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Broad-Host-Range Plasmid pRK340 Delivers Tn5 into the *Legionella pneumophila* Chromosome

MARK G. KEEN, E. D. STREET, AND PAUL S. HOFFMAN*

Department of Microbiology and Immunology, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163

Received 30 November 1984/Accepted 12 March 1985

Transposon Tn5 was introduced into *Legionella pneumophila* on plasmid pRK340, which is temperature sensitive for plasmid maintenance. The presence of plasmid DNA was confirmed by agarose gel electrophoresis and by conjugal transfer of the plasmid to *Escherichia coli*. Tn5 insertions were obtained by culturing *L. pneumophila* at the nonpermissive temperature (43°C) on buffered charcoal-yeast extract agar containing kanamycin. Of the 260 kanamycin-resistant colonies picked, 220 failed to conjugate pRK340 to *E. coli*. Plasmid DNA was not visualized from eight randomly picked Tn5-containing strains, and Southern hybridizations indicated that Tn5, but not pRK340, inserted into multiple sites in the *Legionella* chromosome. In addition, the streptomycin resistance determinant on Tn5 was expressed in *L. pneumophila*.

Legionella pneumophila and related species are fastidious facultative intracellular parasites for which determinants of virulence have not been clearly established. Continued propagation of virulent strains on laboratory media leads to a loss of virulence (15), which can be restored by passage of the avirulent strains in cell culture (24). Despite transitions between virulence and avirulence, no phenotypic markers have been found which correlate with loss of virulence. Although plasmid-encoded virulence factors have been reported for other gram-negative bacteria (18, 19), no correlation between plasmids and virulence has been made for the legionellae (1, 5). Genetic regulation of the transition from virulence to avirulence in the legionellae may be under control mechanisms similar to those recently described for *Bordetella pertussis* (23). Weiss and Falkow (23) demonstrated that the phase change (reversible conversion from virulence to avirulence) of *B. pertussis* was controlled by a chromosomal *trans*-acting gene product. However, before progress can be made towards resolving mechanisms of pathogenesis for the legionellae, it will be necessary both to develop a workable genetic system and to identify specific markers associated with virulence.

Since transposon mutagenesis has proved to be an effective tool in studies of pathogenesis, we set out to develop a means for delivering transposons into the chromosome of *L. pneumophila*. It has recently been reported that *Pseudomonas* plasmids of the IncP-1 incompatibility group can be transferred to several *Legionella* species via conjugation (8). Chen et al. (6) have introduced Tn5 into several *Legionella* species on the broad-host-range suicide plasmid pJB4JI, but they did not demonstrate transposition. In this study we show that pRK340, a temperature-sensitive derivative of the IncP-1 plasmid RK2, can effectively deliver Tn5 to the chromosome of *L. pneumophila*.

All strains and plasmids used in this study are listed in Table 1. *L. pneumophila* strains were maintained on ACES (*N*-(2-acetamido)-2-aminoethanesulfonic acid)-buffered charcoal-yeast extract (BCYE) agar (17), and batch cultures were grown in BYE medium (without charcoal) and harvested as previously described (12). All cultures were tested for purity by microscopic examination and by their inability to grow on brucella agar (Difco Laboratories, Detroit, Mich.). *Escherichia coli* strains were propagated in superbroth and on LB agar (13) supplemented with diaminopimelic acid (DAP, 0.1 mM) and the appropriate antibiotics. *Pseudomonas aeruginosa* was cultured on brucella agar.

Plate matings were performed by the method of Bradley et al. (4) on media supplemented with the appropriate antibiotics. Matings from *E. coli* X1849 donors (log phase) to *L. pneumophila* recipients (stationary phase) were done by mixing equal volumes of cells. The mixture was incubated statically for 15 to 30 min and plated onto BCYE agar without antibiotics, and once growth was evident, the patches were replica plated onto selective media. The BCYE medium selects against *E. coli* X1849 by lacking DAP and containing an inhibitory concentration of cysteine. The conjugal transfer of plasmid pRK340 from a DAP-requiring *E. coli* strain to *L. pneumophila* was initially done at 30°C because the stability of pRK340 at 37°C was unknown. Subsequent experiments verified that pRK340 was stably maintained at 37°C. Plasmid R68.45 was also introduced into *L. pneumophila* via conjugation. The legionellae are particularly sensitive to kanamycin (20 μg/ml), and selection of transconjugants on kanamycin-containing BCYE medium proved to be effective. Spontaneous kanamycin-resistant (Km^r) mutants of *L. pneumophila* were not observed in this study. Although previous studies indicated that transfer of IncP-1 plasmids from *E. coli* to *L. pneumophila* occurred at frequencies between 10^{-3} and 10^{-5} (6, 8), we experienced a much lower frequency of transfer. Although conjugal transfer of pRK340 to *L. pneumophila* occurred at a low frequency ($>10^{-6}$; data not shown), once the plasmid was established, it was stably maintained.

Plasmid-containing *Legionella* strains were mated with *E. coli* HB101 and scored for growth on LB agar supplemented with kanamycin (50 μg/ml), tetracycline (25 μg/ml), and streptomycin (100 μg/ml). All Km^r *Legionella* strains readily transferred pRK340 and R68.45 back to *E. coli*. Plasmid-containing exconjugates and control strains of *L. pneumophila* were examined for the presence of plasmids by the method of Kado and Liu (11). After extraction of plasmid DNA, samples were subjected to electrophoresis on horizontal 0.5% agarose gels with a Tris-borate buffer as described previously (13). The *Legionella* strains contained plasmid

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Relevant phenotype[a]	Source or reference
L. pneumophila		
Philadelphia 1	Serovar 1, avirulent	L. Pine, Centers for Disease Control
Philadelphia 1(pRK340)	Km^r Sm^r	This laboratory
Philadelphia 1(R68.45)	Cb^r Km^r	This laboratory
P. aeruginosa		
PAO2(R68.45)	Cb^r Km^r Tc^r Cma	(9)
E. coli		
HB101	Sm^r	(3)
X1849	Nal^r, DAP requirement	(10)
HB101(pRK290)	Sm^r Tc^r	(7)
HB101(pRK340)	::Tn*5* Tc^r, Ts maintenance	M. Brasch, University of Texas
X1849(R68.45)	Cb^r Km^r Tc^r, DAP requirement	This laboratory
X1849(pRK340)	::Tn*5* Tc^r, DAP requirement, Ts maintenance	This laboratory

[a] Cb^r, Carbenicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; Cma, chromosomal mobilization ability; Nal^r, naladixic acid resistance; DAP, diaminopimelic acid; and Ts, temperature sensitive.

DNA (R68.45 and pRK340; Fig. 1). No differences were observed in the electrophoretic mobilities between plasmids extracted from strains of *L. pneumophila* and those extracted from control strains of *E. coli* and *P. aeruginosa*, indicating that plasmid rearrangement had not occurred in *L. pneumophila*. The higher-molecular-weight band observed with pRK340 DNA obtained from *L. pneumophila* may correspond to open circular DNA, or it could be just an artifact of the preparation.

To determine whether plasmid pRK340 could be used to deliver Tn*5* into the *Legionella* chromosome, strains containing pRK340 were grown under nonselective conditions at 30°C in BYE broth and plated directly onto BCYE agar plates supplemented with 20 μg of kanamycin per ml and incubated at the nonpermissive temperature (43°C). Colonies arising on BCYE agar plates after 36 to 48 h were picked and propagated on medium containing kanamycin at 37°C. The plasmid-borne tetracycline resistance determinant of pRK340 was found to be poorly expressed in *L. pneumophila*. Although the legionellae are sensitive to tetracycline, the excess ferric iron added to BCYE medium probably inactivates the tetracycline, and our attempts to score plasmid-containing *Legionella* strains on BCYE medium with tetracycline in the absence of ferric pyrophosphate were inconsistent. To determine whether plasmids were lost from *L. pneumophila* at 43°C, 260 Km^r colonies were mated with *E. coli* HB101. Of these colonies, 220 failed to transfer resistance markers. Since all control strains of *L. pneumophila* carrying pRK340 transferred the plasmid to *E. coli* at a high frequency, we assumed this failure was due to curing of the plasmid. The mating experiment was carried out several times, and in no instances did any of the 220 strains containing Tn*5* inserts subsequently transfer kanamycin resistance to *E. coli*. Eight strains were picked at random from the 220 putative Tn*5* inserts for evaluation by plasmid isolation and agarose gel electrophoresis. None of the eight randomly picked strains contained plasmid DNA (Fig. 1). However, these experiments could not rule out the possibility that both plasmid and Tn*5* had integrated into the *Legionella* chromosome.

To resolve this possibility, we examined these strains by Southern hybridization (20) for integrated plasmid DNA and integrated Tn*5* DNA sequences. Chromosomal DNA was prepared from the Tn*5* insert strains and from control strains of *L. pneumophila* containing either no plasmid or pRK340 as described by Marmur (14). Purification of plasmid pRK290 (an RK2 derivative with no Km^r determinant) was by the protocol of Birnboim and Doly (2). pSV2neo DNA (21) was a gift from G. Kitchingman, St. Jude Children's Research Hospital. This plasmid contains sequences from the Km^r determinant of Tn*5*. The chromosomal DNA was restricted with *Eco*RI (New England BioLabs, Inc., Boston, Mass.) and subjected to horizontal agarose gel electrophoresis (0.85% agarose). Nick translation of pSV2neo and pRK290 was performed as described previously (13). The pSV2neo probe hybridized with Tn*5* sequences in the chromosome of the six strains examined (Fig. 2). These results also show that the transposon integrated into at least three different chromosomal locations. Similar experiments after DNA digestion with *Cla*I also supported this conclusion (data not shown). The *Cla*I-digested samples revealed four different chromosomal locations, although one of these locations contained a doublet. Several of the insertions were in fragments of identical size, and additional studies will be needed to resolve whether independent insertions can occur in a specific site or whether the insertions arose from a common clone. When DNA from strains containing Tn*5* inserts was probed for pRK340 plasmid DNA, no evidence for plasmid sequences could be found. The plasmid probe hybridized strongly with pRK340 DNA in the control sample. pSV2neo DNA did not hybridize with pRK290 DNA.

Tn*5* contains a streptomycin resistance gene which is not expressed in enteric bacteria, but is expressed in nonenteric bacteria (16). We examined *L. pneumophila* for the expression of Tn*5*-encoded streptomycin resistance. When plasmid (pRK340)-containing strains and strains containing Tn*5* inserts were examined for streptomycin resistance, these strains were found to be resistant to concentrations inhibitory to the parent strain. In addition, strains containing

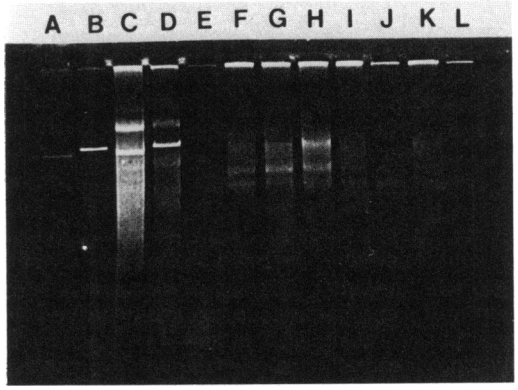

FIG. 1. Agarose gel electrophoresis of plasmid- and Tn*5* insertion-containing strains of *L. pneumophila*. Lane A, *E. coli* X1849(pRK340); lane B, *P. aeruginosa* PAO2(R68.45); lane C, *L. pneumophila* Phil-1(pRK340); lane D, *L. pneumophila* Phil-1(R68.45); lanes E through L, Km^r *L. pneumophila* isolates containing Tn*5* insertions. Linear chromosomal DNA fragments are seen in lanes depicting the strains containing inserts.

pRK340 were more resistant to streptomycin than were strains containing Tn*5* inserts (Fig. 3). This characteristic may be attributable to multiple copies of the streptomycin resistance gene in pRK340-containing strains (22). We have isolated several spontaneous streptomycin-resistant mutants of *L. pneumophila* which are resistant to much higher levels of streptomycin (200 μg/ml) than what is due to expression of the Tn*5*-encoded resistance (15 μg/ml).

We have demonstrated that the temperature-sensitive plasmid pRK340 can be used to deliver Tn*5* into the chromosome of *L. pneumophila*. The high efficiency of curing of the plasmid at the nonpermissive temperature ensures that a high percentage of Km^r survivors will contain Tn*5* insertions. The results from Southern hybridizations demonstrate that Tn*5* integrates into the *Legionella* chromosome at several sites, which may be an indication of random insertion. One limitation of this method is an inability to distinguish between the survivors containing plasmid and the plasmidless strains containing inserts. Although differences in streptomycin resistance appear in disk diffusion tests, the use of differential resistance to streptomycin as a screening method (plasmid versus insert) in replica-plating experiments has not proved reliable. We have recently added Tn*3* (ampicillin resistance) to pRK340 and are presently evaluating the use of ampicillin resistance as a marker for the plasmid. Studies with R68.45 indicate that ampicillin is a strong selectable marker in *L. pneumophila* (8).

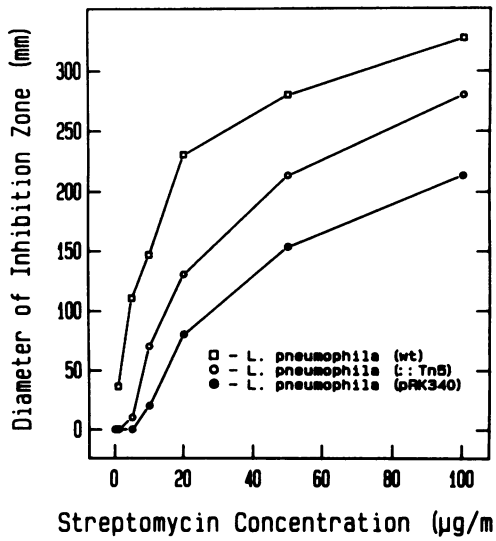

FIG. 3. Quantitation of streptomycin resistance by the disk diffusion assay. All points are the means of triplicate assays on BCYE agar plates.

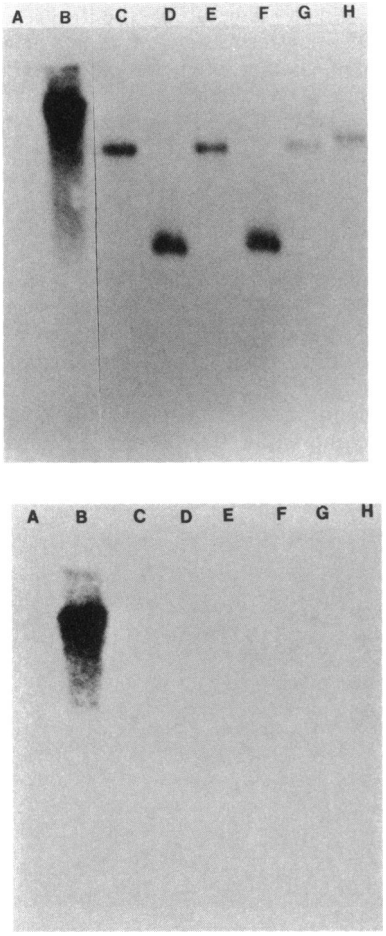

FIG. 2. Hybridization of ^{32}P-labeled pSV2neo (upper panel) and pRK290 (lower panel) to *Eco*RI digests of DNA purified from *L. pneumophila* strains. Lanes A, DNA from wild-type strain of *L. pneumophila*; lanes B, DNA from *L. pneumophila* containing pRK340; lanes C through H, *L. pneumophila* strains containing Tn*5* insertions.

To our knowledge, this is the first report of successful transposition of Tn*5* into the chromosome of *L. pneumophila*. Although we have demonstrated that Tn*5* integrates into multiple sites on the *Legionella* chromosome, this method does have certain limitations. The low frequency of conjugal transfer of pRK340 to *L. pneumophila* precludes the use of the independent mating method as a means for generating independent Tn*5* insertions. In addition, the method used in this study will give rise to a population of nonindependent clones, as suggested by the Southern hybridization data (Fig. 2). However, this method should be useful for the selection of mutants with positively selectable phenotypes. We are presently examining as donors other strains of *E. coli*, as well as *Legionella* strains, in an effort to improve the conjugation frequencies. The ability to obtain specific mutants by transposon insertion mutagenesis should lead to resolution of many unanswered questions regarding this interesting facultative intracellular pathogen.

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