

Natural Competence for DNA Transformation by *Legionella pneumophila* and Its Association with Expression of Type IV Pili

BARBARA J. STONE AND YOUSEF ABU KWAIK*

Department of Microbiology and Immunology, University of Kentucky
Chandler Medical Center, Lexington, Kentucky 40536-0084

Received 18 September 1998/Accepted 18 December 1998

We have recently described the expression of two pili of different lengths on the surface of *Legionella pneumophila* (B. J. Stone and Y. Abu Kwaik, *Infect. Immun.* 66:1768–1775, 1998). Production of long pili requires a functional *pilE_L* locus, encoding a type IV pilin protein. Since type IV pili in *Neisseria gonorrhoeae* are associated with competence for DNA transformation, we examined the competence of *L. pneumophila* for DNA transformation under conditions that allowed the expression of type IV pili. We show that *L. pneumophila* is naturally competent for DNA transformation by isogenic chromosomal DNA and by plasmid DNA containing *L. pneumophila* DNA. Many different *L. pneumophila* loci are able to transform *L. pneumophila* after addition of plasmid DNA, including *gspA*, *ppa*, *asd*, and *pilE_L*. The transformation frequency is reduced when competing DNA containing either *L. pneumophila* DNA or vector sequences is added to the bacteria, suggesting that uptake-specific sequences may not be involved in DNA uptake. Competence for DNA transformation correlates with expression of the type IV pili, and a *pilE_L* mutant defective in expression of type IV pili is not competent for DNA transformation. Complementation of the mutant for competence is restored by the reintroduction of a cosmid that restores production of type IV pili. Minimal competence is restored to the mutant by introduction of *pilE_L* alone. We conclude that competence for DNA transformation in *L. pneumophila* is associated with expression of the type IV pilus and results in recombination of *L. pneumophila* DNA into the chromosome. Since expression of type IV pili also facilitates attachment of *L. pneumophila* to mammalian cells and protozoa, we designated the type IV pili CAP (for competence- and adherence-associated pili).

Legionella pneumophila is the causative agent of Legionnaires' disease. Infection by *L. pneumophila* occurs after introduction of bacteria from an environmental source into a human host after inhalation of contaminated aerosols (see references 1 and 5 for recent reviews). The bacteria are then able to replicate within phagosomes of mononuclear phagocytes and possibly epithelial cells (36, 37). Multiple bacterial factors appear to be required for attachment of *L. pneumophila* to protozoan and mammalian cells. Complement-dependent and complement-independent mechanisms have been described for attachment of *L. pneumophila* to macrophages and lung fibroblasts (10, 22, 40). Mutants defective in attachment to both protozoan and mammalian cells have recently been described (19–21, 26).

One of the *L. pneumophila* attachment factors that has been recently described is the pili (50). Expression of pili by *L. pneumophila* was originally identified by electron microscopy (41, 42). The pili can be categorized as short (0.1 to 0.6 μm) or long (0.8 to 1.5 μm) (50). Expression of long pili on the bacterial surface is abolished by an insertion mutation in the *pilE_L* locus, encoding a type IV pilin protein (50). The *pilE_L* mutant has a 50% reduction in attachment to the protozoan *Acanthamoeba polyphaga* and a 50 to 65% reduction in attachment to human alveolar epithelial cells and U937 macrophage-like cells.

In addition to *L. pneumophila*, type IV pilin genes have been identified in a number of pathogenic bacteria, such as *Neisseria gonorrhoeae* (35), *Neisseria meningitidis* (39), *Pseudomonas*

aeruginosa (28), enteropathogenic *Escherichia coli* (15), and *Vibrio cholerae* (17, 46). Type IV pili are characterized by a region of homology at the amino-terminal end of the amino acid sequence (27, 52). Protein components involved in biogenesis of type IV pili have been implicated in protein secretion, twitching motility, and filamentous phage assembly (27, 52). In *N. gonorrhoeae*, factors required for type IV pilus expression on the bacterial surface are also required for natural competence for DNA transformation (for a recent review, see reference 18).

Genetic exchange between naturally competent bacteria has been described for a number of bacteria, including *N. gonorrhoeae* (49), *Bacillus subtilis* (54), *Haemophilus influenzae* (9), *Streptococcus pneumoniae* (8), *Acinetobacter calcoaceticus* (29), and others (see reference 33 for a review). Competence for transformation can be constitutive, as is the case for *N. gonorrhoeae* (49). Competence can also be induced as a function of growth conditions, such as in *H. influenzae* (48). Natural transformation requires uptake of extracellular DNA prior to incorporation of the DNA in the bacterial genetic repertoire. DNA uptake in the gram-negative bacteria *N. gonorrhoeae* (16, 23) and *H. influenzae* (47) is recognition sequence dependent while DNA uptake in the gram-positive bacteria *B. subtilis* and *S. pneumoniae* (reviewed in reference 33) and in the gram-negative bacterium *A. calcoaceticus* (32, 38) is recognition sequence independent. Processing of DNA prior to transformation (reviewed in reference 33) allows entry of linear DNA molecules into the bacterium in a single-stranded form prior to recombination into the chromosome in a *recA*-dependent manner. Circular DNA molecules are rendered linear prior to similar processing and recombination, although reconstitution of replicating plasmids can also occur.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Kentucky Chandler Medical Center, Lexington, KY 40536-0084. Phone: (606) 323-3873. Fax: (606) 257-8994. E-mail: yabukw@pop.uky.edu.

TABLE 1. Bacterial strains and plasmids

Species, strain, serogroup, or plasmid	Relevant properties	Reference(s) or source
Bacteria		
<i>L. pneumophila</i>		
AA100		3
AA400	AA100; <i>asd</i> ::Kan ^r	25
BS100	AA100; <i>pilE_L</i> ::Kan ^r	50
BS200	AA100; Nal ^r	This study
Serogroup 1	<i>pilE_L</i> ⁺	50
Serogroup 2	<i>pilE_L</i> ⁺	50
Serogroup 3	<i>pilE_L</i> ⁺	50
Serogroup 4	<i>pilE_L</i> ⁺	50
Serogroup 5	<i>pilE_L</i> ⁺	50
Serogroup 6	<i>pilE_L</i> ⁺	50
Serogroup 7	<i>pilE_L</i> ⁺	50
Serogroup 8	<i>pilE_L</i> ⁺	50
Serogroup 9	<i>pilE_L</i> ⁺	50
Serogroup 10	<i>pilE_L</i> ⁺	50
Serogroup 11	<i>pilE_L</i> ⁺	50
Serogroup 12	<i>pilE_L</i> ⁺	50
Serogroup 13	<i>pilE_L</i> ⁺	50
<i>L. wadsworthii</i>	<i>pilE_L</i> ⁺	50
<i>L. santarosae</i>	<i>pilE_L</i> ⁺	50
<i>L. cherii</i>	<i>pilE_L</i> ⁺	50
<i>L. longbeachae</i>	<i>pilE_L</i>	50
<i>L. gormanii</i>	<i>pilE_L</i>	50
<i>L. micdadei</i>	<i>pilE_L</i>	50
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169</i> (φ80 <i>lacZΔM15</i>) <i>hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	24
Plasmids		
pBC SK+	ColE1 origin, <i>lacZα</i> , Cm ^r	Stratagene (La Jolla, Calif.)
pBC-K	pBC SK+; Cm ^r Kan ^r	This study
pUC4K	pBR322 origin; Amp ^r Kan ^r ; source for the Kan ^r marker	Pharmacia Biotech (Piscataway, N.J.)
pBJ120	pBC SK+; Cm ^r <i>pilE_L</i> ⁺	50
pBJ114	pBC SK+; Cm ^r <i>pilE_L</i> ::Kan ^r	50
pBOC20	<i>oriV oriT sacB</i> Cm ^r	6
pBJ115	pBOC20; Cm ^r <i>pilE_L</i> ::Kan ^r	50
pGS-K	pBOC20; Cm ^r <i>gspA</i> ::Kan ^r	4
pJA2A-K2	pBOC20; Cm ^r <i>ppa</i> ::Kan ^r	2
pOHBOC1	pBOC20; Cm ^r <i>asd</i> ⁺	25
pOHBOC1-K	pBOC20; Cm ^r <i>asd</i> ::Kan ^r	25
pOHBOC2	pBOC20; Cm ^r <i>asd</i> ⁺	25
pTLP6	<i>oriV oriT rpsL cos</i> ^r Cm ^r	7, 34
pCC3	pTLP6; Cm ^r <i>pilE_L</i> ⁺	50

In this report, we describe the phenomenon of natural competence for DNA transformation in *L. pneumophila*. Transformation of *L. pneumophila* occurs after addition of exogenous DNA, either *L. pneumophila* chromosomal DNA or plasmid DNA containing *L. pneumophila* DNA. Transformation is dependent upon recombination of donor DNA into the chromosome. Similar to the case for *N. gonorrhoeae* (11, 14, 49), transformation in *L. pneumophila* is induced under conditions similar to those for expression of type IV pili. Transformation in *L. pneumophila* requires the type IV pilin locus (*pilE_L*), which is also essential for expression of type IV pili.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *L. pneumophila* serogroups and strains, *Legionella* species, and *E. coli* strain used in this study are listed in Table 1. Strain BS200 is a spontaneous nalidixic acid-resistant (Nal^r) mutant generated from passage of strain AA100 on buffered charcoal-yeast extract (BCYE) agar supplemented with 30 μg of nalidixic acid per ml. The plasmids used in this study are also described in Table 1. Plasmid pBC-K was derived from pBC SK+ by inser-

tion of the Kan^r gene from pUC4K into the *EcoRI* site. Cosmid pCC3 was isolated from a chromosomal library of *L. pneumophila* AA100 that contains approximately 45 kb of *L. pneumophila* DNA.

DNA preparation. Chromosomal DNAs from *L. pneumophila* and *E. coli* strains were isolated as previously described (45) or by using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.) as specified by the manufacturer. Plasmid DNA was isolated by using a QIAGEN (Chatsworth, Calif.) Midiprep kit. DNA preparations were resuspended in sterile distilled water and vortexed prior to use.

Bacterial culture and induction of competence. *L. pneumophila* strains obtained from freezer stocks were used directly or grown on BCYE agar for 48 h at 37°C prior to culture for induction of competence or examination of pilus expression by electron microscopy. Bacteria were grown in 5 ml of buffered yeast extract (BYE) broth without shaking at 37°C for 4 days in 15-ml capped plastic tubes. Prior to addition of chromosomal DNA or plasmid DNA, 4.6 ml of BYE broth was removed from the culture without disturbing the bacteria settled at the bottom of the culture tube. DNA was added to the bacterial culture (unless otherwise specified) to a final concentration of 400 μg of chromosomal DNA per ml or 40 μg of plasmid DNA per ml in a final volume of 0.5 ml, gently mixed, and returned to 37°C for an additional 2 days. Viable counts were determined for total bacteria on BCYE agar and for transformed bacteria on BCYE agar containing the appropriate antibiotics (20 μg of kanamycin per ml, 2.5 μg of chloramphenicol per ml, or 30 μg of nalidixic acid per ml).

L. pneumophila AA400 was grown in the presence of 40 μg of diaminopimelic acid (DAP) per ml. DAP was also added during transformation of AA100 with pOHBOC1-K and during growth for total viable counts. *L. pneumophila* cultures grown under alternate growth conditions were all started from *L. pneumophila* cultures grown on plates. Bacteria were resuspended in 1 ml of sterile water at approximately 10⁹ CFU/ml, and 20 μl (in BYE broth) or 200 μl (in water) was added to each 5-ml culture. Cultures were incubated at 24, 30, or 37°C. Aerated cultures and cultures grown on agar prior to electron microscopic examination or addition of DNA were grown for 2 days at 37°C. Transformation experiments performed in the presence of competing plasmid DNA were performed as described above except that 4 or 80 μg of competing plasmid DNA per ml was added to the bacteria and gently mixed just prior to addition of 4 μg of pBJ114 DNA per ml.

Data analysis. The transformation frequency was calculated as the number of antibiotic-resistant CFU per milliliter/total CFU per milliliter. For transfer of the *asd* gene into AA400, transformation frequency was measured as CFU per milliliter for cells grown on BCYE without DAP/CFU per milliliter for cells grown on BCYE with DAP. All experiments were performed with duplicate samples and performed two or more times, depending on the degree of variability. Data presented are from representative experiments, and error ranges are calculated from duplicate samples.

RESULTS

***L. pneumophila* is naturally competent for DNA transformation.** We have recently described the expression of type IV pili by *L. pneumophila*. Since type IV pili have been associated with natural DNA transformation in *N. gonorrhoeae* (11), *L. pneumophila* was examined for competence after growth of the bacteria under conditions which promoted expression of pili (50). Bacteria were grown without aeration for 4 days in BYE medium (as described in Materials and Methods). DNA transformation was first attempted with chromosomal DNA isolated from strain BS200, a spontaneous Nal^r isogenic mutant of *L. pneumophila* AA100. The ability of *L. pneumophila* wild-type strain AA100 to become Nal^r was examined after addition of BS200 chromosomal DNA. The bacteria and DNA were allowed to incubate for 2 days prior to enumeration of transformants. The Nal^r phenotype from BS200 was transferred to AA100 at a frequency of approximately $1.4 \times 10^{-6} \pm 7.1 \times 10^{-8}$ with the addition of 400 μg of chromosomal DNA per ml. Incubation of bacteria with DNA for less than 2 days resulted in a decreased transformation frequency (data not shown). In contrast, addition of the same amount of chromosomal DNA from AA100 to strain AA100 did not result in detection of any transformants ($>10^{-9}$).

DNA transformation of an alternative resistance marker was attempted. The transformation frequency of isogenic chromosomal DNA containing a Kan^r marker, which is 1.7 kb in size, was determined. The Kan^r marker was previously introduced into *L. pneumophila* AA100 by homologous recombination to generate strain BS100 and is present within *pilE_L*, the type IV

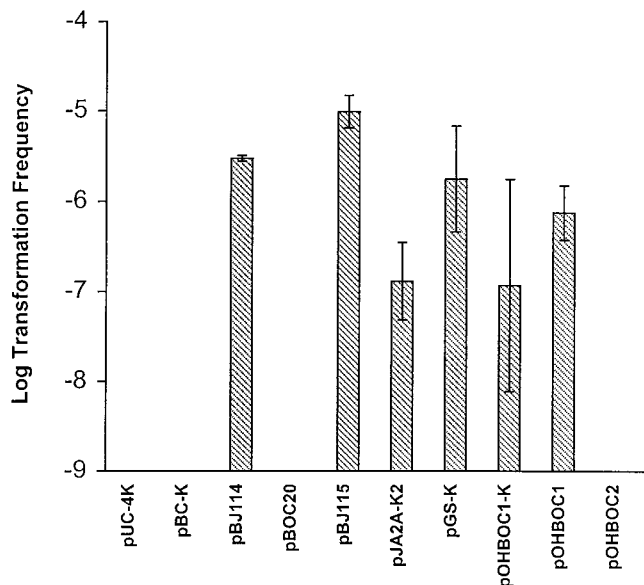


FIG. 1. Transformation of *L. pneumophila* with plasmid DNA. Transformation frequency was measured after addition, at 4 $\mu\text{g/ml}$, of plasmid DNA from pUC4K, pBC-K, pBJ114 (*pilE_L::Kan^r*), pBJ115 (*pilE_L::Kan^r*), pJA2A-K2 (*ppa::Kan^r*), pGS-K (*gspA::Kan^r*), or pOHBOC1-K (*asd::Kan^r*) to *L. pneumophila* AA100. The number of chloramphenicol-resistant transformants was measured after addition of pBOC20. Loss of auxotrophy for DAP was measured after addition of 4 μg of pOHBOC1 or pOHBOC2 plasmid DNA per ml to *L. pneumophila* AA400. The limit of detection for transformation frequency was 10^{-9} . Error bars indicate standard deviations.

pilin locus (50). The transformation frequency after addition of BS100 chromosomal DNA to AA100 was $5.8 \times 10^{-7} \pm 5.0 \times 10^{-7}$, which is comparable to the frequency of transformation after addition of BS200 DNA (NaI^r). Addition of AA100 chromosomal DNA to strain AA100 did not result in any detectable transformation ($<10^{-9}$). In all transformation experiments, variability (up to 10-fold) between experiments with regard to transformation frequency was seen. However, the competence phenotype was consistent in all experiments. Therefore, results shown are representatives of a typical experiment.

Transformation requires *L. pneumophila* sequences. The ability of plasmids obtained from an *E. coli* background to generate transformants of *L. pneumophila* was determined to examine potential barriers to uptake of exogenous DNA. All plasmids were obtained from the same *E. coli* strain prior to transformation experiments to eliminate differences in the methylation status of the DNA, which may affect transformation frequency. Plasmids obtained from *E. coli* are able to replicate autonomously in *L. pneumophila* after introduction by electroporation (data not shown). Plasmid pBJ114 contains approximately 2 kb of *L. pneumophila* chromosomal DNA (including the *pilE_L* open reading frame) with a Kan^r marker in the same location within *pilE_L* as in strain BS100. The transformation frequency was determined for transfer of the Kan^r marker. pBJ114 added to *L. pneumophila* AA100 at 4 $\mu\text{g/ml}$ resulted in a transformation frequency of 3×10^{-5} (Fig. 1). The transformation frequency was dose dependent (Fig. 2) and saturable, as addition of more than 4 μg of plasmid DNA per ml did not increase the transformation frequency (data not shown). However, plasmids pUC4K (the original source of the Kan^r marker) and pBC-K (containing the same vector as pBJ114 and the Kan^r marker), which have no *L. pneumophila* DNA, were unable to transfer to AA100 a Kan^r phenotype (Fig. 1). Therefore, the presence of *L. pneumophila* DNA is

required for transformation after addition of exogenous plasmid DNA.

Transformation of *L. pneumophila* DNA leads to recombination with the chromosome. The inability of plasmids pUC4K and pBC-K to transfer a Kan^r phenotype to *L. pneumophila* is not due to an inherent inability of *L. pneumophila* to maintain these plasmids, since they can be electroporated into *L. pneumophila* and subsequently maintained. Since the replicative status of plasmid DNA after uptake by competent *L. pneumophila* was unknown, the ability of competent *L. pneumophila* to maintain plasmid DNA was determined. Our results showed that no Cm^r or Kan^r transformants were obtained after addition of exogenous pBC-K DNA. Interestingly, transformants obtained after addition of pBJ114 DNA to AA100 were able to grow in the presence of kanamycin but not chloramphenicol, suggesting a recombination event between the *L. pneumophila* DNA from pBJ114 and the chromosome. Southern analysis of chromosomal DNA obtained from 17 Kan^r transformants and probed with *pilE_L* indicated replacement of the *pilE_L* locus with the *pilE_L::Kan^r* locus from pBJ114 (data not shown). Therefore, transformation of *L. pneumophila* with pBJ114 is related to the presence of *L. pneumophila* chromosomal DNA sequences and resulted in homologous recombination of the DNA into the chromosome.

Uptake of DNA by *N. gonorrhoeae* and *H. influenzae* requires the presence of specific sequences within the DNA (16, 23, 47), while *S. pneumoniae* and *B. subtilis* do not require the presence of recognition sequences for DNA uptake (33). To help determine if specific *L. pneumophila* DNA sequences were required for uptake of DNA, transformation of *L. pneumophila* with plasmid DNA was measured in the presence of competing DNA. Plasmid pBJ114 (4 $\mu\text{g/ml}$) was added to *L. pneumophila* after addition of competing DNA. Plasmids pBJ120 (containing the *pilE_L* locus) and pBC-K (containing no *L. pneumophila* DNA) were able to reduce the frequency of transformation in a dose-dependent manner. Addition of pBC-K or pBJ120 reduced transformation equally well. Addition of 4 μg of DNA per ml reduced transformation frequency 5- to 10-fold, while 80 μg of plasmid DNA per ml reduced transformation 10- to 100-fold. Addition of 400 μg of chromosomal

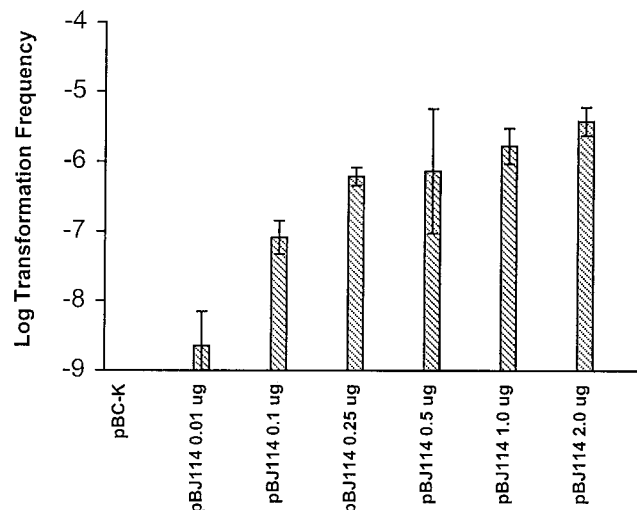


FIG. 2. DNA concentration dependence of transformation with plasmid DNA. Transformation frequency was measured after addition of the indicated amount of plasmid DNA. The limit of detection for transformation frequency was 10^{-9} . Error bars indicate standard deviations.

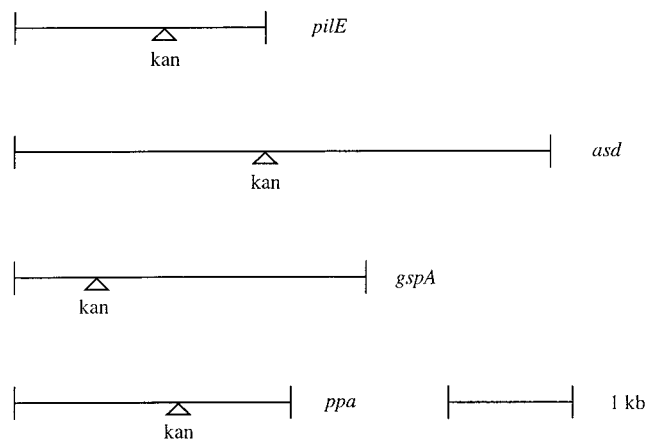


FIG. 3. Position of the Kan^r marker in cloned DNA fragments. The relative position of the marker is shown for pBJ114 and pBJ115 (*pilE*), pOHBOC1-K (*asd*), pGS-K (*gspA*), and pJA2A-K2 (*ppa*). A 1-kb fragment is shown for scale.

DNA from *L. pneumophila* AA100 or *E. coli* DH5 α per ml completely abolished transformation. Because both plasmids and both sources of chromosomal DNA could reduce the transformation frequency, it is unlikely that *L. pneumophila* has a mechanism to recognize specific sequences prior to uptake of DNA. Rather, the specificity of transformation of DNA seems to be related to the frequency of recombination. Therefore, the inability to detect transformation after addition of pUC4K or pBC-K plasmid DNA is probably due to an inability of heterologous DNA to recombine into the *L. pneumophila* chromosome.

Transformation of *L. pneumophila* with *gspA*, *ppa*, and *asd* loci. Whether the acquisition of DNA by *L. pneumophila* was specific only for the *pilE*_L locus was addressed by attempting transformation of *L. pneumophila* after addition of exogenous plasmid DNA harboring various *L. pneumophila* loci: *gspA*, *ppa*, and *asd*. Synthesis of a global stress protein (GspA) can be induced under various in vitro stress conditions as well as during intracellular infection within macrophages (1, 2, 4). The *ppa* locus encodes a pyrophosphatase that is also induced during intracellular infection (2). Production of aspartate- β -semialdehyde dehydrogenase (Asd) is required for peptidoglycan biosynthesis in *L. pneumophila* (25). All of these loci were interrupted by a Kan^r marker and introduced into *L. pneumophila* on a pBOC20-based vector. The relative position of the Kan^r marker in each cloned DNA fragment is shown in Fig. 3. Transformation of AA100 was examined for each plasmid and compared to transformation after addition of pBJ115, a pBOC20-based plasmid containing the *pilE*_L locus interrupted by a Kan^r insertion. The *pilE*_L, *gspA*, *ppa*, and *asd* loci from the plasmids were all able to be recovered by *L. pneumophila* (Fig. 1). In contrast, the vector pBOC20 was unable to transfer a detectable phenotype to AA100. Therefore, transformation of *L. pneumophila* may occur after addition of a variety of *L. pneumophila* loci on plasmid DNA. The frequency of transformation was variable between different experiments (up to 10-fold), but transformation ability was consistent in all experiments. The relative amount of *L. pneumophila* chromosomal DNA on each transforming plasmid did not correlate to the transformation frequency. The differences between the transformation frequencies of the plasmids were not significant except for comparison of pBJ114 and pBJ115 with pJA2A-K2 (unpaired *t* test, $P < 0.05$).

All of the loci examined contained at least 2.0 kb of chromosomal DNA which had been disrupted by the insertion of

the Kan^r marker. The ability of *L. pneumophila* to be transformed by plasmid DNA without a resistance marker disrupting the *L. pneumophila*-derived DNA was also assessed. Two different plasmids containing the *asd* locus were tested for the ability to transform *L. pneumophila* AA400. AA400 was generated from strain AA100 by the insertion of a Kan^r marker into *asd* and is auxotrophic, requiring addition of DAP for growth (25). The transformation frequency after addition of plasmids containing the uninterrupted *asd* locus to AA400 (regenerating the wild-type phenotype) was determined. pOHBOC1 contains ~4.3 kb of chromosomal DNA, while pOHBOC2 contains ~1.4 kb, including 209 bp upstream and 209 bp downstream of the open reading frame of *asd*. Although AA400 was transformed at a frequency of 8.2×10^{-7} after addition of pOHBOC1 DNA, transformation was not detected after addition of pOHBOC2 or pBOC20 (Fig. 1). Thus, transformation of *L. pneumophila* occurred independently of the presence of the Kan^r marker on the plasmid. However, the inability of AA400 to be transformed after addition of pOHBOC2 DNA may be due to a requirement for the presence of a minimum amount of DNA for recombination.

Dependence of competence upon culture conditions. The frequency of transformation of *L. pneumophila* AA100 to NaI^r after addition of isogenic BS200 chromosomal DNA was examined at different phases of growth. The transformation frequency of AA100 increased over time when the bacteria were grown under conditions required for pilus expression (37°C, broth culture, without aeration). Transformation was undetectable when DNA was added to the culture at day 0 (transformants were sampled after a 2-day incubation with the DNA [see Materials and Methods]) and peaked at 4 days after inoculation of cultures (Fig. 4, bottom panel). Transformation was undetectable when DNA was added to cultures grown for 6 days (Fig. 4, bottom panel). Bacterial viability did not decrease significantly during the duration of the experiment (Fig. 4, top panel).

To determine if the transformation frequency could be altered by changes in culture conditions, transformation of *L. pneumophila* AA100 was examined after addition of pBJ114 DNA under various culture conditions. The transformation frequency was measured after bacterial culture at different temperatures to determine if competence was affected by temperature. Transformation was apparent after incubation of the bacteria at 37, 30, and 24°C (Table 2). No consistent difference in the transformation frequencies measured at different temperatures was observed. Also, the presence of 5% CO₂ during culture at 37°C had no effect (Table 2). The ability of *L. pneumophila* to become competent in an aqueous environment, as might be relevant in the environment, was measured. Bacteria were grown on solid medium and resuspended in double-distilled sterile water for 4 days at 37 or 24°C, and pBJ114 DNA was added. However, no transformation was detected (Table 2). Therefore, as determined with the culture conditions tested, *L. pneumophila* is transformation competent when grown at 24 to 37°C in BYE broth without aeration.

Because recognition sequence-dependent transformation in *N. gonorrhoeae* requires factors involved in type IV pilus biogenesis (18), the correlation between type IV pilus expression and competence in *L. pneumophila* was examined. The ability of *L. pneumophila* to be transformed after addition of chromosomal DNA was originally determined for bacteria grown under conditions which allow expression of type IV pili (50). Therefore, transformation of *L. pneumophila* was measured after growth of bacteria under conditions where no type IV pili are expressed. Expression of type IV pili by *L. pneumophila* was not evident as determined by electron microscopic exam-

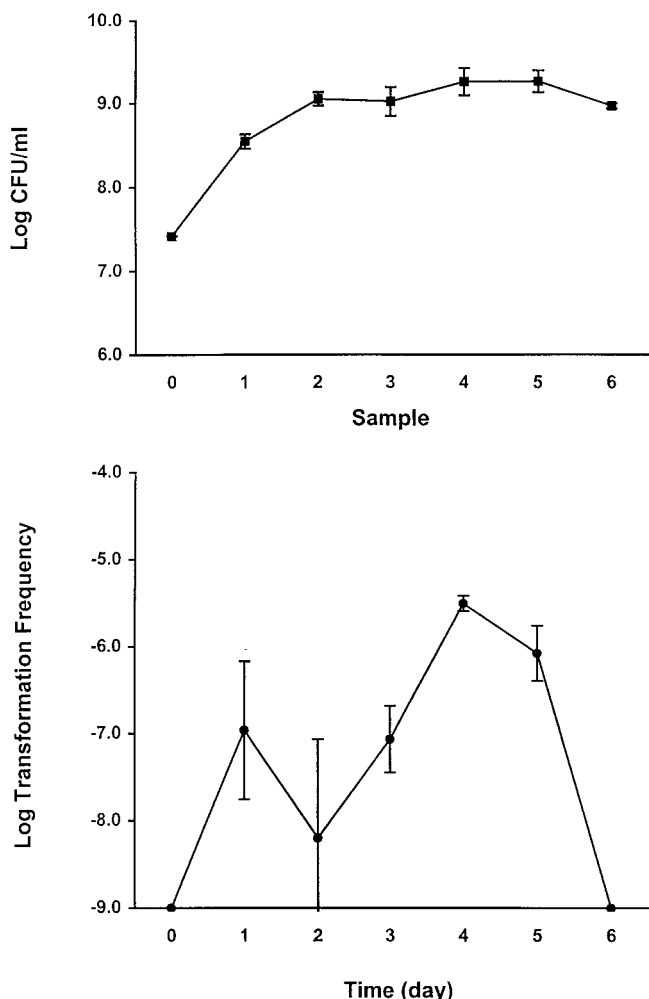


FIG. 4. Induction of competence in *L. pneumophila*. (Top) Viable counts before addition of DNA. Sample numbers correspond to samples taken at the time points indicated in the bottom panel. (Bottom) *L. pneumophila* BS200 chromosomal DNA (400 μ g/ml) was added to cultures at the time points indicated, and the cultures were allowed to incubate for an additional 2 days before transformation frequency was determined. No transformants were detected after addition of DNA at days 0 and 6. The limit of detection for transformation frequency was 10^{-9} . Error bars indicate standard deviations.

ination after culture of the bacteria in BYE broth with aeration at 37°C or on BCYE agar at 37°C (data not shown). No transformation of *L. pneumophila* was detected after addition of pBJ114 when the cultures were aerated or when *L. pneumophila* was grown on solid medium (Table 2). Therefore, the transformation-competent phenotype and expression of type IV pili are induced under similar culture conditions.

A *pilE_L* mutant is not competent for DNA transformation. The gene products required for type IV pilus expression in *N. gonorrhoeae* are essential for uptake sequence-dependent competence resulting in DNA transformation (18). Since type IV pilus expression in *L. pneumophila* and natural transformation competence are induced under similar growth conditions, the requirement for type IV pilus expression in *L. pneumophila* for transformation competence was examined. In contrast to wild-type strain AA100, the *pilE_L* mutant BS100 is unable to express type IV pili (50). Therefore, the requirement of *L. pneumophila* transformation for the *pilE_L* locus was determined with BS100 and compared to transformation of AA100.

In contrast to AA100, the *pilE_L* mutant BS100 was unable

to be transformed after addition of chromosomal DNA from strain BS200 (transformation frequency of $>10^{-9}$). To confirm that type IV pili were required for competence, the ability of the wild-type *pilE_L* locus to restore competence to BS100 was determined. Cosmid pCC3 contains the *pilE_L* locus and restores expression of type IV pili to strain BS100 (50). The cosmid also restored transformation frequency of BS100 to wild-type levels ($1.9 \times 10^{-6} \pm 7.1 \times 10^{-8}$). Since pCC3 contains a large fragment of chromosomal DNA from *L. pneumophila*, restoration of competence to BS100 was determined with pBJ120, a plasmid that contains only one complete open reading frame, *pilE_L*. Introduction of pBJ120 into BS100 minimally restored transformation frequency ($7.6 \times 10^{-8} \pm 1.0 \times 10^{-7}$). Neither the pTLP6 nor the pBC vector was able to restore competence to BS100 (transformation frequency of $<10^{-9}$). However, BS100 harboring pBJ120 does not express type IV pili (50). We cannot exclude the possibility that low levels of *PilE_L* on the surface of *L. pneumophila* may result in both a minimal transformation frequency and reduced numbers of type IV pili, which would be difficult to detect by electron microscopic examination, particularly since only 10% of wild-type bacteria express the type IV pili (50). Nevertheless, the type IV pilin gene is sufficient to restore a minimal level of competence for DNA transformation.

Prevalence of natural competence for transformation in legionellae. The prevalence of the *pilE_L* locus in *L. pneumophila* serogroups and in various *Legionella* species has been recently determined by Southern analysis (50). The locus was present in all 13 *L. pneumophila* serogroups (analyzed under high-stringency conditions) and showed variable hybridization for 16 *Legionella* species examined (analyzed under low-stringency conditions). The natural transformation competence of representatives of the *L. pneumophila* serogroups and *Legionella* species was determined after addition of plasmid DNA (pOHBOC1-K) containing a Kan^r marker inserted within *asd* obtained from *L. pneumophila* AA100. The *asd* locus was chosen because the gene was likely to be present in all species, since none of the *Legionella* species tested were auxotrophic prior to transformation. This was further confirmed by DNA hybridization with *asd* as a probe. In situ hybridizations of all tested serogroups and strains showed hybridization to all 13 serogroups (data not shown). Four of six *Legionella* species tested hybridized to *asd* as well (*L. wadsworthii* and *L. santicrucis* did not hybridize).

TABLE 2. Effect of culture conditions on transformation frequency

Plasmid	Culture medium	Temp (°C)	Other culture condition	Transformation frequency ^a
pBC-K	BYE	37		10^{-9}
pBJ114	BYE	37		$3.9 \times 10^{-7} \pm 1.3 \times 10^{-7}$
pBC-K	BYE	30		10^{-9}
pBJ114	BYE	30		$9.5 \times 10^{-6} \pm 2.0 \times 10^{-6}$
pBC-K	BYE	24		10^{-9}
pBJ114	BYE	24		$1.6 \times 10^{-7} \pm 1.4 \times 10^{-8}$
pBC-K	BYE	37	Aeration	10^{-9}
pBJ114	BYE	37	Aeration	10^{-9}
pBC-K	BYE	37	CO ₂	10^{-9}
pBC-K	BCYE	37	CO ₂	10^{-9}
pBJ114	BCYE	37		10^{-9}
pBC-K	Water	37		10^{-9}
pBJ114	Water	37		10^{-9}
pBC-K	Water	24		10^{-9}
pBJ114	Water	24		10^{-9}

^a Transformation frequency was measured after addition of 40 μ g of plasmid DNA per ml.

As expected, transformation was detected for *L. pneumophila* AA100 (serogroup 1). However, even though all serogroups hybridized with a probe containing the *pilE_L* locus in high-stringency Southern hybridizations, transformation was detected only for *L. pneumophila* serogroup 5, at a frequency of $1.4 \times 10^{-6} \pm 4.2 \times 10^{-7}$. All other *Legionella* species tested were not transformable whether they hybridized to the type IV pilin gene or not (transformation frequency, $<10^{-9}$). The inability of these *Legionella* species to be transformed after addition of the *asd*-containing plasmid suggests that the *pilE_L* locus is not functional. However, the chromosomal DNA from *L. pneumophila* AA100 may be a barrier to homologous recombination in different serogroups or species if the *asd* loci are not similar enough to allow homologous recombination. Alternatively, DNA uptake or recombination by other serogroups or species may be hindered by alternative restriction systems not encountered in *L. pneumophila* AA100.

DISCUSSION

This is the first description of natural competence for transformation in *L. pneumophila*. The ability of *L. pneumophila* to become competent for DNA transformation is dependent on growth conditions and is induced under conditions similar to those for expression of type IV pili on the bacterial surface. Disruption of the *pilE_L* locus of *L. pneumophila* results in loss of type IV pili and also abolishes induction of competence. Restoration of both expression of pili and transformation competence is accomplished by reintroduction of the wild-type *pilE_L* locus on a cosmid. Since type IV pili of *L. pneumophila* are also involved in adherence to protozoan and mammalian cells, the pili have been designated CAP, for competence- and adherence-associated pili.

PilE_L is similar to type IV pilin proteins found in *N. gonorrhoeae*, *P. aeruginosa*, and *Moraxella bovis*, among others (50). However, only in *N. gonorrhoeae* has a connection between the type IV pilus structure and natural competence for DNA transformation been observed (18). While *B. subtilis* and *H. influenzae* both maintain DNA uptake mechanisms with components that resemble proteins found in association with type IV pilin systems (13, 30, 53), neither has been shown to produce any type IV pilus structure. In addition, many pathogenic species, such as *P. aeruginosa*, produce type IV pili (51) but have not found to be naturally competent. Therefore, *L. pneumophila* is the second example of a bacterium which requires a type IV pilin locus for both pilus expression and natural competence for DNA transformation.

In *N. gonorrhoeae*, all of the gene products required for type IV pilus biogenesis are also required for DNA uptake (18). These include PilE, the major pilus structural component (35), and PilC, an adhesin (43) which has been localized to the tip of the pilus (44) and also to the outer membrane (18). Interestingly, transformation requires the presence of PilE and PilC but not necessarily the assembled pilus structure (18). A similar phenomenon may be present in *L. pneumophila*. Reintroduction of only the *pilE_L* open reading frame into the *pilE_L* mutant consistently restored competence, but at a minimal level compared to complementation with a cosmid harboring the locus. The *pilE_L* gene alone did not restore expression of type IV pili, suggesting perhaps reduced levels of PilE_L at the bacterial surface. Incomplete complementation may be due to inefficient transcription of *pilE_L* from the plasmid or may represent the requirement for additional loci that were disrupted by a polar effect in the *pilE_L* mutant. Regardless, the *pilE_L* gene alone is sufficient for restoration of minimal levels of

transformation, and the *pilE_L* locus is required for both type IV pilus expression and transformation competence.

Acquisition of DNA fragments by bacteria can be dependent upon particular sequences found within the fragment or can be independent of these sequences. In *N. gonorrhoeae*, for example, type IV pilus-dependent uptake is species specific and requires the presence of a 10-bp sequence frequently found as part of transcription terminators within gonococcal chromosomal DNA (16, 23). However, transformation can also occur independently of such sequences in the absence of pili (12). The exact function of such sequences during DNA uptake is unknown. However, other mechanisms for DNA uptake appear to be independent of particular sequences, as is the case in *S. pneumoniae* (33), *B. subtilis* (33), and *A. calcoaceticus* (32, 38). Since *L. pneumophila*-derived DNA on a plasmid, vector DNA, *L. pneumophila* chromosomal DNA, and *E. coli* chromosomal DNA were capable of reducing the transformation frequency of a plasmid containing *L. pneumophila* DNA, the presence of an uptake mechanism specific for *L. pneumophila* sequences is unlikely. In this respect, *L. pneumophila* would be different from *N. gonorrhoeae* in the requirement for recognition sequence-specific DNA uptake during type IV pilin-dependent transformation.

However, it is unknown if uptake of vector DNA without the presence of *L. pneumophila* DNA occurs. We have been unable to differentiate between a lack of DNA uptake and DNA degradation after uptake because of the low transformation frequency and high levels of background during attempted radiolabelled-DNA uptake experiments (data not shown). Our failure to detect transformation of vector DNA may therefore be due either to failure of *L. pneumophila* to internalize the DNA or to degradation of DNA after uptake. Since vector DNA and *E. coli* chromosomal DNA block transformation, it is most likely that vector DNA is taken up and degraded.

In other bacterial transformation systems, transforming DNA is taken up as single-stranded DNA fragments and thus requires recombination onto the bacterial chromosome or recircularization of DNA into a plasmid (33). In *L. pneumophila*, there is a barrier to transformation for plasmid DNA that does not contain *L. pneumophila* chromosomal DNA. This barrier is not due to a general failure to maintain plasmid DNA, since electroporated plasmids are maintained in *L. pneumophila*. However, Southern hybridization analysis confirmed that transformation of a plasmid containing *L. pneumophila* DNA is a result of homologous recombination (of *L. pneumophila* DNA but not the vector) into the *L. pneumophila* chromosome. It is probable that plasmid DNA taken up by the bacteria is unable to recircularize and replicate autonomously and therefore that homologous recombination must occur for transformation to be detected.

Transformation of *L. pneumophila* after addition of plasmid DNA was dependent on culture conditions which correlated with growth conditions used during previous characterization of CAP pili (50). Temperature-regulated pilus expression has previously been described for *L. pneumophila* (31). Pili were found to be differentially expressed at 30°C but not at 37°C when grown on solid media. Expression correlated with transcription of *pilBCD*, whose products are homologues of *P. aeruginosa* PilB, PilC, and PilD (31), which are required for type II protein secretion and pilus biogenesis (52). As reported here, growth of *L. pneumophila* at 30°C in broth did not result in a consistent difference in transformation frequency when compared to growth at 24 or 37°C. However, as there was variability between experiments (up to 10-fold), and because type IV pilus expression is low (10% of that for wild-type bacteria), it is not possible to evaluate slight differences in

transformation frequency that may have occurred due to temperature regulation of pilus expression.

It is possible that the variability seen during transformation experiments could be caused by nuclease activity present in the culture medium during introduction of DNA into *L. pneumophila* or by different levels of shearing of DNA in individual DNA preparations. Two different chromosomal DNA isolation procedures were used to prepare DNA. However, variability between experiments was seen regardless of the isolation procedure used. Because of the observed variability, the inconsistent differences between transformation efficiencies were difficult to interpret. However, despite the variability in frequency seen, the ability of *L. pneumophila* to become competent for transformation after addition of exogenous DNA and the dependence of transformation upon the *pilE_L* locus were very consistent for all experiments.

The discovery that *L. pneumophila* is naturally competent for DNA transformation will be beneficial in genetic manipulations of the bacteria. However, the consequences of the ability of *L. pneumophila* to acquire genetic elements by natural competence for DNA transformation are unknown. The ability to incorporate DNA into the bacterial chromosome from the extracellular environment is an important mechanism for horizontal exchange of genetic information. Considering the ubiquitous presence of *L. pneumophila* in the environment, natural competence for DNA transformation may allow incorporation of genes into the bacterial chromosome which could increase environmental fitness or increase the ecological niches available to the microorganism.

ACKNOWLEDGMENTS

We thank Omar Harb and Lian-Young Gao for critical analysis of the manuscript.

B. J. Stone was supported by National Research Service Award CA09509. Y. Abu Kwaik is supported by NIH grant AI38410.

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