TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and features[a]	Reference or source
L. pneumophila		
80-045	Serogroup 1, wild-type isolate	48
LAM0101	80-045 *laiA*::Km^r	This study
LAM0102	LAM0101 with pMMBLG0503	This study
LAM0103	LAM0101 with pMMB207C	This study
E. coli		
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F′*proAB* $lacI^q$Z DM15 Tn*10* (Tet^r)]	Stratagene
XLOLR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 thi-1 recA1 gyrA96 relA1 lac*[F′ *proAB* $lacI^q$ZΔ M15 Tn*10* (Tet^r)]	Stratagene
DH10B	F^- *mcrA* (*mrr-hsdRMS-mcrBC*) ϕ*80lacZ* Δ M15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*) *7697 galU galK* λ^- *rpsL nupG*	Invitrogen
Plasmids		
pUC19	*oriR* (ColE1) MCS Ap^r	56
pACYC177	*oriR* (P15A) Km^r	1a
pBK-CMV	*oriR* (ColE1) MCS Neo^r Km^r	Stratagene
pCR-XL-TOPO	*oriR* (ColE1) *ccdB* MCS Km^r $Zeocin^r$	Invitrogen
pLAW344	*sacB* MCS *oriT* (RK2) Cm^r *loxP oriR* (ColE1) Ap^r *loxp*	54
pMMB207C	RSF1010 derivative, IncQ $lacI^q$ Cm^r *Ptac oriT* MCS *mobA*::Km^s	32
pBKLG04	Km^r, contains the region from base 1477 to base 4542 of *laiA* on the phagemid pBK-CMV	This study
pCRLG06	Km^r, contains the region from base 1 to base 3913 of *laiA* on pCR-XL-TOPO	This study
pLAWLG0501	Cm^r, contains the region between the BamHI and SphI sites of pUCLG0501 in the EcoRV site of pLAW344	This study
pMMBLG0503	Cm^r, contains the region from base 1 to base 4542 of *laiA* between the SmaI and SalI sites of pMMB207C	This study
pUCLG05	Ap^r, contains the region from base 2318 to base 4542 of *laiA* between the HincII and XbaI sites of pUC19	This study
pUCLG06	Ap^r, contains the region from base 1 to base 3609 of *laiA* between the PstI and HincII sites of pUC19	This study
pUCLG0501	*laiA*::Km^r of pUCLG05	This study
pUCLG0503	Ap^r, contains the region from base 1 to base 4542 of *laiA* between the PstI and HincII sites of pUC19	This study

[a] MCS, multiple cloning sites; Neo, neomycin.

Plasmids pUC19 (56) and pCR-XL-TOPO (Invitrogen) were used as vectors for cloning the *L. pneumophila* genomic DNA. The vector pLAW344 (54) was used for allelic exchange, whereas pMMB207C (32) was used for complementation studies with *L. pneumophila*. pLAW344 and pMMB207C were kindly supplied by H. Miyamoto (School of Medicine, University of Occupational and Environmental Health, Japan).

Antimicrobial agents for the selection of *L. pneumophila* were purchased from Sigma and used at the following concentrations: kanamycin (Km), 25 μg/ml; and chloramphenicol (Cm), 5 μg/ml. The antibiotic concentrations for *E. coli* were as follows: Km, 50 μg/ml; ampicillin (Ap), 50 μg/ml; and Cm, 25 μg/ml.

Nucleic acid manipulations. All nucleic acid manipulations were done according to standard molecular biology techniques (49). The restriction enzymes used were purchased from Takara (Shiga, Japan). PCR amplifications were carried out with the GeneAmp PCR System 2400 (Perkin-Elmer Biosystems). DNA sequencing was performed with an ABI 310 DNA sequencer (Applied Biosystems). Genetyx-Mac (Genetyx) and BLAST Search (National Center for Biotechnology Information) programs were used for analysis and homology searches. Homology searches were also carried out using the sequence data published by the *Legionella* Genome Project (Columbia Genome Center [http://genome3.cpmc.columbia.edu/~legion/]).

Absorption of sera. Human sera used in the study were separated from the blood of five patients who recovered from Legionnaires' disease and from that of two healthy people. To obtain convalescent-phase sera of mice, five A/J mice (SLC, Hamamatsu, Japan) were intranasally infected with *L. pneumophila* 80-045. After 60 days, the convalescent-phase sera were obtained from mice that recovered from the infection. To remove antibodies that react with *E. coli* and phage proteins, the sera were absorbed with an *E. coli*-phage lysate as described in the instruction manual of the picoBlue immunoscreening kit (Stratagene). The pools of the absorbed sera obtained from five patients, two healthy people, and five mice were used for immunoscreening.

To obtain a monospecific antibody for LaiA, convalescent-phase sera of mice were absorbed with both a living and heat-killed *laiA* mutant (LAM0101). LAM0101 was washed twice with phosphate-buffered saline (PBS) and collected by centrifugation at 3,500 rpm for 15 min. Mouse sera were incubated with the living bacteria at 50°C for 2 h. The sera were gathered by centrifugation, reabsorbed with heat-killed LAM0101 which were boiled in a water bath for 1 h, and incubated for an additional 2 h at 50°C. After incubation, the sera were collected by centrifugation and filtered with a 0.22-μm filter (Corning).

Construction and immunoscreening of an *L. pneumophila* expression library. A Lambda ZAP II custom genomic DNA expression library (Stratagene) was constructed according to the manufacturer's instructions. After growth of *L. pneumophila* 80-045 on BCYE agar at 37°C for 2 days, genomic DNA was isolated, purified, and partially digested with Sau3AI. Fragments varying from 3 to 6 kb were isolated through sucrose gradient centrifugation and ligated to BamHI-digested Lambda ZAP II arms. Subsequently, the 80-045 expression library was screened with the convalescent-phase mouse sera as described in the protocols provided with the picoBlue Immunoscreening Kit (Stratagene). Inserted DNA fragments of the clones that reacted with the convalescent-phase sera were excised as pBK-CMV phagemid plasmids by simultaneous infection of helper phage in XL1-Blue MRF′ cells.

SDS-PAGE and Western blot analysis. Bacterial cells were lysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl, pH 6.8, containing 2% SDS, 6% β-mercaptoethanol, and 10% glycerol) and boiled at 100°C for 10 min. A solution of 10 μl of each sample was laid on an 8% or 12% resolving gel. Proteins were separated using SDS electrophoresis and electroblotted onto Hybond ECL nitrocellulose membrane (Amersham). The membrane was probed with antisera, and the signals were detected with ECL Western blotting detection reagents (Amersham). In some experiments, *L. pneumophila* cells were treated with 0.4 mg/ml proteinase K (PK)

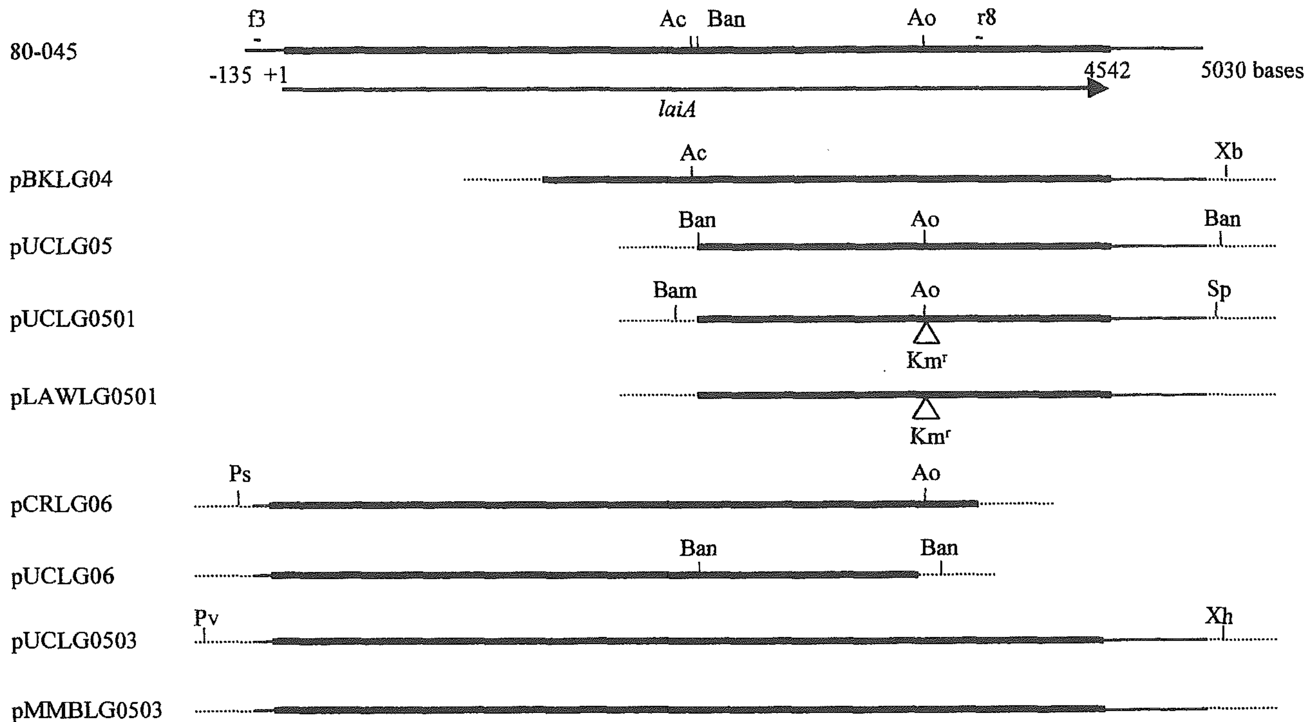

FIG. 1. Schematic diagrams of *laiA* in the chromosome of *L. pneumophila* 80-045 and of plasmids constructed in this study. The chromosome of 80-045 is presented as a solid line, and the vectors are presented as broken lines. The region corresponding to the *laiA* gene is shown as heavy lines. The putative translational start codon of the LaiA protein is indicated as 1. The arrow indicates the direction of transcription of the *laiA* gene. Sites for primers f3 and r8 used in PCR analysis are shown. Triangles show the insertion of the Km^r cassette. Abbreviations for the restriction enzymes: Ac, AccII; Ao, Aor51HI; Bam, BamHI; Ban, BanII; Ps, PstI; Pv, PvuII; Sp, SphI; Xb, XbaI; Xh, XhoI.

and/or 0.01% Triton X-100 at 37°C for 30 min before lysis with the sample buffer as described by Hazlett et al. (20).

Construction of plasmids for allelic exchange. The constructed plasmids used in this study are shown in Fig. 1. First, a DNA fragment of the recombinant plasmid pBKLG04, which contained the 3′ terminus (from the base 1477 to 4542) of *laiA* in the pBK-CMV phagemid vector, was digested with XbaI and AccII (base 2318 of *laiA*). Thereafter, the fragment containing part of *laiA* was cloned into the XbaI and HincII sites of pUC19 to generate pUCLG05. To knock out the *laiA* gene, a Km resistance (Km^r) cassette from pACYC177 (1a) was inserted. The Km^r cassette was ligated to the Aor51HI site (base 3609 of *laiA*) of pUCLG05, and the resultant plasmid was called pUCLG0501. Subsequently, the pUCLG0501 DNA was digested with BamHI and SphI. The fragment, which carried the *laiA* gene containing the Km^r cassette, was blunt ended and ligated to the EcoRV site of pLAW344 (54) to generate pLAWLG0501. Lastly, pLAWLG0501 DNA was introduced into 80-045 by electroporation to mediate allelic exchange, and the strain was plated on BCYE agar containing Km after growth in AYE at 37°C for 5 h (54). Km^r Suc^r Cm^s colonies, which should be Km^r mutants of the *laiA* gene on the chromosome, were selected, and one of them was named LAM0101.

Construction of plasmids for complementation. To obtain a fragment that contained the entire gene, a portion of the 5′ terminus of *laiA* was amplified by PCR and ligated to the 3′ terminus. The DNA fragment corresponding to the 5′ terminus of *laiA* (approximately 3.9 kb in length) was amplified by PCR with primers f3 (5′-CTGCCCTGCTCTTTATAC-3′) and r8 (5′-CGAGTTACTCGC AAAACTCG-3′), using total cellular DNA from 80-045 cells as the template. The nucleotide sequence of primer f3, which is located upstream of the *laiA* gene, was determined by the sequence of an *L. pneumophila* Philadelphia-1 isolate (http://genome3.cpmc.columbia.edu/~legion/). The PCR product was cloned into pCR-XL-TOPO (Invitrogen) to produce pCRLG06. After digestion of pCRLG06 DNA with PstI and Aor51HI (base 3609 of *laiA*), the fragment corresponding to the 5′ terminus of *laiA* was cloned into the PstI and HincII sites of pUC19 to generate pUCLG06. Subsequently, the fragment containing the 3′ terminus of the *laiA* gene was ligated to the 5′ terminus; the BanII fragment (from base 2326 of *laiA* to the BanII site of the vector) of pUCLG06 was replaced by the 3′-terminal fragment of *laiA*, which was derived from a BanII fragment of pUCLG05. The constructed plasmid containing the entire *laiA* gene was named pUCLG0503. The pUCLG0503 DNA was digested with PvuII and XhoI, and the obtained fragment was cloned into the SmaI and SalI sites of the pMMB207C vector to generate pMMBLG0503. The sequence of *laiA* of pMMBLG0503 was confirmed to be the same as that of 80-045, and expression of the *laiA* gene in pMMBLG0503 was confirmed by Western blot analysis with patient sera. pMMBLG0503 and empty pMMB207C DNAs were introduced into LAM0101 to generate LAM0102 and LAM0103, respectively.

Growth and motility of bacteria in the laboratory. Growth of *L. pneumophila* was examined as follows. *L. pneumophila* was inoculated from a BCYE agar plate into 5 ml of AYE broth at a concentration of 10^8 CFU/ml. After overnight incubation at 37°C, the bacteria were suspended in 5 ml of fresh medium at an optical density at 600 nm of 0.3 and further cultured. The optical density of the culture was measured at 600 nm every 2 h for 24 h. The presence of pili and flagella in *L. pneumophila* was assessed by transmission electron microscopy after negative staining, and motility was assessed using microscopy (6).

Cell culture method. A549 cells (JCRB0076), a type II alveolar epithelial cell line, and U937 cells (JCRB9021), a human monocytic cell line, were obtained from the Japanese Collection of Research Bioresources cell bank (Osaka, Japan). All of the cells were maintained in RPMI 1640 medium (Invitrogen) plus 10% heat-inactivated fetal calf serum (HyClone). For experiments, the A549 cells were seeded into 24-well flat-bottom tissue plates (Corning) at a concentration of 5×10^5 cells/ml and incubated for 18 h at 37°C in a humid atmosphere containing 5% CO_2 in air. The U937 cells were seeded into 24-well plates at a concentration of 1×10^6 cells/ml and differentiated into macrophages by incubating for 48 h in RPMI medium with 10^{-8} M phorbol 12-myristate 13-acetate in the same atmosphere.

Adhesion and invasion assays. A suspension of *L. pneumophila* culture in RPMI 1640 medium was added to A549 monolayers at multiplicities of infection of 20 to 2,000 and was further cultured for 1.5 h. The monolayers were washed twice with PBS to remove nonadherent bacteria. For the adhesion assay, the cells were peeled from the well by addition of 0.1% Triton X-100 and sonicated with Bioruptor UCD-200T (Cosmo Bio, Tokyo, Japan) at 130 W for 24 s. A series of

10-fold dilutions of the sonicated solution was plated on BCYE plates, and the number of bacteria was counted. For the invasion assay, after washing twice with PBS, 0.5 ml of the culture medium supplemented with 100 μg/ml of gentamicin (Wako Pure Chemical Industries, Osaka, Japan) was added to each well and incubated for 2 h to kill extracellular bacteria. The cells were washed again with PBS, and the number of intracellular bacteria was determined as described above. To correct for the variation in level between experiments, adhesion or invasion of the tested strains was indicated as a ratio to that of the wild 80-045 isolate [i.e., relative adhesion = (percent adhesion of test strain/percent adhesion of 80-045) × 100%].

Intracellular growth assay. Approximately 1×10^6 bacteria were added to each well inoculated with U937 cells. After incubation with the bacteria for 1 h at 37°C, the cells were washed twice with PBS and treated with 100 μg/ml of gentamicin for 2 h. The initial time point ($t = 0$) is the first time point immediately after gentamicin treatment and represents intracellular bacteria that were enumerated following treatment of the host cell with Triton X-100. Infections were allowed to proceed until the indicated time. U937 cells were then lysed, and bacteria that grew intracellularly were enumerated on BCYE plates.

Mouse infection and LD_{50}. For examination of the difference in virulence of *L. pneumophila* strains, 6-week-old male A/J mice (SLC, Shizuoka, Japan), in whose peritoneal macrophages *L. pneumophila* is able to grow (57), were used. After anesthetization by injection under the skin with a mixture of ketamine hydrochloride (60 mg/kg; Sigma) and xylazine (12 mg/kg; Sigma), A/J mice were infected by intranasal inoculation with 40 μl of *L. pneumophila*. Lungs of mice were removed at 1, 24, and 48 h after the inoculation and homogenized. The homogenate was diluted with saline and plated on BCYE plates for determination of bacterial number. Mortality of mice was monitored for 30 days after the infection, and the 50% lethal dose (LD_{50}) was calculated by the Reed-Muench method (41). All animal experiments complied with the National Institute of Infectious Diseases guidelines regarding the use of animals in research.

Statistical analyses. All in vitro experiments were carried out in triplicate and repeated at least three times. The significance of the results was analyzed using Student's t test. In the LD_{50} experiments, the significance of survival curve differences was examined by both log rank test and Wilcoxon test methods. Differences were considered significant at a P value of <0.05.

Nucleotide sequence accession numbers. Nucleotide sequences reported in this paper are available in the GenBank database under accession no. AB107985, AB107986, AB107987, AB185448, and AB196938.

RESULTS

Identification of novel *L. pneumophila* antigens reacted with convalescent-phase sera by immunoscreening of the 80-045 expression library. In order to identify antigens that were expressed during infection caused by *Legionella*, a lambda ZAPII phage expression library of 80-045 was generated and screened with the convalescent-phase mouse sera. Forty-five positive clones appeared from approximately 100,000 plaques, and the positive lambda ZAP phages were converted into the phagemid form in XL1-Blue MRF′. In order to confirm the expression, total proteins of bacteria harboring the phagemids were reacted with convalescent-phase sera of Legionnaires' disease patients. Twelve clones were positive (data not shown).

Subsequently, the nucleotide sequences of the inserts were determined and the putative open reading frames (ORFs) within them were identified. The fragments corresponding to each ORF of the 12 recombinant phagemids were independently subcloned into the relevant sites of pUC19. Expression of these ORFs was tested again by immunoreaction with the convalescent-phase patient sera. Nine clones that reacted, i.e., N1, N2, N4, N5, N8, N25, N31, N32, and N34 (isolate numbers), were selected (data not shown). Clones N2, N4, N31, and N32 and clones N5, N8, and N34 expressed the same antigentic proteins. Protein expression of the representative positive clones N1, N5, N25, and N31 was further examined by reaction with sera of healthy people. Three clones, N1, N5, and N31, expressed proteins that reacted only with the patient sera (data not shown).

The ORF of N1 encoded a 29-kDa polypeptide which contained 253 amino acid residues and lacked the 5′- and 3′-terminal ends. The ORF of N5 encoded a 33-kDa polypeptide which consisted of 292 amino acid residues. The two peptides corresponded to Lpg2803 and Lpg2327 of *L. pneumophila* Philadelphia-1 (3). The two genes were named *lg*N1-1 and *lg*N5-1 for *L. pneumophila* genes N1-1 and N5-1, respectively. The ORF of N31 encoded a 134-kDa polypeptide, which contained 1,021 amino acid residues, at the 3′-terminal portion of a presumed open reading frame. The C terminus of the polypeptide shared homology (41% similarity over 454 residues) with the integrin analogue of *Saccharomyces cerevisiae* (25). This result suggested that the gene might be involved in adhesion of *L. pneumophila*. The gene was named *laiA* for *L. pneumophila* adhesion molecule homologous to the integrin analogue of *S. cerevisiae*. The role of *laiA* in infection was further investigated in this study.

Cloning of the entire *laiA* gene from the 80-045 isolate. The DNA fragment containing the entire *laiA* gene of strain 80-045 was cloned into pMMB207C. The sequence was determined in both directions by using appropriate primers synthesized according to the analyzed sequences. The *laiA* gene consisted of 4,542 nucleotides and was predicted to encode a 1,513-residue polypeptide with a theoretical molecular mass of 170 kDa. A homology search revealed that the *laiA* gene shared 80.5% identity at the nucleotide level with the corresponding gene (*lpg2157*) of Philadelphia-1. There was 76.9% identity between the amino acid residues encoded by the two genes. Furthermore, five paralogs of *lpg2157* (*lpg2153*, *lpg2154*, *lpg2156*, *lpg0234*, and *lpg2509*) were present on the chromosome of Philadelphia-1 (3). We also determined the nucleotide sequences of these *laiA* paralogs of 80-045, which corresponded to those of Philadelphia-1. Three of them, *laiB*, *laiC*, and *laiE*, were located upstream of *laiA*, whereas the other two genes, *laiD* and *laiF*, were located separately in another region of chromosome (Fig. 2A). The *laiB*, *laiC*, *laiD*, *laiE*, and *laiF* genes consisted of 5,763, 4,605, 4,545, 1,131, and 1,149 nucleotides, respectively. They were predicted to encode 1,920-, 1,534-, 1,514-, 376-, and 382-residue polypeptides with theoretical molecular masses of 217, 173, 172, 43, and 43 kDa, respectively. At the nucleotide level, the *laiA*, *laiB*, *laiC*, and *laiD* genes shared approximately 80% homology with each other, but they had lower homology (45% to 49%) with the *laiE* and *laiF* genes.

During the preparation of this paper, Luo and Isberg (27) reported five paralogs, i.e., *sdeA*, *sdeB*, *sdeC*, *sidE*, and *sdeD*, of *L. pneumophila* strain LP02, a derivative of Philadelphia-1. The five genes corresponded to and were identical to *lpg2157*, *lpg2156*, *lpg2153*, *lpg0234*, and *lpg2509*, respectively (3). Those authors indicated that these proteins are translocated by a Dot/Icm type IV secretion system (27, 47, 51). At the amino acid level, LaiD, LaiE, and LaiF shared higher homology (98.3%, 97%, and 99%, respectively) with the corresponding SidE (Lpg0234), Lpg2154, and SdeD (Lpg2509) polypeptides. LaiC and LaiB possessed 89% and 83.1% homology with the corresponding polypeptides SdeC (Lpg2153) and SdeB (Lpg2156), respectively. However, there was 76.9% homology between LaiA and SdeA (Lpg2157). Compared to the other

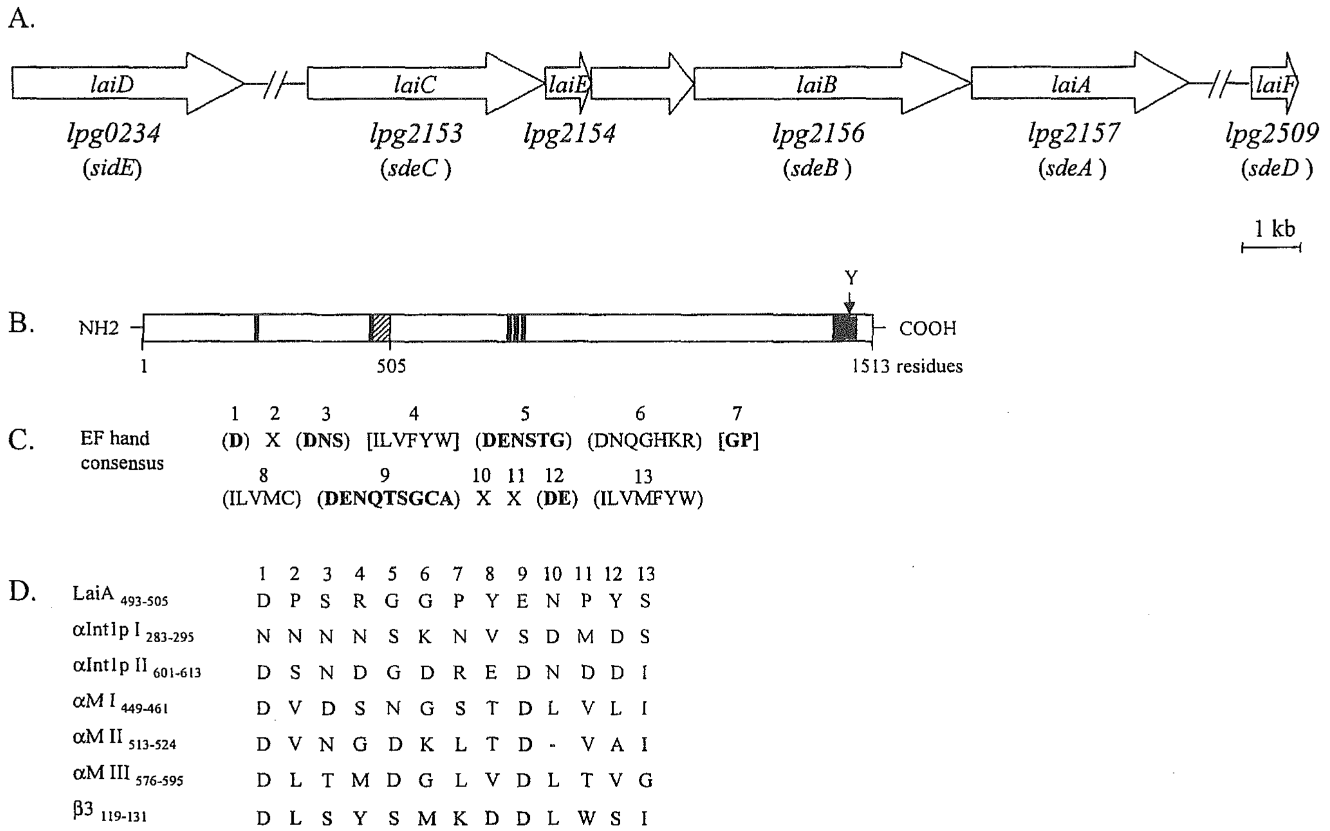

FIG. 2. (A) Schematic representation of the chromosomal region containing *laiA* and its five paralogs in *L. pneumophila* 80-045. The corresponding genes of Philadelphia-1 (3) and Lp02 (27) are presented under the predicted ORFs. (B) Domain structure of LaiA. The putative divalent-cation-binding site is shown as a hatched box, the transmembrane domain is shown as a gray box, and the DXSX motifs are shown as black boxes. (C and D) Comparison of divalent-cation-binding motifs. (C) The consensus amino acid sequences for the 13 amino acid residues of EF hand divalent-cation-binding motifs (1). Parentheses, acceptable amino acids; brackets, unacceptable amino acids; X, any amino acid. Cation-coordinating sites are shown in boldface. (D) Alignment of the putative cation-binding site of LaiA, two cation-binding sites in *C. albicans* αIntlp, three cation-binding sites in α_M, and one cation-binding site in β_3 (14, 15, 24). The standard single-letter code is used. A dash indicates a gap. The figure was designed according to the work of Hostetter (24).

paralogs, *laiA* (*sdeA*, *lpg2157*) seems to be more diverse in the various *L. pneumophila* isolates.

Characteristics of the LaiA polypeptide. We searched LaiA for the characteristic motifs present in the integrins of *Candida albicans* and vertebrate cells (15, 24). We could not find the putative I domain of integrins and integrin analogues in LaiA. However, LaiA carried five homologues of potential MIDAS motifs (DXSX) for the coordination of divalent cations. Two of them were located at the N terminus, and the remaining three were in the middle of the LaiA protein (Fig. 2B). Chou-Fasman analysis (Genetyx) indicated that LaiA has multiple α-helices, which are present in integrins and integrin analogues (data not shown). The divalent-cation-binding sites in the α subunits are known to facilitate the ligand binding of integrins to the tripeptide Arg-Gly-Asp (RGD) (15, 24). One putative divalent-cation-binding site was found at residues 493 to 505 of the LaiA polypeptide. Comparison to the consensus sequence for the divalent-cation-binding site (Fig. 2C) revealed that there were four mismatches of amino acids at positions 7, 8, 12, and 13 (Fig. 2D). Similar to the case for the other integrins, a transmembrane domain (hydrophobic sequence) was located at amino acids 1436 to 1463 of LaiA as determined by Hydo-Cluster analysis (Genetyx), and a single tyrosine residue was also present in the C-terminal end (Fig. 2B). Thus, LaiA contained several characteristic integrin motifs.

Isolation of a *laiA* mutant by allelic exchange. To examine the involvement of the *laiA* gene in infection caused by *L. pneumophila*, a *laiA* mutant (LAM0101) was constructed by introduction of a Km^r cassette. LAM0101 was selected, and the presence of the Km^r cassette in the *laiA* gene on the chromosomal DNA was confirmed by PCR and DNA sequence analysis.

Characteristic phenotypes of LAM0101 were compared with those of 80-045 under laboratory conditions. The colony morphology, growth rate in AYE broth, and sodium sensitivity of LAM0101 were not significantly different from those of the wild isolate (data not shown). Furthermore, there were no obvious differences in the presence of pili and flagella or in motility between 80-045 and LAM0101 in AYE broth (data not shown).

Exposure of LaiA to the cell surface of *L. pneumophila*. To determine the cellular location of the LaiA protein, the PK accessibility method was employed. Total cellular proteins from 80-045, which were treated with PK, PK and Triton

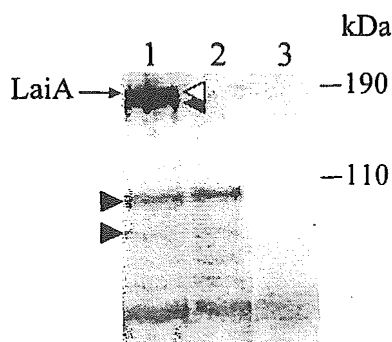

FIG. 3. Immunoblot of the total proteins of *L. pneumophila* with mouse monospecific anti-LaiA convalescent-phase sera. After suspension in SDS-PAGE sample buffer, samples were electrophoresed on an 8% running gel, blotted, and reacted as described in Materials and Methods. An open arrowhead indicates the full-length LaiA protein. Bands shown by closed arrowheads seem to be the degraded fractions of LaiA. Positions of molecular mass markers are indicated on the right. Lane 1, total cellular protein of 80-045; lane 2, total cellular protein of PK-treated 80-045; lane 3, total cellular protein of LAM0101.

X-100, or Triton X-100 only, were analyzed by Western blotting with the monospecific anti-LaiA antibody. As shown in Fig. 3, a band corresponding to the full-length LaiA protein was located at a molecular mass of approximately 170 kDa (lane 1). There were several bands that reacted with the LaiA-specific antibody (lane 1). These seemed to be degradation products of LaiA, because no bands were detected in the total cellular protein of LAM0101 (lane 3). After digestion of intact 80-045 bacteria with PK, the band corresponding to the full-length LaiA protein disappeared (lane 2). The same result as seen in lanes 1 and 2 was obtained when the 80-045 cells were treated with Triton X-100 only and with both PK and Triton X-100 (data not shown). These results suggest that a portion of LaiA that is sensitive to PK was exposed to the cell surface.

Adhesion and invasion of the *L. pneumophila* 80-045 isolate depends on the *laiA* gene. The ability of *L. pneumophila* to adhere to and invade A549 epithelial cells was examined. *L. pneumophila* 80-045 adhered to and invaded the A549 cells at rates of approximately 7×10^{-3} and 3×10^{-6} with respect to the number of inoculated bacteria, respectively. The adherence rate of the *laiA* mutant was 70% lower than that of the wild type (Fig. 4A). The level of adhesion of LAM0101 was complemented to that of 80-045 by addition of pMMBLG0503 but not by addition of the empty pMMB207C vector (Fig. 4A). The invasion ability of LAM0101 was also 70% lower than that of the 80-045 isolate (Fig. 4B). The *laiA*-complemented strain LAM0102 recovered the level of invasive ability of the wild isolate (Fig. 4B). These results suggest that the *laiA* gene is involved in the adhesion of 80-045 to A549 alveolar epithelial cells and that adhesion may be one of the most important prerequisites for invasion of *L. pneumophila*.

The gentamicin sensitivities of *L. pneumophila* wild-type 80-045, LAM0101, LAM0102, and LAM0103 were compared by incubating the strains with gentamicin at 100 μg/ml for 2 h. No significant differences in the susceptibility to gentamicin were found (data not shown).

The *laiA* gene does not affect intracellular multiplication of *L. pneumophila* in macrophage cells. Intracellular growth of 80-045 and LAM0101 in the human monocytic U937 cells was examined (Fig. 5). The number of *laiA* mutant cells in the U937 cells was approximately 40% of that of the wild type strain at 0 h (the first time point following the gentamicin treatment to kill extracellular bacteria). The numbers of the two strains growing in the U937 cells increased approximately 1,000 and 100,000 times at 24 and 48 h after infection, respectively. The rates of bacterial growth in U937 cells were not significantly different for 80-045 and LAM0101. This result shows that the *laiA* gene does not significantly affect the growth rate of *L. pneumophila* in U937 cells.

***laiA* affects the virulence of *L. pneumophila* 80-045 in the A/J mouse model.** In order to elucidate whether the reduction of adherence to epithelial cells in the *laiA* mutant affects the virulence of *L. pneumophila*, we performed two assays: elimination of bacteria from mouse lungs and mouse lethality after

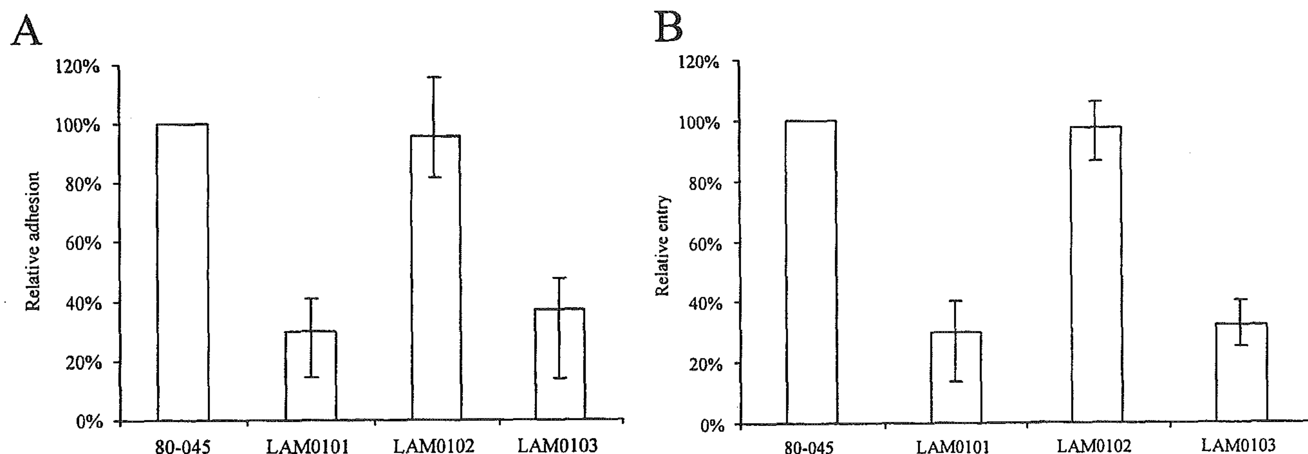

FIG. 4. Ability of *L. pneumophila* to adhere to (A) and invade (B) A549 alveolar epithelial cells. Data points and error bars represent the means and standard errors. Shown here are the results with the wild *L. pneumophila* strain 80-045, the *laiA* mutant strain LAM0101, the *laiA*-complemented (pMMBLG0503) strain LAM0102, and LAM0103, the strain carrying the empty pMMB207C vector. All experiments were performed more than three times with triplicate cultures in each experiment.

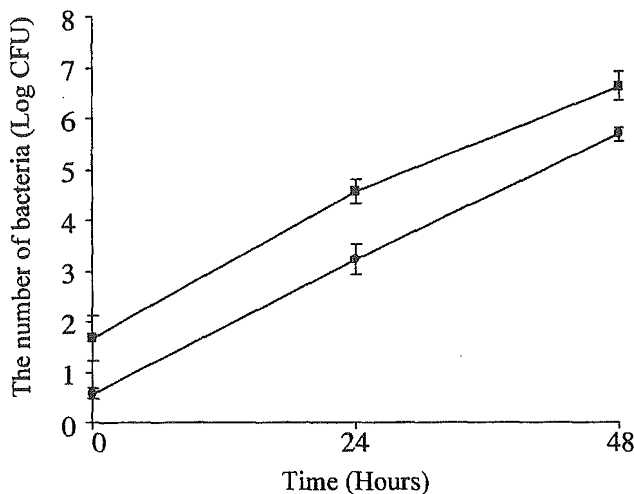

FIG. 5. Growth of *L. pneumophila* in U937 human monocytic cells. Differentiated U937 cells (1×10^6 cells/well) were infected with approximately 1×10^6 bacteria/well for 1 h. The data shown are representative of those from three experiments, which showed similar results. Error bars indicate the standard errors of the mean. The growth rates of 80-045 (squares) and LAM0101 (circles) at 0, 24, and 48 h after infection showed no significant difference.

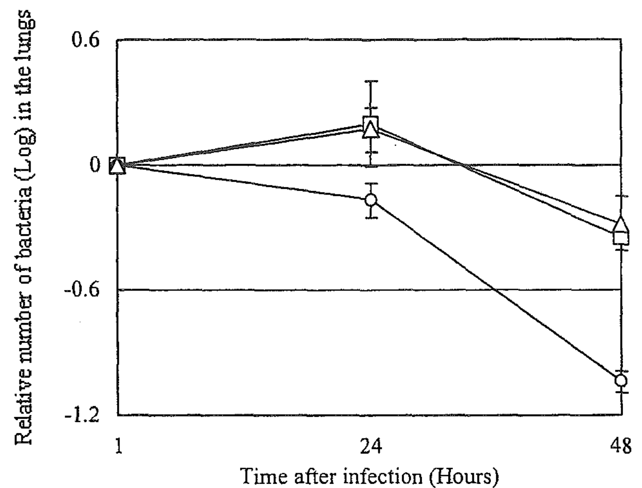

FIG. 6. Elimination of *L. pneumophila* from the lungs of intranasally inoculated mice. A/J mice were inoculated with 10^6 cells of *L. pneumophila*. In each experiment, three mice were used for the test at the 1-h infection point and four mice each were used for the 24- and 48-h infection points. At the time points indicated, the mice were sacrificed and the number of *L. pneumophila* in the lungs was determined as described in Materials and Methods. The number of bacteria at 1 h postinoculation was set at 1, and the numbers at the 24- and 48-h time points are presented as the relative ratio. Shown here are the results for the wild isolate 80-045 (squares), the *laiA* mutant LAM0101 (circles), and the *laiA*-complemented strain LAM0102 (triangles). Results indicate the means ± standard deviations of the bacterial counts at the indicated time point. The numbers of LAM0101 bacteria at the 24- and 48-h time points are significantly less than those of 80-045 and LAM0102. Three independent experiments showed similar results.

intranasal inoculation. LAM0102 lost pMMBLG0503 (Cm^r) in mouse lungs under nonselective conditions for the plasmid. Approximately 1/2 and less than 1/500 of total bacterial counts surviving in the lungs retained the plasmid at 48 and 96 h after infection, respectively (data not shown). Therefore, we examined the elimination of bacteria from lungs within 48 h after infection and did not determine the LD_{50} of LAM0102 for mice.

A representative result for the elimination of *Legionella* from mouse lungs is presented in Fig. 6. The numbers of LAM0101 cells at the 24- and 48-h time points were significantly less than those of 80-045 and LAM0102. The results for mouse lethality after infection with 80-045 and LAM0101 are summarized in Table 2. The LD_{50}s of 80-045 and LAM0101 for the A/J mice were calculated to be 2.2×10^6 and 2.3×10^7 CFU, respectively, demonstrating that the *laiA* mutant was less virulent than the 80-045 wild-type strain. These results show that the *laiA* gene is involved in the virulence of *L. pneumophila* 80-045 in the A/J mouse model.

DISCUSSION

L. pneumophila can multiply intracellularly within a wide range of host cells, including protozoa and macrophages and/or epithelial cells of animals. Although the pathway for a productive *L. pneumophila* infection is a multistep process, the life cycle of the bacteria can be simply presented in two stages, replication and transmission (35). Based on the virulence traits, most of the virulence genes of *L. pneumophila* can be divided into two distinct classes: one is for the promotion of replication, and the other is for transmission. LaiA, identified in this study, appears to be involved in transmission, but not in replication, of *L. pneumophila*.

Adhesion and invasion are considered to be the primary mechanisms for the pathogenesis of intracellular pathogens, and recent observations have suggested that the initial entry plays an important role in subsequent intracellular survival and replication of *L. pneumophila* (4, 6). It has been reported that the epithelial cells also play a crucial role in the infection caused by *L. pneumophila* (34). Although the mechanism of entry is not well understood, several factors are involved in the adhesion of *L. pneumophila*, including opsonization of complement (26, 29, 37) and some specific antibodies (23, 26, 37). In addition to these factors, nonopsonic adhesive molecules (16, 44), such as a type IV pilus (52), are also involved in the adherence. More recently, two gene clusters, *enh1* and *enh2*, have been reported to enhance the entry of *L. pneumophila* into cells (6). The *rtxA* gene, which is present at the *enh1* locus, is involved in adhesion to macrophages and epithelial cells and also in virulence of *L. pneumophila* (5, 6). LaiA was involved in adherence and entry into epithelial cells (Fig. 4) and also in virulence of *L. pneumophila* infection in mice (Fig. 6 and Table 2). This study provides new evidence that adhesion and entry of *L. pneumophila* are critical steps for *Legionella* virulence and that LaiA is directly involved in these essential steps.

Integrins have multiple functions in adhesion, morphogenesis, and signaling in vertebrate cells. The functions of the integrin homologues of cyanobacteria and *S. cerevisiae* have not been well studied, whereas the integrin homologue of *C. albicans* was determined to be involved in adhesion, filamentous growth, and virulence (14, 15). A549 cells were established from a human lung cancer and have the characteristics of well-differentiated type II alveolar epithelial cells (33). The

TABLE 2. Survival of A/J mice infected with 80-045 and LAM0101 and their LD_{50} values[a]

Strain	No. of surviving mice/total no. of mice after inhalation of the indicated number of bacteria							LD_{50} (CFU)
	3.7×10^8	1×10^8	3.7×10^7[c]	1×10^7[c]	3.7×10^6[c]	1×10^6	3.7×10^5	
80-045	ND[b]	ND	0/10	0/10	7/17	14/16	10/10	2.2×10^6
LAM0101	0/10	1/12	5/11	12/15	10/10	10/10	ND	2.3×10^7

[a] Survival of A/J mice was monitored for 30 days after infection. The targeted amount of bacteria inhaled into lungs of mice is indicated; the actual doses of 80-045 and LAM0101 inhaled into lungs were approximately 1.1- and 1.23-folds the targeted doses, respectively. The LD_{50} was calculated with actual doses.

[b] ND, not determined.

[c] $P < 0.05$ for 80-045 versus LAM0101. The statistical significance of survival curve differences was examined by both log rank test and Wilcoxon test methods.

alveolar wall is lined with type II epithelial cells that are hemispherically projected into the alveolar space and also have many microvilli at their surface. The type II alveolar epithelial cells are predicted to have no phagocytic activity (50). However, since the ability of *L. pneumophila* to invade A549 cells was correlated with adhesion of the *laiA* mutant, we propose that adhesion of *L. pneumophila* subsequently induces phagocytosis of type II epithelial cells for bacterial entry. Although the mechanism of signaling is still not understood, the attachment of LaiA may induce cytoskeletal rearrangements and other changes (7) necessary for the induction of phagocytosis of the cells.

Integrins, which are heterodimeric transmembrane proteins, are present on vertebrate cells (24), and integrin homologue genes have been found in *Xenopus* (39), cyanobacteria (30), *S. cerevisiae* (25), and *C. albicans* (14, 15). LaiA seems to be an integrin-like polypeptide, based on sequence motif similarities. Homology of LaiA of 80-045 with Lpg2157 of Philadelphia-1 (3) was also not very high (less than 80%). It is also known that the vertebrate integrins have only limited sequence identity among different species. This suggests that the *laiA* genes are quite diverse in different species and strains. The origin of integrins does not appear to be confined to eukaryotic cells but may have an evolutionary root in simpler organisms. It has been suggested that *L. pneumophila* synthesizes gene products, such as the RtxA molecule (5), which mediate attachment and/or invasion via an interaction with the β_2 integrin expressed on epithelial cells. Additionally, involvement of an integrin-like protein in adherence may provide some insights into a novel adhesion mechanism in bacterial infection. *L. pneumophila* may recognize proteins with the RGD tripeptide synthesized by the epithelial cells through the LaiA integrin-like protein, which is expressed on the surfaces of bacteria. It will be interesting to determine whether adhesion molecules, such as RtxA and LaiA, recognize different types of epithelial cells for adhesion. If so, this might explain the preferential sites of *Legionella* infection. This possibility should be investigated in the future.

Within the past decade, several elegant molecular biology techniques have been developed for the detection and characterization of virulence genes (2, 19, 42). Every approach has its advantages and disadvantages for the detection of virulence genes. To clarify the pathogenesis of bacteria, the development of new techniques and the integrated use of these approaches are absolutely necessary. A novel virulence gene, *laiA*, of *L. pneumophila* was detected in this study. This gene, which has homology to an integrin analogue gene of *S. cerevisiae* (25), encodes a protein that is involved in *L. pneumophila* adhesion to and entry into A549 alveolar epithelial cells and also in virulence in an A/J mouse model. Many questions regarding the pathogenicity of *L. pneumophila* remain, and the mechanisms of LaiA and its paralogs in *L. pneumophila* virulence require further investigation. Results of such studies may give new insight into infections caused by these pathogenic bacteria.

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Legionella pneumophila Serogroup 1 Isolates from Cooling Towers in Japan Form a Distinct Genetic Cluster

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Abstract: **Thirty-one epidemiologically unrelated *Legionella pneumophila* serogroup 1 isolates (10 from cooling towers, 10 from public spas and/or hot spring baths, and 11 from patients) were analyzed by pulsed-field gel electrophoresis (PFGE) and sequence-based typing (SBT) using 6 loci, *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA*. The results of PFGE and SBT analysis indicated that all 10 isolates from cooling towers clustered into a unique type, which was distinct from strains of other environmental sources.**

Key words: ***Legionella pneumophila*, Molecular typing, Pulsed-field gel electrophoresis (PFGE), Sequence-based typing (SBT)**

Legionella pneumophila serogroup 1 is the major causative agent associated with legionellosis in Japan and the rest of the world. *L. pneumophila* is widely found in the environment, particularly in man-made water systems, which are often sources of bacterial infection. In Japan, hot springs and public baths are more probable sources of legionellosis than cooling towers (2).

Pulsed-field gel electrophoresis (PFGE) is considered to be one of the most discriminative epidemiological methods for subtyping *L. pneumophila* strains and for elucidating the sources of infection (4, 17, 19). On the other hand, sequence-based typing (SBT), which was recently developed, is also a powerful epidemiological method. SBT of *L. pneumophila* serogroup 1 isolates in Europe (the EUL collection) has been reported (6–8). Six genes, *flaA*, *mompS*, *pilE*, *asd*, *mip*, and *proA*, some of which are probably under selective pressure, were shown to be useful genetic markers for epidemiological typing (6, 7). The *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* genes encode the flagellum protein subunit (10), type IV pilin (20), aspartate-β-semialdehyde dehydrogenase (9), macrophage infectivity potentiator protein (5), the major outer membrane protein composed of 28- and 31-kDa subunits (11; GenBank/EMBL/DDBJ accession no. AF078136, E. Cristoph and W. Ehret, unpublished data), and the extracellular zinc metalloprotease (3), respectively. In this study, we used PFGE and SBT to analyze *L. pneumophila* serogroup 1 isolates from Japan and compared the usefulness of these techniques.

Materials and Methods

Bacterial strains. All *L. pneumophila* serogroup 1 isolates used in this study were independently obtained from a wide variety of Japanese locations and designated with a NIIB (National Institute of Infectious Diseases, Department of Bacteriology) series of numbers (10 from cooling towers, 10 from public spas and/or hot spring baths, 7 from patients with bath-related infections, and 4 from patients infected at unknown sources). The 4 patients' cases were reported as sporadic cases[NIIB 79 (18), NIIB 80 (12), NIIB 81 (14), NIIB 83 (16)] (Fig. 1). Clinical isolates were derived from 2 female and 9 male pneumonia patients between the ages of 43 and 71. Determination of *L. pneumophila* and serogroup 1 was carried out by PCR specified for the *L. pneumophila mip* gene (15) and a slide agglutination test using monovalent serum for *L. pneumophila* serogroup 1 (Denka Seiken, Tokyo). These isolates were cultivated on a buffered charcoal yeast extract (BCYE) agar (Difco, Detroit, Mich., U.S.A.) plate for 3 days at 35 C. The cultured colonies were directly used

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Abbreviations: EWGLI, European Working Group for *Legionella* Infections; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; SBT, sequence-based typing.

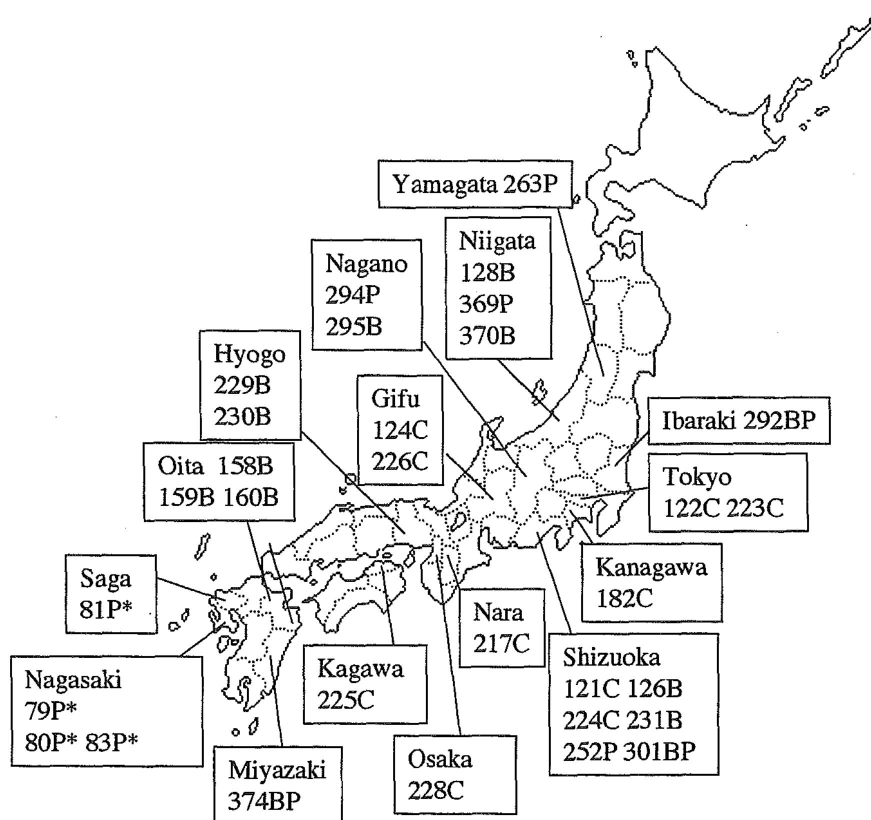


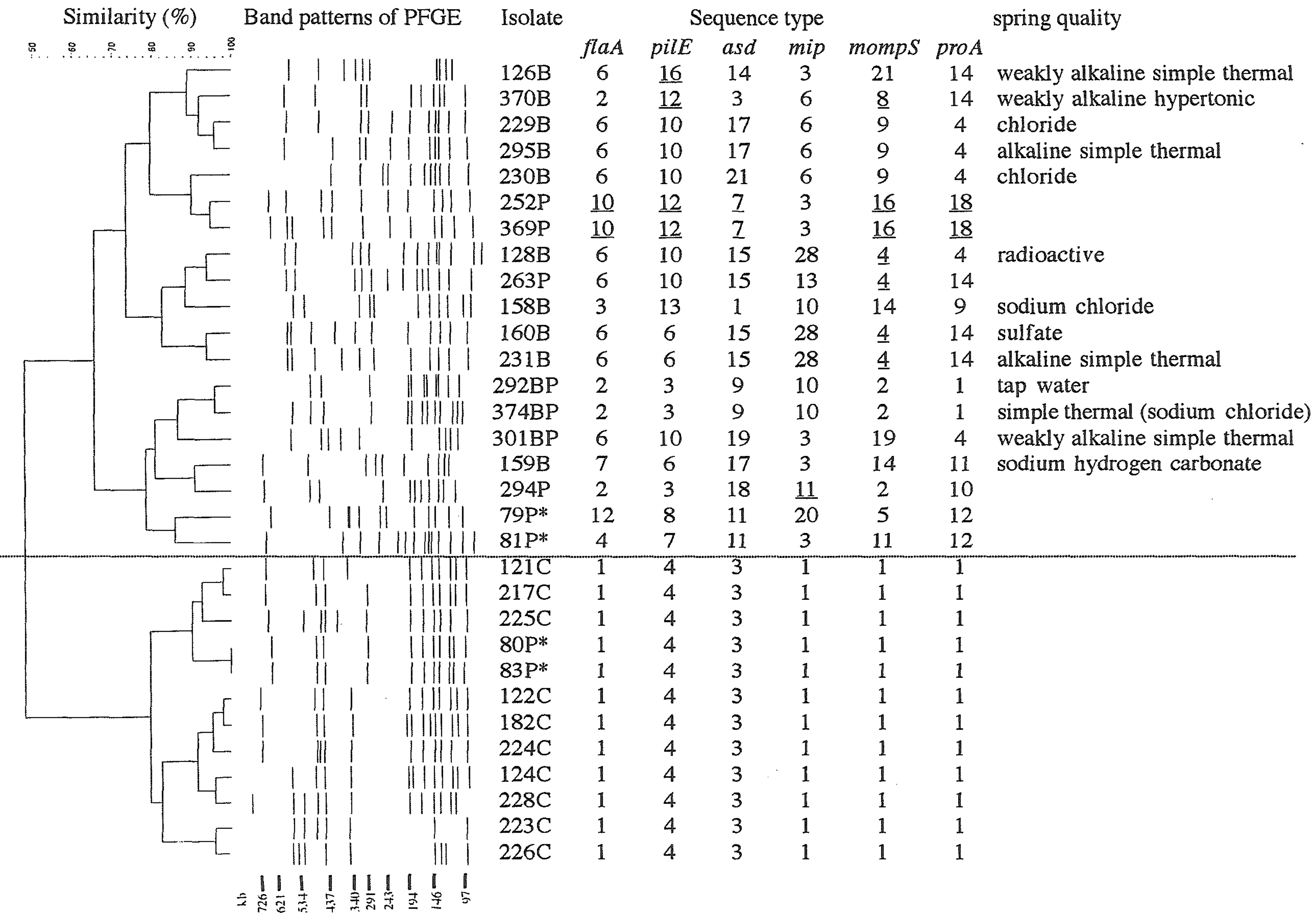
Fig. 1. Isolates of *L. pneumophila* serogroup 1 in Japan were used in this study. All isolates are epidemiologically unrelated to each other. The names of these isolates are indicated on the Japanese map along with the names of the prefectures where they were isolated. The terminal capital letters after the NIIB-numbers represent the following: C, the isolates from cooling tower water; B, the isolates from spa and/or hot spring bath water; P, the isolates from patients assumed to be infected at spas and/or hot spring baths; P*, the isolates from patients infected at unknown sources; BP, the representative isolates obtained from both patients and bath water in an outbreak. The P isolates are indicated on the prefectures where the patients visited, and P* isolates are indicated on the prefectures where the hospitals of patients' admission were located.

for PFGE and SBT.

PFGE. Preparations for PFGE typing were carried out as described previously (13) with a slight modification. The DNA samples were digested with 20 U of restriction enzyme *Sfi*I (New England Biolabs, Boston, Mass., U.S.A.) at 50 C. PFGE was performed on 1% agarose gels with 0.5× TBE running buffer using a CHEF DRII (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) system. The initial switching time of 5 sec was linearly increased to a final switching time of 50 sec over 21 hr at 6 V/cm and 14 C (1). A dendrogram of the PFGE pattern based on Ward's method (21) was constructed using the Fingerprinting II software (Bio-Rad Laboratories).

SBT. The primers used for SBT of *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* were the same as those used by Gaia et al. (7, http://www.ewgli.org). The apex of the microtip was brought in contact with a colony on an agar plate, and the tip was immersed in 10 μl of distilled water in microtubes. The microtubes were treated at 100 C for 10 min and briefly centrifuged. The supernatant was used as the DNA template. Amplification by PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif., U.S.A.). The reaction mixture and conditions used were the same as those described by Gaia et al. (8, http://www.ewgli.org). *Taq* polymerase was purchased from Applied Biosystems.

PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). Both strands of the amplicons were sequenced with a model 310 ABI sequencer (Applied Biosystems). The nucleotide sequences obtained were trimmed based on the data for *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* presented by Dr. Norman Fry (http://www.ewgli.org). Putative novel variants found in this study were submitted to the curators of the EWGLI SBT database for verification and assignment



Isolate	*flaA*	*pilE*	*asd*	*mip*	*mompS*	*proA*	spring quality
126B	6	16	14	3	21	14	weakly alkaline simple thermal
370B	2	12	3	6	8	14	weakly alkaline hypertonic
229B	6	10	17	6	9	4	chloride
295B	6	10	17	6	9	4	alkaline simple thermal
230B	6	10	21	6	9	4	chloride
252P	10	12	7	3	16	18	
369P	10	12	7	3	16	18	
128B	6	10	15	28	4	4	radioactive
263P	6	10	15	13	4	14	
158B	3	13	1	10	14	9	sodium chloride
160B	6	6	15	28	4	14	sulfate
231B	6	6	15	28	4	14	alkaline simple thermal
292BP	2	3	9	10	2	1	tap water
374BP	2	3	9	10	2	1	simple thermal (sodium chloride)
301BP	6	10	19	3	19	4	weakly alkaline simple thermal
159B	7	6	17	3	14	11	sodium hydrogen carbonate
294P	2	3	18	11	2	10	
79P*	12	8	11	20	5	12	
81P*	4	7	11	3	11	12	
121C	1	4	3	1	1	1	
217C	1	4	3	1	1	1	
225C	1	4	3	1	1	1	
80P*	1	4	3	1	1	1	
83P*	1	4	3	1	1	1	
122C	1	4	3	1	1	1	
182C	1	4	3	1	1	1	
224C	1	4	3	1	1	1	
124C	1	4	3	1	1	1	
228C	1	4	3	1	1	1	
223C	1	4	3	1	1	1	
226C	1	4	3	1	1	1	

Fig. 2. Cluster dendrogram by PFGE and sequence-based types of *L. pneumophila* serogroup 1 isolates in Japan. Allele numbers are according to the EWGLI SBT database for *L. pneumophila* (http://www.ewgli.org), and new alleles which were determined in this research are underlined. *flaA*, *pilE*, *asd*, *mompS*, and *proA* of NIIB 252, *pilE* of NIIB 126, *mip* of NIIB 294, and *mompS* of NIIB 128 and NIIB 370 have been lodged in the DDBJ sequence database under Accession no. AB215085, AB215086, AB215087, AB215093, AB215088, AB231162, AB215089, AB215090, and AB215091, respectively. Two independent experiments of PFGE showed a similar result. Characteristics of hot spring water were quoted from the reference of the hot spring.

of new allelic numbers according to the curators' instructions (http://www.ewgli.org).

Results

PFGE

The 31 epidemiologically unrelated isolates were discriminated into 30 PFGE types (Fig. 2). The dendrogram of the PFGE pattern of isolates from cooling towers formed a distinct genetic cluster. The cluster is quite different from the other cluster of isolates derived from public spas and hot spring baths. This result suggests that the PFGE pattern reflects the habitat of isolates rather than their geographic location.

SBT

We applied SBT to the same *L. pneumophila* serogroup 1 isolates as those used in the PFGE analysis. The sequences of *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* were determined. The 31 isolates were divided into 8, 9, 11, 8, 11, and 8 types based on the sequences of *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA*, respectively (Fig. 2). As a result, the 31 isolates were divided into 16 SB types in total. A noteworthy finding was that all 10 isolates from cooling towers clustered into a unique type. The other 17 isolates (10 from public spas and/or

A. *flaA* (182bp)

Allele type	30	34	48	49	87	100	108	141	162	180
1	C	T	C	A	T	A	C	T	T	G
2	C	T	T	A	T	A	T	T	T	A
3	C	C	T	C	A	G	T	T	T	A
4	T	T	T	A	T	A	T	T	C	G
6	C	T	T	A	T	A	T	C	T	A
7	T	T	T	A	T	A	T	T	T	A
10	C	T	T	A	T	A	C	T	T	A
12	C	T	T	A	T	A	T	T	C	A
	S	N	S	N	S	N	S	S	S	S

(Header: Sites of variable nucleotides)

B. *asd* (473bp)

Allele type	67	76	112	130	156	199	253	270	276	282	319	355	362	367	428	436	469
1	C	G	A	T	C	A	T	C	G	T	G	G	G	G	A	C	A
3	C	A	A	C	C	G	T	T	G	C	A	G	G	G	A	T	A
7	T	G	A	C	C	A	T	C	G	T	A	A	A	G	G	C	G
9	C	A	A	C	C	G	T	C	G	C	A	G	G	G	A	C	G
11	C	G	G	T	C	A	T	C	A	T	G	G	G	G	A	C	A
14	C	A	A	C	C	A	C	C	G	T	A	G	G	G	A	C	G
15	C	G	A	C	C	A	C	C	G	T	G	G	G	G	A	C	G
17	C	G	A	T	T	A	T	C	G	T	A	G	G	G	A	C	G
18	C	A	A	C	C	G	T	C	G	C	A	G	G	A	A	C	G
19	C	A	A	C	C	A	C	C	G	T	G	G	G	G	A	C	G
21	C	G	A	T	C	A	T	C	G	T	A	G	G	G	A	C	G
	S	S	S	S	N	S	S	S	S	S	S	S	N	S	N	S	S

(Header: Sites of variable nucleotides)

C. *pilE* (333bp)

Allele type	5	6	7	9	10	13	14	31	32	33	35	41	48	51	88	93	105	135	153	159	162	186	201	204	219	225	228	237	241	255	264	270	273	276	303	318	324
3	T	C	A	T	C	A	T	A	G	C	G	A	C	G	A	A	A	C	C	T	C	T	T	T	C	T	A	C	C	T	G	C	G	C	G	C	C
4	T	C	A	T	C	G	T	A	G	C	G	A	C	G	A	C	A	C	C	T	C	T	T	T	T	T	G	C	T	T	G	C	G	C	A	C	T
6	T	C	C	T	C	G	T	A	G	C	G	A	C	G	A	A	A	C	C	T	C	T	T	T	T	T	G	C	T	T	T	C	G	C	A	T	T
7	T	C	A	T	C	G	T	A	G	C	G	A	G	A	A	A	A	C	C	T	T	T	C	C	T	T	G	C	T	G	T	C	A	C	A	C	T
8	T	C	A	T	C	G	T	A	G	C	G	A	G	G	A	A	A	C	C	T	C	T	C	C	T	T	G	C	T	G	T	C	A	C	A	C	T
10	T	C	A	T	C	G	T	A	G	C	G	A	C	G	A	A	A	A	T	T	T	T	T	T	C	G	A	C	C	T	G	T	G	T	A	C	C
12	T	C	A	T	C	G	T	A	G	C	G	A	C	G	A	A	A	C	C	T	C	T	T	T	T	T	G	C	T	T	T	C	G	C	A	C	T
13	G	T	A	C	A	G	G	A	A	G	A	G	C	G	G	A	C	C	G	G	T	C	T	T	T	T	G	G	T	T	T	C	G	C	A	C	C
16	T	C	A	T	C	G	T	G	G	C	G	A	G	G	A	A	A	C	C	T	C	T	C	C	T	T	G	C	T	G	G	C	G	C	A	C	C
	N	S	N	S	N	N	N	N	N	N	N	N	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

(Header: Sites of variable nucleotides)

Fig. 3. Polymorphic sites in the six gene fragments. A, *flaA*; B, *asd*; C, *pilE*; D, *mip*; E, *proA*; and F, *mompS*. The bp-length in parenthesis following the gene name indicates the size of nucleotides in the region used to determine allele type (http://www.ewgli.org). The number indicated above nucleotides is the nucleotide number from the first base of the determined region. The variable sites of nucleotide sequences in the each gene were shown as shaded letters. The polymorphisms that are synonymous (S) and nonsynonymous (N) are shown below each polymorphic-site.

hot spring baths, 7 from patients with bath-related infections) were divided into 13 types. We could not find an obvious correlation between SB types and characteristics of hot spring water. SBT indicated that the isolates obtained from spas and/or hot spring baths and from patients with bath-related infections in Japan were highly diverse, in contrast with the isolates obtained from cooling towers in Japan, which had genetically converged. We characterized SB types of 4 isolates from patients infected at unknown sources. Two of 4 isolates [NIIB 80 (12) and NIIB 83 (16)] were the same SB type as the isolates from cooling towers, and the others [NIIB 79 (18) and NIIB 81 (14)] were different from the known environmental and clinical SB types (Fig. 2).

We found a novel allele from each of *flaA*, *asd*, *mip*, and *proA*, two novel alleles of *pilE*, and three novel alleles of *mompS* in this research. Polymorphic sites of all alleles obtained in this study are indicated in Fig. 3.

Discussion

The 31 epidemiologically unrelated isolates from Japan were discriminated into 30 types by PFGE and divided into 16 types by SBT. The isolates with the same SBT demonstrated similar PFGE patterns. In particular, cooling tower isolates formed a distinct genetic cluster of PFGE patterns and had a common SBT. We furthermore confirmed that SB types of more 4 isolates (NIIB 65-Tokyo, NIIB 237-Gunma, NIIB 239-Tokyo, and NIIB 418-Aichi) derived from independent cooling towers were of the same type, (1, 4, 3, 1, 1, 1) (unpublished data). Using the methods of PFGE and SBT, it may be possible to determine the environmental source of a strain of unknown origin. In fact, 2 of 4 isolates from patients infected at unknown sources were assigned to the cooling tower SB type and considered to derive from cooling towers. The first clinical isolate in

D. *mip* (402bp)

Allele type	46	157	160	253	262	287	298	307	310	400
1	T	T	G	A	T	T	T	C	A	G
3	T	A	G	A	T	G	T	T	T	T
6	T	A	G	A	T	T	C	C	T	T
10	T	A	G	A	T	T	T	C	A	G
11	C	A	A	C	C	T	T	C	A	A
13	T	A	G	A	T	T	T	C	A	G
28	T	A	G	A	T	G	T	C	T	T
	S	S	S	S	S	N	S	S	S	S

(Column headings: Sites of variable nucleotides)

E. *proA* (405bp)

Allele type	27	87	90	96	97	99	102	105	108	114	186	189	207	228	267	279	336	393
1	G	T	T	A	G	T	C	T	A	T	T	A	A	G	C	C	C	T
4	G	C	T	A	G	T	C	T	A	T	T	A	A	G	C	C	C	T
9	A	T	C	A	A	C	T	C	G	C	C	A	G	A	C	C	T	C
10	A	T	T	G	G	C	T	C	G	C	C	A	A	A	C	C	C	C
11	A	T	T	A	A	C	T	C	G	C	C	A	A	A	C	C	C	C
12	A	T	T	A	G	C	T	C	G	C	C	A	A	A	C	T	C	C
14	A	C	T	A	G	T	C	T	A	T	T	G	A	A	C	C	C	T
18	A	T	T	A	A	C	T	G	G	T	C	A	A	A	A	C	T	C
	S	S	S	S	N	S	S	S	S	S	S	S	S	S	N	S	S	S

(Column headings: Sites of variable nucleotides)

F. *mompS* (352bp)

Allele type	2	8	18	19	53	62	74	75	92	122	137	173	182	191	200	208	245	267	281	317	326	327	328	329	333	334	335
1	T	T	G	C	G	T	A	T	T	C	C	T	C	G	T	T	T	G	C	C	C	A	A	C	A	T	T
2	T	C	G	C	G	T	A	T	T	C	C	T	C	G	T	T	T	G	C	C	C	A	A	C	A	T	T
4	T	T	T	T	G	T	G	T	C	C	C	C	C	A	T	T	C	G	C	C	C	A	A	C	A	T	T
5	T	C	G	C	G	T	G	T	T	C	C	T	C	A	C	T	T	A	A	C	C	A	A	C	A	T	T
8	C	T	G	C	G	T	G	T	T	C	C	T	C	A	C	T	T	A	A	C	C	A	A	C	A	T	T
9	T	T	G	C	G	T	A	T	C	C	C	C	C	A	T	T	C	G	C	C	C	A	A	C	A	T	T
11	C	T	G	C	G	A	A	T	T	C	C	T	C	A	C	T	T	A	A	C	C	A	A	C	A	T	T
14	C	T	T	T	G	A	A	C	T	T	T	T	G	A	C	C	T	A	A	T	A	G	G	T	G	T	T
16	C	T	T	T	A	T	A	C	T	T	C	T	T	A	T	T	C	A	A	C	C	A	A	C	A	T	T
19	T	T	T	T	G	T	A	T	C	C	C	C	C	A	T	T	C	G	C	C	C	A	A	C	A	G	A
21	T	T	T	T	G	T	A	T	C	C	C	C	C	A	T	T	C	G	C	C	C	A	A	C	A	T	T
	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	N	N	S	N	S	S

(Column headings: Sites of variable nucleotides)

Fig. 3.

Japan (18) was not assigned to the cooling tower SB type.

Both PFGE and SBT indicated that the spa-bath-isolates from Japan were highly diverse. The water used in public spa baths in Japanese resorts is mostly obtained from hot springs. The characteristics of hot spring water, such as chemical substances, pH, and temperature, are highly variable. The genetic diversity of the spa bath-strains may reflect the wide variety of their habitats.

The 152 *L. pneumophila* strains currently in the EUL collection, which is comprised of clinical and environmental isolates obtained from 10 European countries, are divided into 62 types by SBT. To date >90 sequence types have been identified (personal communication Dr. Norman Fry). We found that only 4 SB types were common between Europe and Japan. One of these was the SB type (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*)=(1, 4, 3, 1, 1, 1), which was the cooling tower-type identified in our study. This type was most commonly isolated in Europe (6). This type has been isolated from water samples from European spa pools, hotels and hospitals, although it is not known if isolates of this type have been found in European cooling towers. One of the common types was (2, 3, 9, 10, 2, 1), which was the second most frequently detected isolate in Europe (6). The strains with the sequence type (1, 4, 3, 1, 1, 1), which might inhabit cooling towers, are probably spreading around the world.

Although PFGE is the most widely used technique and is generally accepted to be highly effective in discriminating genomic differences, it may have certain drawbacks with regard to interlaboratory reproducibility (4). SBT appears to be less effective at discriminating between strains than PFGE. On the other hand, SBT shows excellent reproducibility, and it is easy to create a database of SBT, because SBT does not require the interpretation of gel images. Furthermore, the 6 genes used in SBT were genetically stable even after several passages of bacteria in amoebae or on agar (7, 8). We have to consider the advantages and limitations of both methods and apply the most suitable method according to the requirements.

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Outbreak of Legionnaires' disease on a cruise ship linked to spa-bath filter stones contaminated with *Legionella pneumophila* serogroup 5

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SUMMARY

In January 2003, two cases of Legionnaires' disease associated with a ship's cruise were registered in the database of National Epidemiological Surveillance of Infectious Diseases. A 70-year-old male heavy smoker with mild emphysema contracted the disease during a cruise. *Legionella pneumophila* serogroup (sg) 5 was isolated from the patient's sputum and the ship's indoor spa. The isolate from the spa matched the patient's isolate by genotyping performed by pulsed-field gel electrophoresis (PFGE). The second case was in a 73-year-old female. During epidemiological investigation, a third case of Legionnaire's disease in a 71-year-old male was subsequently diagnosed among passengers on the same ship on the following cruise. Environmental investigation revealed that porous natural stones (*Maifanshi*) in the filters of the spas had harboured *L. pneumophila*, a phenomenon which has not been reported except in Japan. This is the first documented evidence of *L. pneumophila* sg 5 infection on a ship and of porous stones as a source of *Legionella* infection.

INTRODUCTION

Legionnaires' disease is a severe form of atypical pneumonia often caused by *L. pneumophila*, which in the United States from 1980 to 1998 accounted for 91·4% of clinical isolates [1]. Of the *L. pneumophila* isolates whose serogroup (sg) was known, 85% were sg 1, and only 1·9% were sg 5. In Japan from 1999 to 2002, 91% of clinical isolates for Legionnaires' disease were *L. pneumophila*; and among known serogroups, 65% and 3·8% were *L. pneumophila* sg 1 and sg 5 respectively [2]. Similarly, most ship-associated cases have been ascribed to infection by *L. pneumophila* sg 1, and isolation of sg 5 has been rare [3], only one being previously reported, in a Pacific cruise out of Sydney [4]. A molecular link between the disease and a source of infection on a ship has been documented in only two clusters caused by *L. pneumophila* sg 1 [5, 6].

In this report, we describe an outbreak of Legionnaires' disease caused by *L. pneumophila* sg 5 which was molecular-epidemiologically linked to the spa baths during the two cruises on the same ship.

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MATERIALS AND METHODS

Description of the cases

Case 1 (the index case in this cluster)

A 70-year-old Japanese man had been a passenger on a cruise ship with his wife from 27 December 2002 to 5 January 2003. They had never stayed ashore overnight. He had used the men's indoor spa bath at least seven times during the cruise. He presented with dry cough and mild fever since 1 January and with diminished appetite and general fatigue since 6 January [7]. On 13 January, he was admitted to the emergency room with severe dyspnoea. *L. pneumophila* sg 5 was isolated from the patient's sputum collected on the day of admittance, and urinary antigen was detected by Binax NOW *Legionella* immunochromatographic test (Binax Inc., Portland, ME, USA) in a specimen collected on hospital day 3. He rapidly developed multiple organ failure accompanied by septicaemia. Although the patient was discharged from the intensive care unit on day 22 of his hospitalization, the disease resulted in chronic need for supplemental oxygen.

Case 2

A 73-year-old female passenger on the same cruise ship had complained of cough and fever since 3 January. On 14 January, she was hospitalized for dyspnoea and severe pneumonia [8]. Biotest EIA test (Biotest AG, Dreieich, Germany) and Binax NOW *Legionella* immunochromatographic test detected urinary antigen of 'serogroups 1 and/or 5'. Culture and PCR of a sputum specimen were negative. By microplate agglutination test, single-serum antibody titres against *L. pneumophila* sg 1 to sg 6 were also negative. In spite of macrolide and aminoglycoside antibiotic therapy and steroid pulse therapy, she developed a pulmonary thromboembolism but on 16 March 2003 was finally discharged from the hospital. She had used the women's indoor spa bath in the cruise. Her home bath was not a spa, and the bath water was emptied after use every day. She had used neither pool nor spa except for the women's indoor spa and possibly also the whirlpool spa on the ship. There was no fountain in the public gardens near her home. No legionella was isolated from the female patient's home bath.

Case 3

A 71-year-old male passenger on the subsequent cruise from 7 to 9 January 2003, had presented with cough since 11 January and with fever of 38–39 °C since 12 January. He consulted a physician and was diagnosed without roentgenography as having bronchitis [9]. He had used the men's indoor spa bath twice a day for 3 days, but neither a whirlpool spa nor a warm pool during the cruise. After the epidemiological investigation had begun, he was informed of the occurrence of ship-associated Legionnaires' disease and visited the local community hospital on 30 January. By Biotest EIA test, his urinary antigen of probable 'serogroup 5' was positive and he was diagnosed as having Legionnaires' disease and was hospitalized on the same day. Sputum culture and paired sera test were both negative. Pathological examination revealed bronchitis and interstitial pneumonia. He was discharged on 15 February. His home bath was not a spa and he had not travelled except for the cruise.

Infection of other passengers

The cruise ship was a 26500-ton Japanese passenger ship with capacity for 696 passengers and 204 crew members. The first cruise of the ship was in 1998. There were 238 passenger rooms on the ship, of which 40 rooms had baths and the others showers. On 28 January 2003, based on one confirmed case and a probable source of infection in the cruise ship's environment, 1821 of the 1833 passengers who had travelled in a total of five separate cruises on the same ship from 27 December 2002 to 19 January 2003 were informed of the occurrence of Legionnaires' disease associated with the ship. All were advised to consult a physician in case of illness. This resulted in the finding of case 3, and an additional 18 passengers with illness (two with pneumonia, and the other 16 with fever, cough, nausea, and/or diarrhoea) after the cruises. However, the two other pneumonia patients were diagnosed as not having Legionnaires' disease, and no further case was found. The numbers of passengers on the first and second cruises, where confirmed cases were found, were 459 and 514 respectively. About 200 passengers enjoyed the indoor spas every day and the male : female ratio of indoor spa users was 3 : 2. Users of the whirlpool spa on the deck were fewer than 10 a day.

Environmental sampling

On 23 December 2002, three water samples collected from spas and one cabin's bath on the ship were tested by a private inspection company. These specimens

were obtained before any remedial decontamination. Further water samples collected from the spas on 14 January 2003 were tested at a legally incorporated foundation. All environmental samples mentioned above had been voluntarily collected by the ship's company for *Legionella* tests before the confirmation of the first patient. On 28 January 2003, when the outbreak was first suspected, a public health centre in Hyogo prefecture, where the ship was docked, performed environmental investigations. Sterile swabs were dipped in sterile saline, rotated against a 10-cm^2 surface area, kept and transported in a tube containing 10 ml of saline. Three residual water samples and 12 swabs were collected by public health officers at different sites on the ship, especially the spas. Two samples of porous natural stones (*Maifanshi*) used as filter bed material in the indoor-spa filters were collected on the same day, immersed and transported in sterile water. These stones and their immersion water were tested separately. *Maifanshi* is also called China medical stone. It is found in Inner Mongolia and in Japan, and is a kind of intrusion of granodiorite porphyry composed of aluminosilicates [10]. In recent years, *Maifanshi* have been used in Japan for producing artificial mineral water and as a refrigerator odour killer.

Laboratory methods

Water samples and swab suspensions were concentrated by filtration (0·22 μm pore size). The stones were put into an airtight container and sonicated in saline at 130 W and 20 kHz for 24 s to collect residual biofilms from inside the stones. The turbid extract was concentrated by centrifugation. Diluted and undiluted specimens were plated on buffered charcoal yeast extract (BCYE) α-non-selective and WYO α-selective agars (Eiken Chemical, Tokyo, Japan) [11], with and without heat or acid treatment, following the usual procedures for the isolation of *Legionella* spp. Identification of *Legionella* isolates was performed using species-specific and serogroup-specific monovalent antisera (Denka Seiken, Tokyo, Japan). Some isolates were confirmed by PCR with specific primers for *Legionella* 5S rRNA DNA and the *L. pneumophila mip* gene [primers were prepared according to the sequences from the formerly commercially available EnviroAmp *Legionella* (PerkinElmer Cetus Corporation, Norwalk, CT, USA)]. All available *Legionella* strains isolated from samples were sent to the *Legionella* Reference Centre, Department of Bacteriology in the National Institute of Infectious Diseases in Japan. To show the link between clinical and environmental *L. pneumophila* sg 5 isolates, strains were analysed by use of PFGE of genomic DNA digested with *Sfi*I restriction enzymes [12]. Unfortunately, the isolates from the samples tested at the private inspection company were discarded after testing, which made retests impossible.

To isolate amoebae from the filter stones, 5–6 stones of 1–2 cm in diameter (whole weight, 33–35 g) were washed by inverting the tube for 1 min in a 50-ml centrifuge tube with sterilized water added up to 50 ml. The washing solution was transferred to a new 50-ml centrifuge tube and the volume of the solution was adjusted to 50 ml by adding sterilized distilled water. Five millilitres of the washing solution was concentrated to 1 ml in a 15-ml centrifuge tube by centrifuging at 500 *g* for 5 min. The sediment was resuspended and inoculated onto a non-nutrient agar plate coated with heat-inactivated *Escherichia coli*. Test plates in a plastic bag were incubated at 30 °C and examined for 7 days for growth of amoebae [13].

Some *L. pneumophila* sg 5 strains previously isolated from different origins [Fig. (*b*)] were used for comparison of DNA fingerprints.

RESULTS

Environmental investigations

The circulating system in the men's indoor spa was identical to but independent of that in the women's indoor spa. Each bathtub contained 5·5 m^3 of water. The water in the tubs was changed twice a day. According to the test report of the inspection company on water samples collected on 23 December before the first cruise, water samples from the indoor men's spa and a whirlpool spa on the deck had yielded legionellae, while water from a royal cabin bath had not (Table); but that fact was unfortunately not reported to the ship's company until after the cruise. Because the indoor spa water showed high contamination by legionellae ($1{\cdot}5 \times 10^4$ c.f.u./100 ml, a spa for males), these spas were disinfected by circulation of water with a high concentration of chlorine (5–7 mg/l) for 7 h [9], resulting in decreased *L. pneumophila* sg 5 c.f.u. in water samples collected from an indoor (male) spa and a whirlpool spa on 14 January. However, legionellae were still not eradicated from the spas, and subsequent samples exhibited a legionella concentration of $8{\cdot}0 \times 10$ to $2{\cdot}9 \times 10^3$ c.f.u./100 ml (Table).

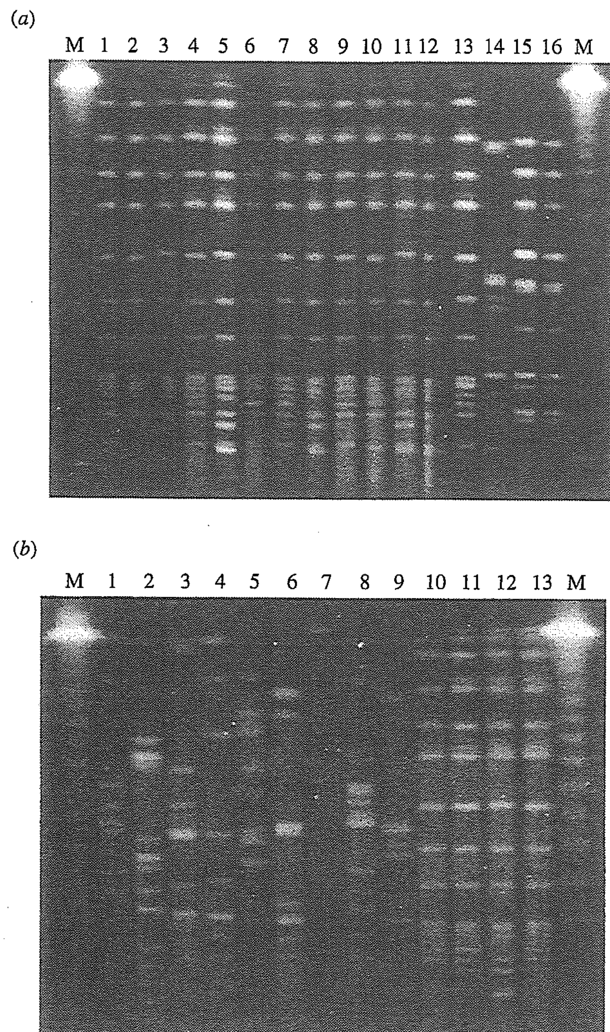

Fig. Macrorestriction analysis of chromosomal DNA derived from *Legionella pneumophila* sg 1 and sg 5 digested with *Sfi*I and separated by pulsed-field gel electrophoresis. (*a*) A comparison of the clinical isolates with the strains isolated from water samples and swabs at different sites of the ship. Lanes 1 and 2, sg 5 isolates from patient (case 1) sputum; lane 3, sg 5 isolate from men's spa water; lanes 4–8, sg 5 isolates from natural stones in men's spa filter; lanes 9–12, sg 5 isolates from natural stones in women's spa filter; lane 13, sg 5 isolate from a strainer of women's spa; lane 14, sg 5 isolate from a whirlpool spa; lane 15, sg 1 isolate from a swab at a strainer for women's spa; lane 16, sg 1 isolate from natural stones in women's spa filter. (*b*) *L. pneumophila* sg 5 strains. Lane 1, NIIB 412, Osaka LG02-11 from a spa bath; lane 2, ATCC 33216 Dallas 1E from a cooling tower; lane 3, NIIB 98 (EY 3420), a clinical isolate in Osaka [22]; lane 4, NIIB104 (EY 3427), a clinical isolate in Kurashiki; lane 5, ATCC 33737 U8W from shower head water; lane 6, NIIB 288 Ishioka 1-2-4 from a spa bath [18]; lane 7, NIIB 330 (ThaiNIH 7811) from a cooling tower; lane 8, NIIB 361 (ThaiNIH 10723) from a cooling tower; lane 9, corresponding to lane 14 of panel (*a*); lane 10, corresponding to lane 1 of panel (*a*); lane 11, corresponding to lane 4 of panel (*a*); lane 12, corresponding to lane 5 of panel (*a*); lane 13, corresponding to lane 6 of panel (*a*). Ms are DNA size markers, lambda ladders (Bio-Rad, Richmond, CA, USA), as indicated on the right and left sides of each electrophoregram.

After a passenger was diagnosed with Legionnaires' disease, administrative inspection of the ship was begun and environmental samples were collected from different sites at the spas and in the male patient's cabin on 28 January. Of the environmental water samples collected on 14 and 28 January, most were negative or soon became negative as a result of the crew's and others' cleaning efforts. The surfaces of the porous stones appeared clean, and no amoebae, often a reservoir of legionellae, were isolated from the stones. But some legionellae were detected in the water surrounding the stones during transit to the testing, and sonication dislodged large amounts of legionellae from inside the porous stones. As a result, it was found that some cleaning of the stones had been achieved, but not eradication of legionella. All 36 isolates from men's spa stones were *L. pneumophila* sg 5, 14 of 15 isolates from women's spa stones were *L. pneumophila* sg 5, with one isolate sg 1. All other isolates from the men's spa and women's spa were identified as *L. pneumophila* sg 5, and a mixture of *L. pneumophila* sg 1 and sg 5 respectively.

L. pneumophila subtyping

We obtained isolates from only case 1. Genomic fingerprints (PFGE) of *L. pneumophila* sg 5 isolates from the patient and various sites of the ship's spas show that the same fingerprint strains as the clinical isolates (lanes 1 and 2) were obtained [Fig. (*a*)] from stones in both spa filters for men (lane 4) and women (lane 10) and from the men's spa water (lane 3). Interestingly, genomic fingerprints of stone isolates revealed a small variation [lanes 4–12, Fig. (*a*)]. Some isolates showing one or two additive band(s) with one band's disappearance in PFGE patterns were observed, suggesting that the porous stones harboured a group of strains derived from a common ancestor. The fingerprints of a *L. pneumophila* sg 5 strain from the water of the whirlpool spa and of a *L. pneumophila* sg 1 strain from the women's spa water (lanes 14 and 15 respectively) were different from the fingerprint of the clinical isolates.

We examined whether the variation in PFGE patterns of *L. pneumophila* sg 5 strains is wide enough to discriminate between clinical and environmental isolates. As shown in Figure (*b*), each *L. pneumophila*

Table. *Isolation and characterization of clinical and environmental* Legionella *strains with the cruise ship*

Sampling site	Specimen (date of sampling)	*Legionella* (c.f.u./100 ml water) (c.f.u./100 g stone)	*Legionella* isolates serogroup	Genomic type of Lp5
Patient from cabin A	Sputum		Lp5	A
Indoor spa for males	Bath water (23 Dec.)	$1{\cdot}5 \times 10^4$	Lost	
	Bath water (14 Jan.)	$2{\cdot}9 \times 10^3$	Lp5	A
	Bath water (28 Jan.)	Not isolated		
	Strainer water (28 Jan.)	Not isolated		
	Natural stones (28 Jan.)	$3{\cdot}3 \times 10^2$	Lp5	A, A′, A″
Indoor spa for females	Bath water (14 Jan.)	$1{\cdot}0 \times 10^3$	Lp1	
	Bath water (28 Jan.)	Not isolated		
	Shower head incrustations (28 Jan.)	Not isolated		
	Natural stones (28 Jan.)	$7{\cdot}1 \times 10$	Lp1, Lp5	A, A′
	Strainer incrustations (28 Jan.)		Lp5	A
Whirlpool spa	Bath water (23 Dec.)	$1{\cdot}4 \times 10^2$	Lost	
	Bath water (14 Jan.)	$8{\cdot}0 \times 10$	Lp5	B
Cabin A (Case 1)	Shower head incrustations (28 Jan.)	Not isolated		
Cabin B (Case 2)	Shower head incrustations (28 Jan.)	Not isolated		
Bath in royal cabin	Bath water (23 Dec.)	Not isolated		

Lp1 and Lp5; *L. pneumophila* serogroups 1 and 5 respectively. Genomic type A, A′, A″ and B; PFGE patterns shown in lanes 1, 5, 7, and 14 in Figure (*a*) respectively.

sg 5 isolate from different origins showed a unique fingerprint, corresponding to the results of a previous report on a cluster of nosocomial Legionnaires' disease caused by *L. pneumophila* sg 5 [14], while the clinical and the environmental isolates in this outbreak showed indistinguishable or quite similar PFGE patterns.

DISCUSSION

Legionnaires' disease has been linked to passenger-ship cruises in several reports, but the source of the infection was clearly demonstrated only in two clusters [5, 6]. In the present cluster, we established that the source of infection was the ship's spa baths rather than the cabin's shower heads or the royal cabin's bath water. Cultures of spa water samples, a strainer swab, and natural stones in filters of the spas yielded *L. pneumophila* sg 1 and sg 5. By analysis with PFGE, the *L. pneumophila* sg 5 isolates from one patient were indistinguishable from the spa water isolates. All three cases were urinary antigen positive. One male patient's illness was confirmed to be due to *L. pneumophila* sg 5 infection by culture; the other male patient's illness was presumed to be due to *L. pneumophila* sg 5 infection because only *L. pneumophila* sg 5 was detected in the men's spa. Whether the causative agent of the female's pneumonia belonged to sg 1 or sg 5 could not be determined because antigen of sg 5 can cross-react with that of sg 1 in the urine antigen tests. Our results of positive urines and negative cultures (cases 2 and 3) are not rare. Generally, obtaining an adequate sputum specimen from patients with Legionnaires' disease is difficult and sensitivity is much higher in urinary antigen detection than in culture [15]. Ruf et al. reported that only two respiratory secretions were culture-positive among 13 urine-positive pneumonia patients [16]. In most situations, the use of both the urinary antigen test plus sputum culture is the best combination.

This cluster has relevant clinical implications for physicians. First, community physicians should always inquire about recent travel as part of their patient's history and consider the possibility of legionellosis, because the disease is often overlooked as a cause of community-acquired pneumonia [5]. Second, urinary antigen detection is a rapid and easy test and can detect most cases of legionellosis caused by *L. pneumophila* sg 1 and others as shown in this outbreak. However, without isolation of clinical strains, the source of infection cannot be definitely confirmed [1]. Therefore, clinical specimens for *Legionella* isolation should always be cultured.

The results of this investigation have broad public health implications. Recently, maintenance of spa baths and adherence to sanitation standards have been advocated for prevention of Legionnaires' disease in Japan and other countries. Such methodology