Isolation and characterization of a conjugative plasmid from *Legionella pneumophila*

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The conjugative properties of an indigenous 85 MDa plasmid (designated pCH1) from *Legionella pneumophila* were studied. To determine if pCH1 was transmissible by conjugation, mating experiments were performed between legionellae that harboured pCH1 and several plasmid-less recipients. Plasmid transfer was monitored by colony hybridization, using a cloned 21·0 kb *Sal*I restriction fragment from pCH1 as a probe. The results from these experiments showed that pCH1 could be conjugatively transferred into several strains of *L. pneumophila* serogroup 1 but not into strain Bloomington-2 (serogroup 3) or *Escherichia coli*. Southern hybridization experiments in which pCH1 DNA was used as a probe showed that pCH1 does not share homology with other indigenous *L. pneumophila* plasmids. There was no detectable DNA homology between pCH1 and *L. pneumophila* chromosomal DNA. Additional mating experiments revealed that pCH1 was unable to mobilize the *L. pneumophila* chromosome. The conjugative transfer of pCH1 into plasmid-less avirulent or virulent serogroup 1 strains did not alter the intracellular growth characteristics of these strains in U937 cells, a human-monocyte-like cell line, or in the amoeba *Hartmannella vermiformis*. These results suggest that pCH1 does not contribute to the ability of *L. pneumophila* to enter or grow within eukaryotic cells.

Introduction

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen capable of entering and multiplying in cultured animal cells (Dreyfus, 1987; Pearlman *et al.*, 1988), free-living amoebae (Fields *et al.*, 1986; Holden *et al.*, 1984; Rowbotham, 1986; Newsome *et al.*, 1985) and human mononuclear phagocytes (Horwitz & Silverstein, 1980). At the present time, little is known about the bacterial factors that promote the intracellular lifestyle of this organism.

Plasmids have been reported to carry genes that contribute to the virulence of a variety of Gram-negative intracellular pathogens including *Yersinia* spp. (Ben-Gurion & Shafferman, 1981; Portnoy *et al.*, 1981, 1984), *Shigella* spp. (Maurelli & Sansonetti, 1988) and *Salmonella* spp. (Gulig, 1990). Several groups have identified plasmids in both clinical and environmental isolates of

L. pneumophila (Johnson & Schalla, 1982; Knudson & Mikesell, 1980; Maher *et al.*, 1983; Mikesell *et al.*, 1981). Early work indicated that the plasmids ranged in size from 23–85 MDa and did not confer an identifiable phenotype on strains that harboured them (Johnson & Schalla, 1982; Maher *et al.*, 1983). Despite the presence of these plasmids in *L. pneumophila*, their contribution to the ability of *L. pneumophila* to enter and grow within eukaryotic cells has never been evaluated.

Previous work from this laboratory (Mintz & Shuman, 1988) using broad host range IncP and IncQ plasmids showed that conjugation is possible in *L. pneumophila*. This finding, along with the identification of an 85 MDa plasmid in numerous *L. pneumophila* serogroup 1 clinical and environmental isolates (Schalla & Johnson, 1982; Maher *et al.*, 1983) suggested that certain indigenous *L. pneumophila* plasmids may be transmissible by conjugation.

In the present study, we evaluated the conjugative and virulence properties of an 85 MDa plasmid (designated pCH1) from *L. pneumophila*. Our results demonstrate that pCH1 is self-transmissible by conjugation among serogroup 1 isolates of *L. pneumophila*. Also, pCH1 does

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Abbreviation: mAb, monoclonal antibody.

Table 1. *Bacterial strains*

Abbreviations: Km^r, kanamycin resistant; Nal^r, naladixic acid resistant; Rif^r, rifampicin resistant; Sm^r, streptomycin resistant; Trp⁻, tryptophan auxotroph; *r*⁻*m*⁺, restriction minus modification plus; *r*⁻*m*⁻, restriction minus modification minus.

Designation	Relevant characteristics	Comments	Source*
<i>L. pneumophila</i>			
Philadelphia-1	Wild-type, clinical serogroup 1 isolate	Plasmid-less	H. Horwitz
Pontiac-1	Clinical serogroup 1 isolate	Plasmid-less	
NY26	Clinical serogroup 1 isolate	Plasmid-less	
Ver 5	Clinical serogroup 1 isolate	Plasmid-less	R. Miller
Rockport	Clinical serogroup 4 isolate	Plasmid-less	M. Horowitz
CH-1	Environmental serogroup 1 isolate	Contains an 85 MDa plasmid	J. F. Plouffe
RH-1	Environmental serogroup 1 isolate	Contains 45 and 85 MDa plasmids	
UH-2	Environmental serogroup 1 isolate	Contains a 45 MDa plasmid	
UPH-1	Environmental serogroup 1 isolate	Contains a 33 MDa plasmid	M. Horwitz
OLDA	Clinical serogroup 1 isolate	Contains an 85 MDa plasmid	
LpP ₂	Environmental serogroup 1 isolate	Contains an 85 MDa plasmid	
LpP ₃	Environmental serogroup 1 isolate	Contains 45 and 85 MDa plasmids	R. Miller
SF3256	Environmental serogroup 1 isolate	Contains an 85 MDa plasmid	
SF3257	Environmental serogroup 1 isolate	Contains an 85 MDa plasmid	
Albuquerque-1	Clinical serogroup 1 isolate	Contains an 85 MDa plasmid	—
Atlanta-1	Clinical serogroup 1 isolate	Contains a 62 MDa plasmid	—
Atlanta-4	Clinical serogroup 1 isolate	Contains a 24 MDa plasmid	—
AM511	Sm ^r <i>r</i> ⁻ <i>m</i> ⁺	Derived from Philadelphia-1	A. Marra
CS267	Sm ^r Rif ^r <i>r</i> ⁻ <i>m</i> ⁺	Spontaneous Rif ^r mutant of AMSII	This study
CZ3001	AM511::Tn5mob Km ^r Sm ^r	—	This study
Bloomington-2	Clinical serogroup 3 isolate	Plasmid-less	M. Horowitz
CS2	Serogroup 3 Sm ^r <i>r</i> ⁻ <i>m</i> ⁺	Plasmid-less, spontaneous Sm ^r mutant of Bloomington-2	This study
CS302	Serogroup 3 Sm ^r Rif ^r Trp ⁻ <i>r</i> ⁻ <i>m</i> ⁺	Derived from CS2	This study
Knoxville-1	Avirulent serogroup 1 clinical isolate	Plasmid-less	R. Miller
CS323	Serogroup 1, Sm ^r Rif ^r	Derived from Knoxville-1	This study
<i>E. coli</i>			
DH5α	Nal ^r <i>r</i> ⁻ <i>m</i> ⁺	—	C. Collins

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not contribute to the ability of *L. pneumophila* to enter or grow within eukaryotic cells.

Methods

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. *L. pneumophila* was routinely grown in Albumin Yeast Extract (AYE) broth and on ACES-buffered Charcoal Yeast Extract (ABCYE) agar plates at 37 °C as previously described (Mintz & Shuman, 1988). Strains of *Escherichia coli* were grown at 37 °C on L-agar or in L-broth. When necessary, the following antibiotic concentrations were used: streptomycin (Sm), 50 µg ml⁻¹; rifampicin (Rif), 20 µg ml⁻¹; kanamycin (Km), 25 µg ml⁻¹; ampicillin (Ap), 50 µg ml⁻¹ and tetracycline (Tc), 10 µg ml⁻¹.

Isolation and analysis of plasmid DNA. Plasmid DNA was isolated from *L. pneumophila* using the rapid method of Kado & Liu (1981) or the alkaline lysis protocol of Birnboim & Doly (1979). In some experiments, chromosomal DNA was removed from plasmid-containing lysates by centrifugation through ethidium bromide/CsCl gradients (Maniatis *et al.* 1982). Plasmid DNA prepared by the Kado & Liu method was not cut by restriction endonucleases so plasmid DNA isolated by the Birnboim & Doly method was used for restriction endonuclease analysis in this investigation. Plasmid DNA and restriction enzyme digestions of plasmid DNA were analysed by

horizontal agarose gel electrophoresis using 0.4% and 0.7% agarose (Bio-Rad) gels.

Cloning of pCH1 DNA. CsCl-purified pCH1 DNA was digested with the restriction enzyme *Sal*I (Promega), ligated with *Sal*I-cleaved, alkaline-phosphatase-treated pBR322 DNA and transformed into CaCl₂-treated *E. coli* DH5α. Transformants were selected on L-agar supplemented with ampicillin and putative recombinants were identified by their Ap^rTc^s phenotypes. The presence of pCH1 sequences in recombinant plasmids was confirmed by Southern hybridization using ³²P-labelled pCH1 DNA as a probe.

Mating experiments. Donor strain CH-1 (which harbours pCH1 and contains no antibiotic-resistance markers) and antibiotic-resistant plasmidless recipients were grown to early exponential phase in AYE broth at 37 °C. Equal numbers of donors and recipients were incubated together on non-selective ABCYE plates at 37 °C for 18 h as described by Mintz & Schuman (1988). After incubation, cells were removed from the plates, serially diluted in M63 salts buffer (Silhavy *et al.*, 1984) and spread onto ABCYE agar plates that contained appropriate antibiotics (Sm, Rif or Km) to counterselect the donor strain CH-1. In some matings, strains that contained two or more drug markers were used as recipients. In these experiments, ABCYE agar that contained two antibiotics was used to counterselect CH-1. The plates were incubated at 37 °C. Colonies were replica-plated onto nitrocellulose filters, lysed with NaOH, probed with ³²P-labelled pBR322 DNA (containing a 21.0 kb *Sal*I fragment from pCH1) and subjected to

autoradiography. We routinely screened approximately 2000–3000 transconjugants for the presence of pCH1 DNA. Preliminary experiments showed that ^{32}P -labelled pBR322 DNA by itself did not hybridize with pCH1 or *L. pneumophila* chromosomal DNA. Hybridizations were performed under conditions of high stringency (Silhavy *et al.*, 1984). Colonies that hybridized with the probe were picked from master plates and purified twice by passage on selective media. Plasmid DNA was isolated from these colonies, digested with *SalI* and examined by agarose gel electrophoresis. All mating experiments were repeated at least two times.

For mating experiments involving DNAase treatment, equal numbers of donors and recipients were incubated for 18 h at 37 °C on ABCYE agar that contained 1 mg DNAase ml⁻¹. Cells were then removed from the plates and spread onto antibiotic-containing ABCYE agar as described above.

Chromosome mobilization experiments using strain CH-1 as a donor and auxotrophic mutants of strains Bloomington-2 (Gua⁻ and Trp⁻) and Philadelphia-1 (Thy⁻ and Trp⁻) as recipients were done according to Mintz & Schuman (1988).

Colony immunoblast assay. Single colonies of donors, recipients and transconjugants were inoculated onto 0.45 µm nitrocellulose filters (Fisher) placed on ABCYE agar plates and incubated for 24–48 h at 37 °C. Filters were removed from the plates and incubated in Tris-buffered saline (TBS; 50 mM-Tris/HCl, 150 mM-NaCl, pH 7.5) containing 5% (w/v) nonfat dried milk (Carnation) for 2 h at room temperature. The filters were washed several times with TBS and incubated with monoclonal antibody (mAb) 1E6 (1:1000 dilution) on a rotary shaker (New Brunswick) for 1 h at room temperature. mAb 1E6 (kindly provided by W. Johnson, University of Iowa, USA) is specific for serogroup 1 lipopolysaccharide. After incubation, the filters were washed several times with TBS and probed with horseradish-peroxidase-conjugated goat-antimouse IgG antibodies (1:1000 dilution, Cappel) for 1 h at room temperature. After a series of four washes with TBS, filters were immersed in a solution of 0.05% 4-chloro-1-naphthol (Sigma) and 0.015% hydrogen peroxide.

Antibiotic and heavy metal resistance. The ability of pCH1 to confer resistance to antibiotics or heavy metals was tested in the following manner. Single colonies of isogenic plasmidless and pCH1-containing strains of *L. pneumophila* were inoculated onto ABCYE agar and ABCYE agar containing a specific antibiotic or heavy metal. The plates were incubated at 37 °C for 5 d. Resistance to the following heavy metals was tested using the concentrations suggested by Trevors *et al.* (1985): cadmium chloride (1 mM), sodium arsenate (38 mM), cobalt chloride (3 mM), copper sulphate (20 mM), lead nitrate (0.3 mM), nickel chloride (3 mM), zinc chloride (1 mM) and silver nitrate (5 mM). Antibiotics were tested at the concentrations mentioned above.

Infection of U937 cells and *Hartmannella vermiformis* with *L. pneumophila*. Human-monocyte-like cells (U937) were infected with legionellae as previously described (King *et al.*, 1991). U937 cell monolayers were grown in RPMI 1640 (Cellgro) plus 20% (v/v) normal human serum for up to 3 d at 37 °C in 5% (v/v) CO₂. At daily intervals, samples were removed from the infected monolayers, diluted in M63 salts buffer and plated on ABCYE agar to determine numbers of *L. pneumophila*.

Plate-grown legionellae were co-cultured with the amoeba *H. vermiformis* at 35 °C for 7 d according to King *et al.* (1991). At various times, samples were removed from the co-cultures, serially diluted in buffer, and plated on ABCYE agar to determine the numbers of *L. pneumophila*.

Previous studies using U937 cells (King *et al.*, 1991; Pearlman *et al.*, 1988) and *H. vermiformis* (King *et al.*, 1991) demonstrated that any increase in numbers of *L. pneumophila* as measured above represent intracellular growth of the bacterium.

Results

Isolation and characterization of pCH1

The plasmid content of five serogroup 1 strains (CH-1, OLDA, Albuquerque 1, LpP₂ and SF3256) reported to carry 85 MDa plasmids was analysed by agarose gel electrophoresis. Each strain contained a single plasmid and the electrophoretic mobility of the plasmids was identical (data not shown). Digestion of each plasmid with *SalI* or *BamHI* yielded identical fragment patterns (data not shown). These results suggested that strains CH-1, OLDA, Albuquerque-1, LpP₂ and SF3256 harboured the same plasmid, subsequently designated pCH1.

Restriction endonuclease analysis of pCH1 revealed that *EcoRI*, *HindIII* or *HaeIII* generated ≥ 13 restriction fragments. Digestion with *BglI*, *PvuII*, *SalI*, *BglII* or *NheI* yielded 5–7 restriction fragments (data not shown). pCH1 was not cleaved by *NotI*, *SfiI*, *ApaI* or *RsrII*. We also determined that pCH1 has a single *BamHI* site contained within the 11 kb *SalI* restriction fragment (Fig. 1). Attempts to construct a detailed restriction map of pCH1 were hampered by the large number of restriction fragments generated during single and double digests and our inability to clone the majority of the plasmid genome in *E. coli* (see below). Pulse-field gel electrophoresis of *BamHI*-digested plasmid DNA showed that the apparent molecular size of pCH1 is 125–130 kb (data not shown). This is in agreement with the predicted molecular size of pCH1, which was previously determined to be 85 MDa (128 kb).

The results from antibiotic sensitivity tests indicated that pCH1 did not encode resistance to Rif, Sm, Ap, Km or Tc. Neither did the plasmid confer resistance to any of the heavy metals tested. Attempts to cure pCH1 from plasmid-bearing strains by treatment with acridine orange (Riva *et al.*, 1973), ethidium bromide (Bouanchaud *et al.*, 1969), growth at 41 °C or by repeated subculture on ABCYE agar at 37 °C were unsuccessful. This suggested that pCH1 was stably maintained in *L. pneumophila* in the absence of obvious selective pressure.

Cloning of pCH1 DNA

As shown in Fig. 1, the restriction enzyme *SalI* cleaves pCH1 DNA into six distinct restriction fragments. We attempted to isolate and clone each of the six *SalI* fragments from pCH1. Despite repeated attempts using either pBR322 or pACYC184 as cloning vectors, we were only able to isolate the 21.0 kb *SalI* fragment from pCH1. This fragment was cloned into the unique *SalI* site of pBR322 (Fig. 1) and subsequently used as a probe in plasmid transfer experiments.

Fig. 1. Ethidium-bromide-stained 0.4% agarose gel that contains the cloned 21.0 kb *SalI* fragment of pCH1. Lanes: A, λ *HindIII* molecular size standards; B, undigested pCH1 DNA; C, pCH1 DNA digested with *SalI*; D, pBR322 DNA that contains the cloned fragment digested with *SalI*; E, pBR322 DNA digested with *SalI*. Arrow indicates the position of the 21.0 kb fragment of pCH1.

Conjugative transfer of pCH1

To determine whether pCH1 is conjugative, mating experiments between strain CH-1 (which harboured pCH1) and several antibiotic-resistant plasmid-less *L. pneumophila* recipients were done. Since pCH1 does not encode any selectable markers, we assessed the ability of strain CH-1 to conjugally transfer pCH1 by colony hybridization using the cloned 21 kb *SalI* fragment from pCH1 as a probe.

Initial mating experiments, in which strain AM511 was used as the recipient, revealed that several colonies on the selection plates hybridized with the pCH1 probe. The electrophoretic profile of *SalI*-digested plasmid DNA from each of these colonies was identical to that of *SalI*-digested pCH1 DNA (data not shown). This suggested that pCH1 was transferred by a mating

process. However, the possibility existed that colonies that contained pCH1 were spontaneous antibiotic-resistant mutants of strain CH-1 rather than actual transconjugants. To eliminate this possibility, we did additional mating experiments using multiple-antibiotic-resistant recipients in which two antibiotics were used to counterselect donor strain CH-1. The frequency of spontaneous mutation to resistance for two antibiotics for strain CH-1 would be approximately 10^{-12} . However, pCH1 was detected in exconjugants at frequencies ranging from 10^{-3} – 10^{-4} per recipient. These results supported the idea that colonies that contained pCH1 were transconjugants rather than antibiotic-resistant mutants of strain CH-1. This was confirmed by colony immunoblot experiments with putative transconjugants using mAb 1E6, which does not bind to strain CH-1 but is reactive with each of the other serogroup 1 strains used as recipients in this study. In all cases, colonies that hybridized with the pCH1 probe also bound mAb 1E6 (data not shown). This demonstrated that pCH1 was transferred from strain CH-1 to *L. pneumophila* recipients during the mating process. The detection of pCH1 in transconjugants obtained from matings in the presence of DNAase (1 mg ml^{-1}) provided conclusive evidence that pCH1 was selftransmissible by conjugation (data not shown).

A summary of the mating experiments done in this study is presented in Table 2. By determining the number of pCH1-containing colonies among the total number of transconjugants screened, we estimated that pCH1 was transferred at frequencies ranging from 10^{-3} – 10^{-4} per recipient. Of interest, pCH1 could only be conjugally transferred into serogroup 1 recipients. We were unable to detect transfer of pCH1 into a serogroup 3 recipient (strain Bloomington-2) or *E. coli* DH5 α . Additional mating experiments between strain CH-1 and several serogroup 1 or serogroup 3 auxotrophic recipients showed that pCH1 could not promote the chromosomal transfer of the *gua*, *thy* or *trp* loci in *L. pneumophila* (data not shown).

pCH1 does not share homology with *L. pneumophila* chromosomal DNA or other indigenous plasmids

To determine if pCH1 shared DNA homology with other *L. pneumophila* plasmids or with *L. pneumophila* chromosomal DNA, we performed colony hybridization experiments with plasmid-less and plasmid-containing strains of *L. pneumophila*. In these experiments, CsCl-purified pCH1 DNA labelled with ^{32}P was used as a probe. pCH1 DNA did not hybridize with strains of *L. pneumophila* that contained indigenous plasmids other than pCH1 (strains UH-2, UPH-1, Atlanta-1 and Atlanta-4; Fig. 2). This suggested that pCH1 does not share detectable

Fig. 2. Colony hybridization experiments with plasmid-less and plasmid-bearing strains of *L. pneumophila*. Individual colonies from plasmid-less and plasmid-bearing strains of *L. pneumophila* were patched onto nitrocellulose filters, treated with NaOH and probed with ^{32}P -labelled CsCl-purified pCH1 DNA. Colonies that hybridized with the probe were visualized by autoradiography. Only those strains that harboured an 85 MDa plasmid hybridized with the pCH1 probe.

Table 2. Summary of mating experiments

Abbreviations, see Table 1.

Donor	Recipient		Selection	Plasmid transfer detected
	Strain	Phenotype		
CH-1	AM511	Philadelphia-1 serogroup 1 $\text{Sm}^r r^- m^+$	Sm	Yes
CH-1	CS267	Philadelphia-1 serogroup 1 $\text{Sm}^r \text{Rif}^r r^- m^+$	Sm Rif	Yes
CH-1	CZ3001	Philadelphia-1 serogroup 1 :: $\text{Tn}5\text{mob}$ $\text{Km}^r \text{Sm}^r r^- m^+$	Km Sm	Yes
CH-1	CS320	Knoxville-1 serogroup 1 $\text{Sm}^r \text{Rif}^r$	Sm Rif	Yes
CH-1	CS2	Bloomington-2 serogroup 3 $\text{Sm}^r r^- m^-$	Sm	No
CH-1	CS302	Bloomington-2 serogroup 3 $\text{Rif}^r \text{Trp}^- r^- m^-$	Rif	No
CH-1	DH5 α	<i>Escherichia coli</i> $\text{Nal}^r r^- m^+$	Nal	No

DNA homology with other *Legionella* plasmids. In contrast, all of the *L. pneumophila* strains that contained an 85 MDa plasmid hybridized with the probe (LpP₂, LpP₃, OLDA, RH-1, SF3256 and SF3257; Fig. 2).

The plasmid-less serogroup 1 strains Philadelphia-1, Knoxville-1 and UH-1 did not hybridize with pCH1 DNA (Fig. 2). Also, the plasmid-less serogroup 3 strain, Bloomington-2, did not hybridize with pCH1.

Fig. 3. Infection of U937 cell monolayers with isogenic plasmid-less and plasmid-bearing strains of *L. pneumophila*. U937 cell monolayers were infected with *L. pneumophila* according to the methods of King *et al.* (1991). (a) ■, AM511; □, AM511(pCH1). (b) ▲, CS323; △, CS323(pCH1). Each point represents the mean $\pm$ SE for three separate U937 cell cultures.

Additional experiments demonstrated that pCH1 DNA also did not hybridize with the plasmid-less serogroup 1 strains Pontiac-1, NY 26 and Ver 5, or the plasmid-less serogroup 4 strain Rockport (data now shown). This suggests that these strains do not contain chromosomal sequences homologous with pCH1 DNA.

pCH1 does not affect the intracellular growth of L. pneumophila

Using the mating procedure outlined above, we introduced pCH1 into strains AM511 and CS323 to construct isogenic pairs of plasmid-less and plasmid-containing strains. Strain AM511 is virulent and capable of intracellular growth (Marra & Shuman, 1989). In contrast, strain CS323 is avirulent and incapable of intracellular multiplication (C. S. Mintz, unpublished). Both isogenic sets were used to infect monocyte-like U937 cells or the amoeba *Hartmannella vermiformis*. pCH1 did not affect the ability of strain AM511 to multiply in U937 cells (Fig. 3a) or in *H. vermiformis* (data not shown). Moreover, the plasmid did not restore the ability of the avirulent strain CS323 to replicate in either U937 cells (Fig. 3b) or in *H. vermiformis* (data not shown).

Discussion

In this study, we have shown that pCH1, an indigenous *L. pneumophila* plasmid, is self-transmissible by conjugation among serogroup 1 strains of *L. pneumophila*. Prior

to the present study, we (Mintz & Shuman, 1988) as well as others (Dreyfus & Iglewski, 1985; Chen *et al.*, 1986) demonstrated that broad host range plasmids of the IncP and IncQ incompatibility groups could easily be transferred via conjugation between different *L. pneumophila* isolates. Recently, Tully (1991) demonstrated that a 36 MDa plasmid from *L. pneumophila* strain Dodge (serogroup 1) could be transferred by conjugation to other serogroup 1 strains. Our results, along with those of Tully (1991), suggest that conjugation mediated by indigenous plasmids may be a mechanism of genetic exchange for *L. pneumophila*. To date, neither transformation nor transduction has been reported for *L. pneumophila*.

Despite repeated attempts, we were unable to detect conjugative transfer of pCH1 into a serogroup 3 strain (Bloomington-2) of *L. pneumophila*. This was somewhat surprising since strain Bloomington-2 lacks the *LpnII* restriction-modification system of *L. pneumophila* (Marra & Shuman, 1989), and is an excellent recipient in homologous and heterospecific matings involving IncP and IncQ plasmids (Chen *et al.*, 1984; Mintz & Shuman, 1988). It is not clear why we were unable to detect transfer of pCH1 in strain Bloomington-2. It is possible that pCH1 cannot be stably maintained in this strain. Experiments are underway to determine if pCH1 can be transferred by conjugation into strains from other serogroups of *L. pneumophila*. pCH1 could not be transferred from strain CH-1 into *E. coli* strain DH5 α . Since the 36 MDa plasmid described by Tully (1991) could also not be transferred from serogroup 1 strains of *L. pneumophila* into *E. coli* or *Pseudomonas aeruginosa*, it would seem that conjugative *L. pneumophila* serogroup 1 plasmids have a relatively narrow host range.

Colony hybridization experiments revealed that pCH1 did not share detectable DNA homology with the 24, 33, 45 or 62 MDa plasmids harboured by certain serogroup 1 strains of *L. pneumophila*, and that pCH1 was not related to these other *L. pneumophila* plasmids (Fig. 2). Therefore, it is likely that pCH1 belongs to a different incompatibility group than these other *L. pneumophila* plasmids. In support of this idea, the 45 MDa plasmid has been found in two serogroup 1 strains that also harbour pCH1, viz. RH-1 (Maher *et al.* 1983) and LpP₃ (C. S. Mintz, unpublished). Attempts to assign pCH1 to a known incompatibility group have been hampered by the lack of a selective marker on pCH1 and the difficulty associated with introducing plasmid DNA into *L. pneumophila*. Recently, we determined that electroporation is an efficient way of delivering plasmid DNA into *L. pneumophila* (C. S. Mintz & C.-H. Zou, unpublished). This should permit future experiments designed to identify the incompatibility group of pCH1.

It is well-established that the transfer of chromosomal

genes during conjugation results from integration of a conjugative plasmid, such as F, into the donor chromosome (Deich & Green, 1987; Silhavy *et al.*, 1984). Integration of plasmid DNA into the host chromosome usually occurs by recombination between homologous DNA sequences contained on the plasmid and the chromosome (Bartowsky *et al.*, 1987; Brenton *et al.*, 1985). As previously noted, several plasmid-less *L. pneumophila* strains failed to hybridize with the pCH1 probe in colony hybridization experiments. These results indicate that there is no detectable DNA homology between the *L. pneumophila* chromosome and pCH1. This finding could explain the inability of pCH1 to mobilize the *L. pneumophila* chromosome. In the absence of DNA sequence homology, pCH1 would be unable to integrate into the *L. pneumophila* chromosome. Consequently, the unintegrated plasmid would be incapable of promoting the transfer of chromosomal markers. Of interest, Bartowsky *et al.* (1987) suggested that the lack of chromosome mobilizing activity exhibited by a conjugative plasmid from *Vibrio cholerae* was due to the lack of significant homology between the *V. cholerae* chromosome and the plasmid. Thus, although pCH1 is self-transmissible by conjugation, it is not capable of promoting detectable chromosomal gene transfer in *L. pneumophila*.

Virulence plasmids are essential for the pathogenicity of a variety of intracellular pathogens including *Salmonella* spp. (Gulig, 1990), *Shigella* spp. (Maurelli & Sansonetti, 1988) and *Yersinia* spp. (Portnoy *et al.*, 1981, 1984). In the light of these observations, we evaluated the contribution of pCH1 to the ability of *L. pneumophila* to multiply intracellularly in eukaryotic cells. The introduction of pCH1 into the plasmid-less, virulent strain AM511 did not augment or alter the ability of this strain to grow within U937 cells or *H. vermiformis*. Moreover, the presence of pCH1 in the avirulent strain CS323 did not restore its ability to multiply in U937 cells or *H. vermiformis*. These results indicate that pCH1 does not encode factors that contribute to the intracellular growth of *L. pneumophila* within eukaryotic host cells. Our results are consistent with the findings of Bollin *et al.* (1985) who compared the ability of a plasmid-less and plasmid-containing serogroup 1 strain to infect guinea pigs following intraperitoneal injection. The plasmid-containing strain harboured pCH1 and a 45 MDa plasmid. Their results showed that there was no statistically significant difference in the ID₅₀ of the plasmid-less or plasmid-bearing strain. Interestingly, the LD₅₀ of the plasmid-less strain was significantly lower (approximately 10-fold) than that of the plasmid-containing strain. However, it is important to note that the plasmid-less and plasmid-containing strains used in this study were not isogenic. Therefore, it is difficult to

attribute the reduced virulence exhibited by the plasmid-containing strain to the presence of pCH1 or the 45 MDa plasmid. Nonetheless, our results suggest that pCH1 is not required for expression of virulence by *L. pneumophila*.

Although pCH1 cannot mobilize the *L. pneumophila* chromosome, the self-transmissible nature of the plasmid suggests that it may contribute to the exchange of genetic information among serogroup 1 isolates. For example, the acquisition by pCH1 of transposons which code for antibiotic resistance could pose serious problems in the treatment of Legionnaires' disease. Moreover, the presence of transposons on pCH1 could facilitate integration of the plasmid into the *L. pneumophila* chromosome by transposon-mediated recombination (Ichige *et al.*, 1989; Pischl & Farrand, 1983). This, in turn, could promote Hfr-like chromosomal gene transfer in *L. pneumophila*. In support of this notion, we have previously demonstrated that conjugative plasmids that contain transposons can be used to mobilize the *L. pneumophila* chromosome (Mintz & Shuman, 1988).

At the present time, it is not clear what functions are encoded by pCH1. It does not encode resistance to any of the antibiotics or heavy metals tested nor does it contribute to the intracellular growth of *L. pneumophila*. The absence of pCH1 in many environmental and clinical serogroup 1 isolates suggests that any function(s) encoded by the plasmid are not essential for the survival of *L. pneumophila* in aquatic environments or in the infected host.

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