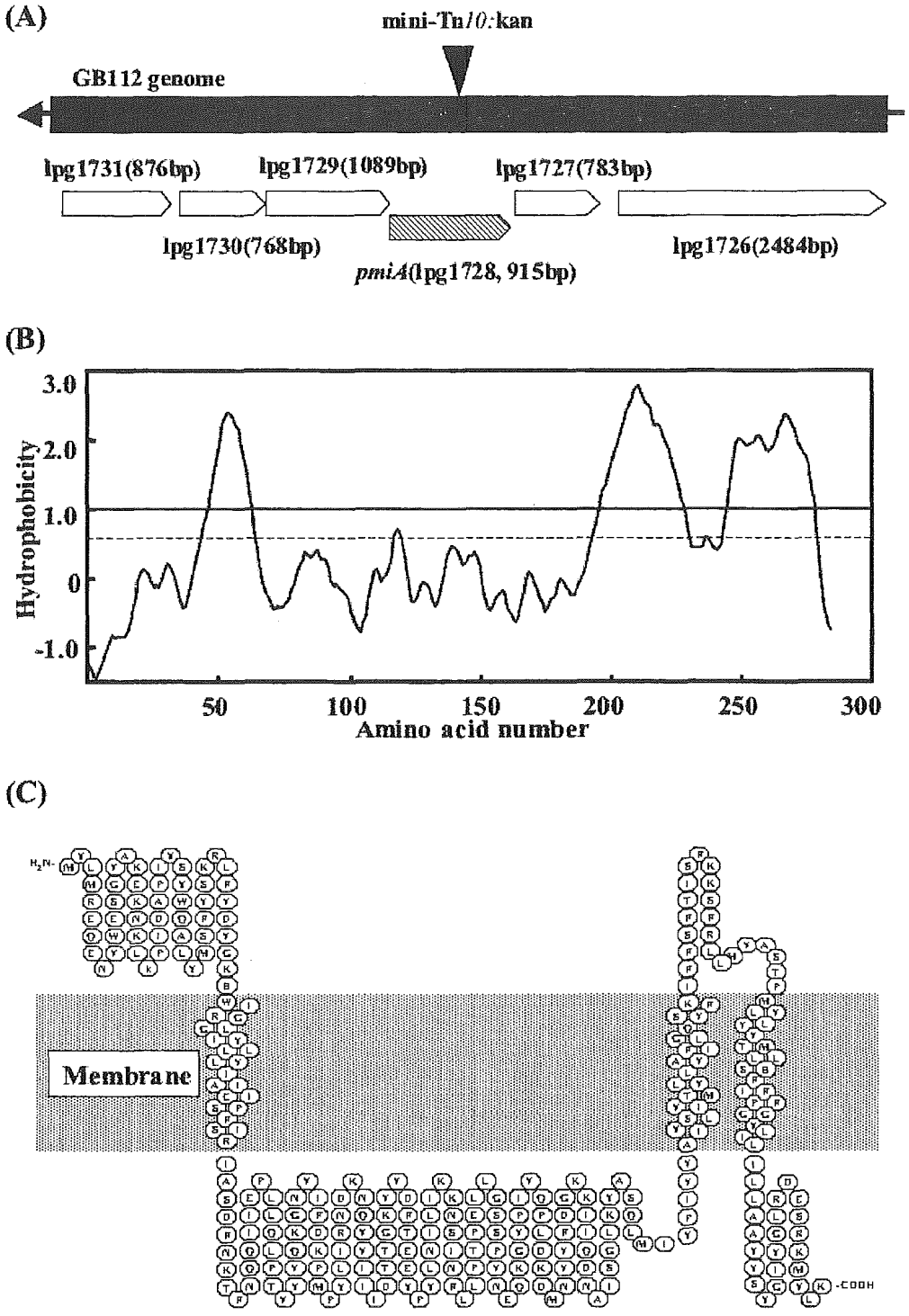

FIG. 5. Genetic and structural analysis of PmiA. (A) Genetic organization of the mutated region in the GB112 mutant. Mini-Tn*10*::*kan* was found to be inserted into a putative 915-bp open reading frame (lpg1728), *pmiA*. In the flanking regions, there are five ORFs up- and downstream of *pmiA* (lpg1731, lpg1730, lpg1729, lpg1727, and lpg1726), whose direction of transcription was the same as that of *pmiA*. (B) Hydropathy profile of the predicted PmiA protein amino acid sequence based on a Kyte-Doolittle analysis with a default window of 21. The solid and dashed lines indicate the cutoff values for certain and putative transmembrane segments, respectively. Negative values indicate relative hydrophilicity. (C) Secondary structure of PmiA predicted by the SOSUI program.

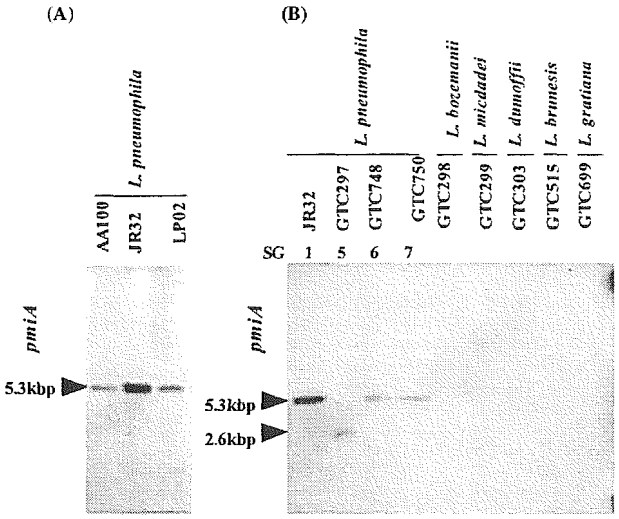

FIG. 6. Southern blot analysis of the *pmiA* gene in *L. pneumophila* serogroup 1 strains (A) and in several serogroups of *L. pneumophila* and other *Legionella* species (B). *pmiA* was detectable in four different serogroups of *L. pneumophila* but was absent from other *Legionella* species, including *L. bozemanii*, *L. micdadei*, *L. dumoffii*, *L. brunesis*, and *L. gratiana*.

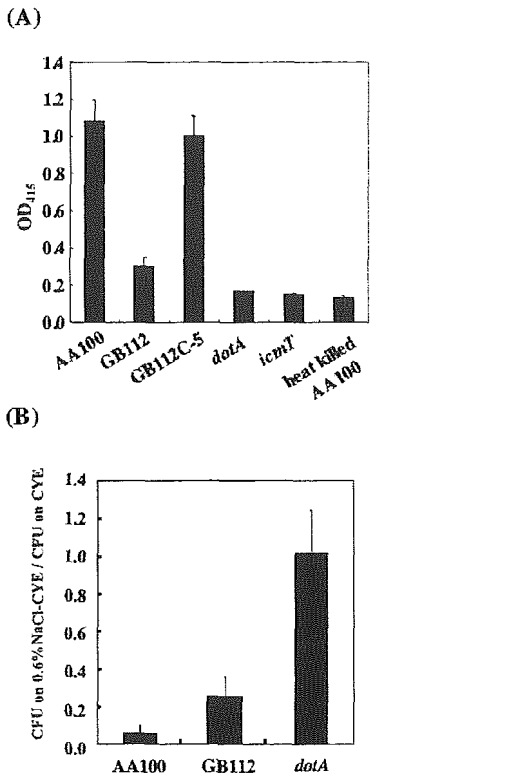

FIG. 7. PmiA is essential for the pore-forming activity but is not required for NaCl sensitivity of *L. pneumophila*. (A) The pore-forming activities of *L. pneumophila* mutant GB112 (*pmiA*), LELA3118 (*dotA*), and GS3011 (*icmT*) were tested by contact-dependent hemolysis of sRBCs. GB112C-5 is a *pmiA*-complemented strain of GB112. Wild-type strain AA100 and heat-killed AA100 were used as positive and negative controls, respectively. (B) NaCl sensitivity of the GB112 mutant. The data are expressed as the ratio of the colony-forming activity after plating on 0.6% NaCl–BCYE agar plates to the colony-forming activity after plating on regular BCYE plates. The AA100 (wild type) and *dotA* strains were used as NaCl-sensitive and NaCl-resistant controls, respectively. The experiments were done three times in triplicate; the data are the data from one representative experiment, and the error bars indicate standard deviations.

rophage cell line. Although the intracellular growth of the *pmiA* mutant within U937 cells shows a partially defective phenotype, LAMP-1 and LAMP-2 colocalize with phagosomes containing the *pmiA* mutant. The acquisition of these late endosomal and lysosomal markers by the *pmiA* mutant-containing phagosomes is correlated with the defect in intracellular survival and replication. It is interesting that despite the colocalization of LAMP-1 and LAMP-2 with phagosomes containing the *pmiA* mutant within U937 macrophages, the bacteria that were able to establish a replicative niche replicated in these cells. It has been reported that the *dotA* and *dotB* mutants reside in a nonlysosomal LAMP-1-positive compartment within mouse-derived bone marrow macrophages (43). The *pmiA* mutant might reside in the same type of phagosome as *dotA* and *dotB* mutants within macrophages.

The *pmiA* gene encodes a putative transmembrane protein that has three membrane-spanning domains based on hydropathy and membrane topology analysis. Interestingly, PmiA has a distinct long hydrophilic region between the transmembrane domains. This region has high similarity to hypothetical proteins of other bacteria possessing type IV secretion systems, such as *Rickettsia* species and *Helicobacter pylori*. A comparison using the protein-protein BLAST (blastp) program revealed that the region between amino acids 36 and 269 of PmiA, which covers the entire long hydrophilic region and the neighboring two transmembrane regions, exhibits similarity (20% identity and 40% similarity) to the corresponding part of hypothetical protein RP489 (accession no. Q9ZD57) of *Rickettsia prowazekii* (9). Interestingly, the corresponding hypothetical protein of *Rickettsia* species is predicted to be a transmembrane protein, and the secondary structure of the whole protein is also similar to that of PmiA (data not shown). The region between amino acids 68 and 155, which covers most of the long hydrophilic region, exhibits similarity (29% identity and 48% similarity) to hypothetical protein jhp0336 (accession no. C71944) of *H. pylori* (8). At least five ORFs both up- and downstream of *pmiA*, which have the same transcriptional direction as *pmiA*, exhibit no similarity with any genes of these bacteria. The genomic positions of the genes encoding each of the corresponding hypothetical proteins of these pathogens are far from the genetic loci that encode the type IV secretion apparatus (data not shown). The functions of these proteins in intracellular survival and replication and whether there is any relationship to the type IV secretion apparatus have not been reported.

We demonstrate here that PmiA is involved in the pore-forming activity which is attributed to the Icm/Dot type IV secretion system (45). The loss of the pore-forming activity in many *icm/dot* mutants, including *dotA*, *icmT*, *icmQ*, and *icmR* mutants, has been demonstrated previously (45, 51, 52). Recently, it has been shown that the IcmQ protein is localized on the bacterial surface shortly after contact with a macrophage

(25). It has been suggested that pore formation in the mammalian cell membrane is caused by membrane insertion of IcmQ after its translocation to the bacterial surface (25). It is possible that PmiA is a component of the Icm/Dot secretion apparatus involved in export of IcmQ.

In summary, we show here that a defect in *pmiA* results in a defect in survival and replication of *L. pneumophila* in U937 macrophages and protozoa. Interestingly, the *pmiA* mutant exhibits a severe defect in intracellular growth within protozoa, in contrast to the partial defect in intracellular growth within macrophages. This defect is associated with trafficking of the *pmiA* mutant-containing phagosome through the endosomal-lysosomal pathway in macrophages and protozoa. The severe defect in the pore-forming activity of the *pmiA* mutant, in addition to a defect in its intracellular trafficking, suggests a potential contribution of the PmiA protein to export of Icm/Dot substrates. However, this potential role of PmiA in export of Icm/Dot substrates must be demonstrated directly.

ACKNOWLEDGMENTS

We thank Howard A. Shuman (Columbia University) and Masahisa Watarai (Obihiro University of Agriculture and Veterinary Medicine) for kind gifts of the *L. pneumophila* strains. We are also grateful to Takashi Fukui, Toshihiko Harada, Maki Kato, and Asano Ikegaya for technical assistance.

This work was supported in part by grant-in-aid 16790258 and the COE Program in the 21st Century from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Grant for Research on Cancer Prevention and Health Services H15-gan-yobou-095 from the Ministry of Health, Labor and Welfare. Y.A.K. was supported by Public Health Service awards RO1AI43965 and R21AI038410-06A1 and by the Commonwealth of Kentucky Research Challenge Trust Fund.

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