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Transfer and Maintenance of Small, Mobilizable Plasmids with ColE1 Replication Origins in *Legionella pneumophila*

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With the mutagenesis of specific, virulence-associated genes of *Legionella pneumophila* as the eventual goal, methods for gene transfer to these bacteria were developed. Following the observations of others that conjugative, broad-host-range plasmids could be transferred from *Escherichia coli* to *L. pneumophila* at low frequency, we constructed a small mobilizable vector, pTLP1, which carries *oriV* from pBR322, *oriT* from pRK2, Km^r from Tn5, and an *L. pneumophila*-derived fragment to permit chromosomal integration. In triparental matings including an *E. coli* with a conjugative (Tra^+) helper plasmid, kanamycin-resistance was transferred from *E. coli* to *L. pneumophila*. Southern hybridization of *L. pneumophila* transconjugants showed that pTLP1 was replicated autonomously. Additional matings of plasmids having deletions or substitutions of pTLP1 sequences confirmed that replication in *L. pneumophila* requires *oriV* only. pTLP1 was maintained in *L. pneumophila* with passage on medium containing kanamycin but was rapidly lost after passage on nonselective medium. This plasmid instability in *L. pneumophila* is most likely due to rapid generation of plasmid-free segregants because of plasmid multimerization and low plasmid copy number. We conclude that mobilizable pBR322-derived plasmids can be used as shuttle vectors to transfer cloned genes to *L. pneumophila*, a feature that can be exploited for the purposes of mutagenesis or genetic complementation.

With the goal of identifying bacterial factors involved in the complex processes of intracellular survival and growth, we sought to develop methods for genetic manipulation of *Legionella pneumophila*, e.g., directed mutagenesis, gene complementation, cloning in *Legionella*. A fundamental requirement in all of these procedures is a method of DNA transfer to the target organism. Accordingly, we began our studies by developing a workable system that permits the introduction of genes, altered *in vitro*, into *L. pneumophila*.

The possible strategies for gene transfer to *L. pneumophila* are limited. In particular, there are no bacteriophages known to infect legionellae, and several attempts in our laboratories to transform *L. pneumophila*, both naturally and using a variety of artificial techniques, have been unsuccessful. In contrast, two groups of investigators have reported that conjugative, broad-host-range plasmids can be transferred to *L. pneumophila* from *Escherichia coli* (Chen *et al.*, 1984; Dreyfuss and Iglewski, 1985), and two other groups have used conjugative plasmids to deliver transposons (Keen *et al.*, 1985; Mintz and Shuman, 1987). Unfortunately, these conjugative plasmids are necessarily large in size and are therefore unsuitable vectors for *in vitro* manipulations. Therefore, to exploit conjugation as a mode of transfer of a small, well-characterized cloning vector, we constructed a derivative of pBR322 that carries *oriT*, the transfer origin of the conjugative plasmid RK2 (Guiney and Yakobson, 1983). In matings using a conjugative helper plasmid, this small, hybrid plasmid was transferred to *L. pneumophila* and, to our surprise, was replicated as an extrachromosomal element.

MATERIALS AND METHODS

Bacterial strains and plasmids. The 130b strain of *L. pneumophila*, a serogroup 1 clinical

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isolate from the Wadsworth V.A. Hospital outbreak (provided by P. Edelstein), or derivatives were used as recipients in all mating experiments. For purpose of counterselection in matings with *E. coli,* a spontaneous nalidixic acid-resistant mutant (Nal^r) of this strain was selected (strain AA101). *L. pneumophila* AA101 was grown on buffered charcoal yeast extract (BCYE)[1] agar containing 25 μg/ml nalidixic acid with or without kanamycin 25 μg/ml (BCYE/Nal/Kan or BCYE/Nal).

pEYDG1, a derivative of pBR322 that carries *oriT* cloned within a functional copy of Tn5 (Yakobson and Guiney, 1984), and pRK212.1, an IncP helper plasmid with an insertionally mutated Km^r gene (Figurski and Helinski, 1979), were kindly provided by D. Guiney (University of California, San Diego). pSMJ90.9 is a plasmid derived from a gene library of *L. pneumophila* (Engleberg *et al.,* 1984) that contains sequences believed to be involved in the production of the bacterial flagella (by virtue of the reactivity of the *E. coli* clone with a monoclonal antibody directed against *L. pneumophila* flagellin; N. C. Engleberg, unpublished observations).

Recombinant DNA techniques. Plasmids were isolated from *E. coli* and *L. pneumophila* using either the alkaline lysis technique (Birnboim and Doly, 1979) or the boiling technique of Holmes and Quigley (1981) as indicated. Restriction endonuclease digestions and DNA ligations were performed according to the specifications of the manufacturers. All ligated plasmid constructs were propagated by transformation into *E. coli* HB101 using $CaCl_2$ treatment.

Filter matings. Triparental matings were performed using a modification of the method of Chikami *et al.* (1985). Briefly, cells of *E. coli* (pRK212.1) and *E. coli* carrying the hybrid plasmid to be transferred were harvested at OD_{550} = 0.6 (approx 10^8 CFU/ml) from LB broth cultures containing ampicillin 50 μg/ml and kanamycin 50 μg/ml, respectively. Cells of *L. pneumophila* AA101 were harvested from confluently inoculated BCYE/Nal plates at 48 h, washed, and suspended in phosphate-buffered saline, pH 7.2 (PBS), at OD_{550} = 0.6. A mixture containing 1.0 ml of each cell suspension was prepared and passed through a 0.45-μm Millipore filter disk. After filtration, the disk was transferred, bacteria side up, to a BCYE agar plate using sterile forceps and incubated at 37°C for no more than 4–5 h to prevent significant *L. pneumophila* replication at this stage. The filter was then washed in 2 ml sterile PBS and samples were plated directly or after dilution on BCYE/Nal and BCYE/Nal/Kan. The identity of all putative transconjugant colonies that emerged on BCYE/Nal/Kan was confirmed by microscopic examination for typical *Legionella* morphology, by failure to grow on LB medium, and by agglutination with a serogroup 1-specific monoclonal antibody. The transfer frequency was estimated by dividing the CFU per milliliter recovered from the mating mixture on BCYE/Nal/Kan by that recovered on BCYE/Nal. The β-lactamase gene of pBR322 was not useful as a selectable marker in *L. pneumophila* in our hands. All manipulations of *L. pneumophila* that carried foreign genes were conducted under BL2 containment.

Southern hybridization. Genomic DNA was isolated from *L. pneumophila* by previously described methods (Engleberg *et al.,* 1984). DNA was cleaved with restriction endonuclease, electrophoresed, and transferred to nitrocellulose membranes using the method of Southern (1975). Plasmids used as probes were radiolabeled with [^{32}P]dATP by nick translation, and hybridizations were conducted under conditions of high stringency. Autoradiography was performed at −70°C using Kodak XRP film and a Dupont Lightning Plus intensifying screen. Densitometry of hybridizing bands was performed on a Zeineh soft laser densitometer (Bio-Med, Fullerton, CA), and baseline-corrected integration below selected peaks was calculated using the Videophoresis II companion software (Bio-Med).

[1] Abbreviations used: BCYE, buffered charcoal yeast extract; BCYE/Nal/Kan, BCYE containing 25 μg/ml nalidixic acid 25 μg/ml kanamycin; BCYE/Nal, BCYE containing 25 μg/ml nalidixic acid; PBS, phosphate-buffered saline; LB broth, Luria–Bertani broth.

RESULTS

Construction of pTLP1. pTLP1 is a 7.9-kb plasmid that carries a ColE1 origin of replication (*oriV*), a selectable antibiotic resistance marker (Km^r), and a fragment of *L. pneumophila* DNA to serve as a site for homologous recombination with the chromosome, given the expectation that the ColE1 replicon would not be propagated autonomously in *Legionella* (Fig. 1). To construct pTLP1, we ligated an 840-bp *Hin*dIII–*Ava*I fragment of pSMJ90.9 to the 3-kb *Hin*dIII–*Ava*I fragment of pBR322. The pSMJ90.9 insert was chosen for this purpose because we thought it unlikely that an interruption of the homologous chromosomal sequences would result in a lethal mutation if plasmid integration occurred. The hybrid plasmid was then reisolated, digested with *Hin*dIII, and ligated with the 4-kb *Hin*dIII fragment of pEYDG1 which contains a transposition-defective fragment of Tn5 with an *oriT* cloned downstream of the Km^r gene.

Transfer of pTLP1 into L. pneumophila. In triparental matings of the nalidixic acid-resistant strain *L. pneumophila* AA101 with *E. coli* (pRK212.1) and *E. coli* (pTLP1), colonies of kanamycin-resistant *L. pneumophila* transconjugants were isolated on BCYE/Nal/Kan after 4–5 days of incubation at 37°C (i.e., 24–48 h later than is usual for *L. pneumophila* AA101 plated on BCYE/Nal). In repeated experiments, transfer of kanamycin resistance occurred reproducibly at a frequency of 10^{-6} to 10^{-5}.

When genomic DNAs from the donor and transconjugant strains were cleaved with *Pvu*II endonuclease and then hybridized with labeled pTLP1, the hybridizing fragments from digests of the plasmid from *E. coli* were conserved in the digests of transconjugant DNA in most isolates (Fig. 2). This observation was repeated using other restriction endonucleases (data not shown) and using DNA from transconjugants obtained after several passages on selective medium (Fig. 2, lane d). In addition, the 3.7-kb chromosomal band that was visualized in the parent *L. pneumophila* AA101 strain digest (i.e., the band having homology to the *L. pneumophila* sequences on pTLP1) was preserved in these transconjugants, suggesting that there had been no plasmid integration at this chromosomal site. The absence of any alteration in the plasmid restriction fragments is strong evidence against plasmid propagation by integration into any other part of the chromosome or by cointegration with pRK212.1 or any previously unrecognized *L. pneumophila* extrachromosomal element. In addition, pTLP1 could be returned from these *L. pneu-*

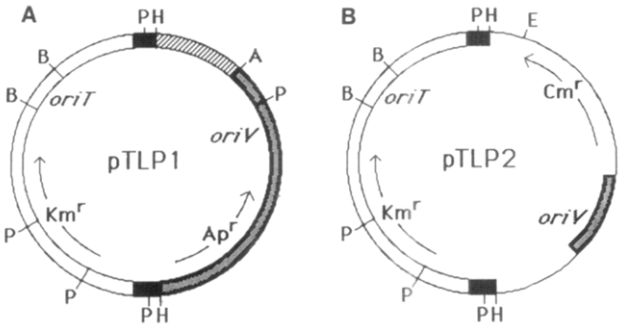

FIG. 1. Physical and restriction maps of pTLP1 (A) and pTLP2 (B). (A) The construction of pTLP1 is described in the text. Fragments contributed by pEYDG1 (□), pSMJ90.9 (▨), and pBR322 (▩) are as indicated. The transpositionally defective remnants of IS50 from pEYDG1 are also indicated (■). Restriction sites for *Hin*dIII (H), *Ava*I (A), *Bam*HI (B), *Eco*RI (E), and *Pvu*II (P) are also indicated. (B) pTLP2 was constructed by ligating the pEYDG1 *Hin*dIII fragment to a Cm^r-bearing derivative of pUC8 (—) provided by T. Serwold-Davis, University of Washington, Seattle. Tn5- and pBR322-derived sequences are indicated as in A.

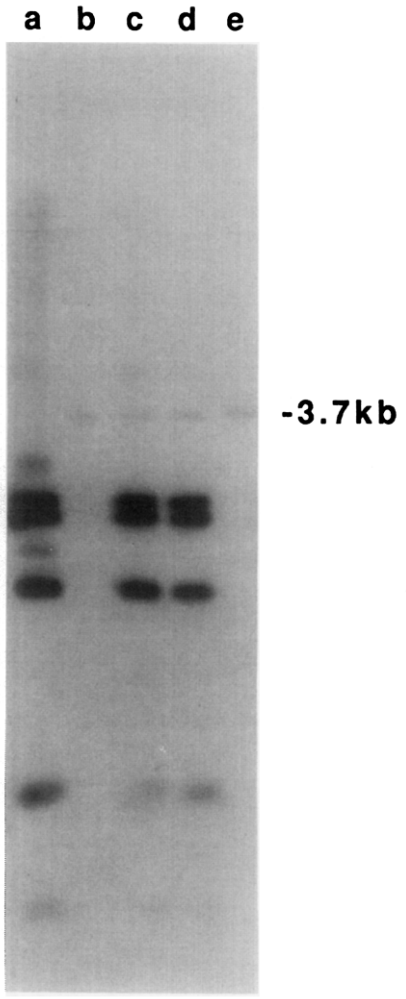

FIG. 2. Southern hybridization analysis of *L. pneumophila* strains probed with radiolabeled pTLP1. All DNA samples have been restricted with *Pvu*II prior to electrophoresis and transfer. Samples include (a) pTLP1 isolated from *E. coli*, (b) *L. pneumophila* AA101, (c) a Km^r transconjugant of AA101, (d, e) the isolate from lane c after several passages on medium with kanamycin (d) and without kanamycin (e). The 3.7-kb band seen in lanes b–e represents the chromosomal fragment carrying the sequences cloned on pSMJ90.9 and subcloned onto pTLP1.

mophila strains to *E. coli* HB101 either by transformation with plasmid DNA isolated by the alkaline lysis method or by conjugation mediated by the helper strain, *E. coli* (pRK212.1), and selection on LB agar with kanamycin. In both experiments, plasmid DNA recovered from recipient *E. coli* was identical to pTLP1 by restriction analysis (data not shown).

Following conjugal transfer of kanamycin resistance into *L. pneumophila*, a few isolates lacked detectable plasmid DNA. In one of these isolates, Southern hybridization suggested that the plasmid had integrated into the chromosome (data not shown). Therefore, although integration of pTLP1 could occur after transfer, it was an uncommon event relative to autonomous plasmid replication.

Physical state of pTLP1 in L. pneumophila. To determine how many copies of pTLP1 were present in each bacterial cell that autonomously replicated the plasmid, we used the autoradiogram shown, in part, in Fig. 2 to compare the relative intensities of a chromosomal band and a plasmid band that contain identical sequences (i.e., the 3.7-kb chromosomal band, known from previous experiments to be single copy, and the *Pvu*II-C band of pTLP1). After integrating below the densitometric peaks in five duplicate lanes of lane c in Fig. 2, the ratio of intensity (*Pvu*II-C band/chromosomal 3.7-kb band) was 13.3 ± 3.2. After correction for vector sequences that are also carried on the *Pvu*II-C band (51%; see Fig. 1), we calculated that there were an average of 6.5 ± 1.6 copies of pTLP1 DNA per cell.

When analyzed by agarose gel electrophoresis in the absence of restriction endonucleases, plasmid bands isolated from *L. pneumophila* and from the retransformed *E. coli* were many times larger than predicted for pTLP1. A Southern hybridization analysis showed that these high-molecular-weight plasmid bands hybridized with the large *Hin*dIII–*Ava*I fragment of pBR322 (selected as a probe to avoid hybridization with the *L. pneumophila* chromosome) (Fig. 3). This finding suggested that pTLP1 recombined to form large, multimeric plasmids after transfer to *L. pneumophila*. In limited endonuclease digestions of pTLP1 isolated from *L. pneumophila*, we found variability in the isomeric state, ranging from predominantly monomers and dimers in bacteria grown confluently on agar for 24–48 h to predominantly multimers of ≥5 in individual colonies at 72 h.

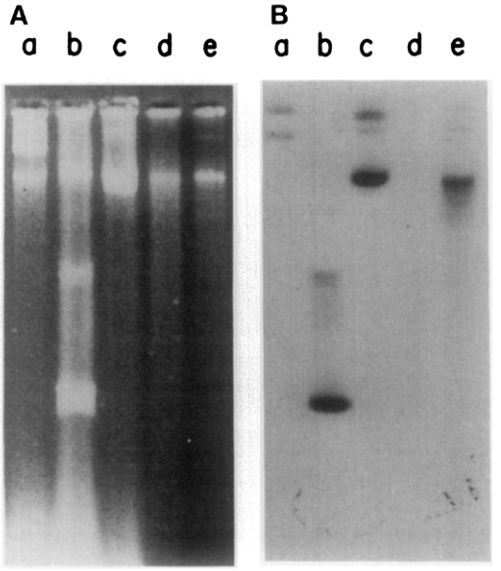

FIG. 3. Agarose electrophoresis (A) and Southern hybridization (B) of plasmid DNA from *L. pneumophila* and *E. coli*. DNA samples in both panels were prepared by the boiling method of Holmes and Quigley (1981) from the following strains: (a) *E. coli* (pRK212.1), (b) *E. coli* (pTLP1), (c) *E. coli* transformed with pTLP1 isolated from *L. pneumophila*, (d) *L. pneumophila* AA101, (e) *L. pneumophila* AA101 (pTLP1). The gel in A was stained with 0.5 μg/ml ethidium bromide and photographed after transillumination with ultraviolet light. After transfer to nitrocellulose membrane, the samples in B were probed with the ^{32}P-labeled *Hin*dIII–*Ava*I small fragment of pBR322, which has homology with both pTLP1 and pRK212.1 but not with *L. pneumophila* DNA (B, lane d). Note that the multimeric forms of pTLP1 seen in B, lanes c and e, were obscured by chromosomal DNA in the corresponding lanes on the stained agarose gel.

Curing of pTLP1. Although pTLP1 could be maintained in *L. pneumophila* indefinitely under selective conditions, passage of the transconjugants on nonselective medium led to the isolation of cured strains at a high frequency (Fig. 2, lane e). We are unable to measure the frequency of loss of pTLP1 precisely because of our inability to adapt the plasmid-bearing *L. pneumophila* to growth in liquid medium with kanamycin. Consequently, to approximate the instability of pTLP1, *L. pneumophila* AA101 (pTLP1) were grown in confluence on BCYE/Nal and were passaged to a fresh plate every 24 h. The confluent growth (which appears as a haze on 24-h plates) was sampled daily and spread for isolation on BCYE/Nal and BCYE/Nal/Kan agar to determine the proportion of plasmid-cured bacteria. The results of this experiment suggest a rapid rate of plasmid cure on nonselective medium (Table 1).

In a complementary study, plasmid-containing *L. pneumophila* were passed from BCYE/Nal/Kan to BCYE/Nal, and after 72 h of incubation, colonies were picked, suspended in PBS, and characterized for kanamycin resistance. In 8 of 10 colonies examined, no Km^r organisms were isolated even in the undiluted suspensions, suggesting a plasmid curing of >99.999%. In the remaining colonies, Km^r organisms represented 2 and 12% of the total recoverable bacteria, indicating that 98 and 88% of the progeny, respectively, were cured after 72 h. These findings, obtained by examination of individual colonies growing on nonselective, semisolid media, are consistent with the results shown in Table 1.

Interestingly, similar analyses performed with an isolate that had been passed repeatedly on selective medium suggested that kanamycin resistance could become stabilized. In two such experiments, significant loss of kanamycin resistance was found in only two of eight colonies and five of eight colonies, respectively. Several of the "stable" isolates obtained in these experiments had no detectable plasmid DNA (data not shown), suggesting that plasmid integration, rather than stabilized plasmid inheritance, may have accounted in

TABLE 1

PROPORTION OF *L. pneumophila* THAT RETAIN pTLP1[a] AFTER PASSAGE ON SELECTIVE AND NONSELECTIVE MEDIA FOR VARIOUS INTERVALS

	Passage time (h)				
Medium	24	48	72	96	110
BCYE/Nal/Kan	30%	67%	31%	ND[b]	ND
BCYE/Nal	14%	4%	1%	0.4%	0.1%

[a] % kanamycin-resistant.
[b] ND, not done.

part for the maintenance of the kanamycin resistance gene when this multiply passed isolate was grown on nonselective medium.

Requirements for pTLP1 replication in L. pneumophila. Since the apparent autonomous replication of a "narrow-host-range" ColE1-type plasmid in *L. pneumophila* was not anticipated, we wanted to determine whether *oriV* was sufficient for plasmid maintenance or whether other exogenous sequences that we introduced were required for the replication of pTLP1 in *L. pneumophila.*

First, because of the possibility that pRK212.1 may have been coincidently transferred to *L. pneumophila* (Fig. 3B, lane e), we wanted to confirm that replication of pTLP1 is not dependent on cointegration with the helper plasmid or on replication functions provided *in trans.* We therefore performed Southern hybridization analysis of DNA from transconjugant strains using pRK212.1 as a probe. These studies showed that although pRK212.1 was present in some transconjugants upon initial isolation, this large plasmid was also unstable and was readily lost after passage under nonselective conditions. However, the loss of pRK212.1 had no effect on the maintenance of pTLP1 as long as the kanamycin selection was maintained (Fig. 4).

Next, we wanted to confirm that replication of pTLP1 in *L. pneumophila* is mediated by *oviV.* To test this hypothesis, we attempted to transfer a series of plasmids with deletions or substitutions of specific sequences on pTLP1. Specifically, we used pEYDG1, which does not possess *L. pneumophila* DNA or sequence homology with *L. pneumophila,* derivatives of pEYDG1 and pTLP1 from which *oriT* is deleted, and pTLP2, which has only 766 bp from pBR322 instead of the 2975-bp fragment cloned on pTLP1 (Fig. 1B). These experiments strongly suggest that plasmid replication in *L. pneumophila* was mediated by a fragment of pBR322 (nt2353-3119) that contained *oriV* (Table 2).

DISCUSSION

We have found that small plasmids having ColE1-type origins can be replicated autonomously in a strain of *L. pneumophila* following conjugal transfer. This was an unexpected finding, since it has been widely accepted that these plasmids are incapable of replication in non-Enterobacteriaceae, such as *Pseudomonas* (Bagdasarian *et al.,* 1979), *Rhizobium* (Ditta *et al.,* 1980), and *Acinetobacter* (Eichenlaub and Steinbach, 1979). In fact, ColE1 replicons have been successfully used as "suicide vectors" to deliver transposons to bacteria of these genera (Sato *et al.,* 1981; Yakobson and Guiney, 1984). Even some enteric bacterial species, such as *Salmonella typhimurium,* may

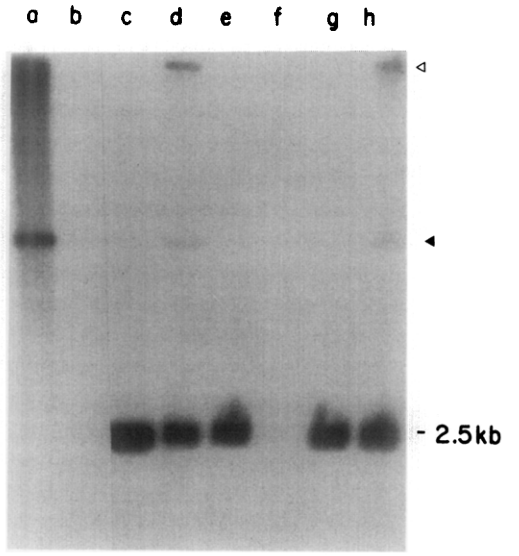

FIG. 4. Southern blot analysis of DNA from *L. pneumophila* transconjugant strains carrying pTLP1 with a probe for pRK212.1. DNA samples were digested with *Pvu*II endonuclease and include (a) pRK212.1 plasmid DNA, (b) *L. pneumophila* AA101, (c) pTLP1 plasmid DNA isolated from *E. coli,* (d) *L. pneumophila* (pTLP1), first passage, (e, f) isolate from lane d after passage on medium with kanamycin (e) or without kanamycin (f), (g, h) two other independent transconjugant isolates after first passage. The open arrow indicates the migration position of linearized pRK212.1; the additional band indicated by the solid arrow is probably due to secondary *Pvu*II endonuclease activity because of the high enzyme concentration used. The 2.47-kb band seen in some lanes is the *Pvu*II-B fragment of pTLP1 that is homologous with the pRK212.1 probe by virtue of the *oriT* sequence. Note that pRK212.1 is present in some primary isolates after conjugation (lanes d and h, but not g), but this large plasmid is lost whether or not kanamycin selection is maintained (lanes e and f).

TABLE 2

TRANSFER TO AND MAINTENANCE OF A SERIES OF SMALL, ColE1-TYPE PLASMIDS IN *L. pneumophila*

Plasmid	Transferred to *L. pneumophila* strain AA101	Plasmid reisolated from *L. pneumophila*
pTLP1	+	+
pEYDG1	+	+
pTLP1Δ*oriT*[a]	−	NA[b]
pEYDG1Δ*oriT*[a]	±[c]	+[c]
pTLP2	+	+

[a] Constructed by deletion of a 760-bp *Bam*HI fragment carrying *oriT*.

[b] NA, not applicable.

[c] This plasmid could not be transferred to strain AA101, but was transferred to and maintained in a variant of AA101 (possibly a restriction-negative mutant) that is a high-frequency conjugal recipient (N. Cianciotto *et al.*, FEMS Letters, in press.). Since only a few transconjugant colonies were isolated in this mating, we assume that the failure to transfer pTLP1Δ*oriT* in a parallel mating has statistical rather than biological relevance.

fail to replicate ColE1 plasmids (Eichenlaub and Steinbach, 1979).

The basis for the "narrow host range" of these plasmids is their requirement for several host replicative functions. Although minimal replication can be initiated *in vitro* from oriV in the presence of only three purified *E. coli* enzymes—RNA polymerase, DNA polymerase I, and RNase H (Itoh and Tomizawa, 1978)—efficient *in vitro* plasmid replication also requires the functions of DNA gyrase, the *dnaB* and *dnaG* gene products, and either DnaA protein or protein i to catalyze primosome assembly (Seufert and Messer, 1987).

Based on a unique set of phenotypic characteristics and a lack of DNA relatedness with other gram-negative bacteria, *L. pneumophila* and other *Legionella* spp. have been classified in the family Legionellaceae (Brenner *et al.*, 1979). Because of the apparent unrelatedness to *E. coli*, we proceeded in our studies under the assumption that, like other unrelated gram-negative organisms, *L. pneumophila* would not provide a suitable set of host factors to initiate replication of *oriV*. We now know that this assumption was incorrect. In retrospect, the true situation might have been suggested by our earlier findings showing that several *L. pneumophila* genes are expressed when cloned in *E. coli* (Engleberg *et al.*, 1984), whereas genes from other distantly related bacteria are often not expressed. This observation implies that at least one of the ColE1 replicative factors, RNA polymerase, has similar substrate specificity in the two species.

In the present study, we also observed that ColE1 replicons were maintained in *L. pneumophila* with antibiotic selection, but that the plasmids were rapidly cured in the absence of antibiotic. In general, plasmid curing is a function of the difference in maximal growth rates of plasmid-bearing and plasmid-free bacteria and the frequency with which plasmid-free segregants appear in culture. Using our current methods, we cannot estimate the growth rates of the two *L. pneumophila* populations. We can, however, speculate about the generation of plasmid-free segregants.

Since pBR322 derivatives lack a *par* locus (Balbas *et al.*, 1986), the distribution of individual plasmids is random between daughter cells, and plasmid maintenance in growing cultures depends on a high copy number (usually 15–30 in *E. coli*). Using densitometry, we estimated that pTLP1 DNA is present in an average of 6.5 copies per *L. pneumophila* cell. However, since one- to two-thirds of *L. pneumophila* growing on BCYE/Nal/Kan (the source of bacteria for our Southern hybridizations) may lack the plasmid (see Table 1), the actual number of duplicates of pTLP1 in those bacteria that carry plasmid DNA may be 1.5- to 3-fold higher (i.e., 10–20 per cell). This corrected estimate of total plasmid DNA copies is similar to copy number expected for these plasmids when propagated as monomers in Rec$^-$ *E. coli*, suggesting that inefficient replication of plasmid DNA is not per se a contributing factor in the high spontaneous cure rate in *L. pneumophila*. A more likely explanation for the cure rate is the multimerization of pTLP1 in *L. pneumophila*, which increases the probability of generating plasmid-free segregants.

In bacteria from 72-h colonies, we found that pTLP1 was isolated in a high-molecular-weight, multimeric form. Similarly, recombination leading to multimerization of ColE1 was observed after transfer to *Proteus mirabilis* 20 years ago (Bazaral and Helinski, 1968; Goebel and Helinski, 1968); it was later shown that this phenomenon occurs only in *recA*$^+$ *E. coli* (Bedbrook and Ausubel, 1976). Since the total amount of plasmid DNA in Rec$^+$ and Rec$^-$ host cells is the same, Rec$^+$ cells with multimerized plasmids will have fewer plasmid replicons per cell. This decrease in the plasmid copy number in Rec$^+$ hosts has been shown to increase plasmid instability by generating plasmid-free segregants at high frequency (Summers and Sherratt, 1984). In *L. pneumophila,* we observed multimers of 5 or more. In these circumstances, the multiplicity of plasmid DNA inferred by comparing a chromosomal DNA sequence with the same sequence cloned on a plasmid may actually represent as few as two or three plasmid replicons per cell. With such a small number of plasmid replicons to distribute randomly to daughter cells, plasmid-free segregants could be generated at a rate as high as 0.25 to 0.5 per generation. At such a rapid rate, cured bacteria would quickly replace plasmid-bearing bacteria in a growing culture, even if there were no differences in the maximal growth rates of the two populations. This rapid emergence of plasmid-free bacteria may also explain, in part, why so many cured bacteria can be isolated from colonies on selective medium (Table 1).

These speculations about plasmid stability in *L. pneumophila* are testable hypotheses. Since multimerization is a RecA-dependent process, we would predict that our *L. pneumophila* isolate has a Rec$^+$ phenotype and that pTLP1 would be stable in a Rec$^-$ *L. pneumophila* mutant. Or, pending the isolation of such a mutant, the addition to pTLP1 of *cer,* which stabilizes ColE1 in Rec$^-$ bacteria by converting multimers to monomers through a RecA-independent mechanism (Summers and Sherratt, 1984), or *par,* which mediates the stable inheritance of the stringent plasmid pSC101 (Skogman and Nilsson, 1984), might increase stability in *L. pneumophila.*

For our purposes, *oriT*-bearing pBR322 derivative plasmids have proved useful. We have recently exploited the feature of plasmid instability to perform site-specific mutagenesis in *L. pneumophila* (using similar plasmids bearing counterselectable markers), and we have used plasmid maintenance under continuous antibiotic selective pressure as a method to *trans*-complement a specific mutant with a cloned gene (N. Cianciotto *et al.,* FEMS Letters, in press). These recent experiments suggest that pBR322-derived plasmids will be useful in a variety of genetic manipulations to study *Legionella* pathogenesis.

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REFERENCES

BAGDASARIAN, M., BAGDASARIAN, M. M., COLEMAN, S., AND TIMMIS, K. N. (1979). New vector plasmids for gene cloning in *Pseudomonas.* In "Plasmids of Medical, Environmental and Commercial Importance" (K. N. Timmis and A. Puhler, Eds.) pp. 411–422. Elsevier/North-Holland, Amsterdam.

BALBAS, P., SOBERON, X., MERINO, E., ZURITA, M., LOMELI, H., VALLE, F., FLORES, N., AND BOLIVAR, F. (1986). Plasmid vector pBR322 and its special-purpose derivatives—A review. *Gene* **50,** 3–40.

BAZARAL, M., AND HELINSKI, D. R. (1968). Characterization of multiple circular DNA forms of colicinogenic factor E1 from *Proteus mirabilis. Biochemistry* **7,** 3513–3519.

BEDBROOK, J. R., AND AUSUBEL, F. M. (1976). Recombination between bacterial plasmids leading to the formation of plasmid multimers. *Cell* **9,** 707–716.

BIRNBOIM, H. C., AND DOLY, J. (1979). A rapid alkaline lysis extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7,** 1513–1523.

BRENNER, D. J., STEIGERWALT, A. G., AND MCDADE, J. E. (1979). Classification of the Legionnaires' disease bacterium: *Legionella pneumophila,* genus novum, species nova of the family Legionellaceae, familia nova. *Ann. Intern. Med.* **90,** 656–658.

CHEN, G. C. C., LEMA, M., AND BROWN, A. (1984). Plasmid transfer into members of the family Legionellaceae. *J. Infect. Dis.* **150,** 513–516.

CHIKAMI, G. K., FIERER, J., AND GUINEY, D. G. (1985). Plasmid-mediated virulence in *Salmonella dublin* demonstrated by use of a Tn5-*oriT* construct. *Infect. Immun.* **50,** 420–424.

DITTA, G., STANFIELD, S., CORBIN, D., AND HELINSKI, D. R. (1980). Broad host range DNA cloning system for gram-negative bacteria: Construction of a clone bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. USA* **77,** 7347–7351.

DREYFUSS, L. A., AND IGLEWSKI, B. H. (1985). Conjugation-mediated genetic exchange in *Legionella pneumophila. J. Bacteriol.* **161,** 80–84.

EICHENLAUB, R., AND STEINBACH, C. (1979). Screening of *Serratia marcescens* strains for extrachromosomal DNA: Detection of a small plasmid useful as a cloning vector in gram-negative bacteria. *In* "Plasmids of Medical, Environmental and Commercial Importance" (K. N. Timmis and A. Puhler, Eds.), pp. 411–422. Elsevier/North-Holland, Amsterdam.

ENGLEBERG, N. C., DRUTZ, D. J., AND EISENSTEIN, B. I. (1984). Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli. Infect. Immun.* **44,** 222–227.

FIGURSKI, D. H., AND HELINSKI, D. R. (1979). Replication of an origin-containing derivative of plasmid pRK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76,** 1648–1652.

GOEBEL, W., AND HELINSKI, D. R. (1968). Generation of higher multiple circular DNA forms in bacteria. *Proc. Natl. Acad. Sci. USA* **61,** 1406–1413.

GUINEY, D. G., AND YAKOBSON, E. (1983). Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. *Proc. Natl. Acad. Sci. USA* **80,** 3595–3598.

HOLMES, D. S., AND QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114,** 193–197.

ITOH, T., AND TOMIZAWA, J. (1978). Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H, and DNA polymerase I. *Cold Spring Harbor Symp. Quant. Biol.* **43,** 409–418.

KEEN, M. G., STREET, E. D., AND HOFFMAN, P. S. (1985). Broad-host-range plasmid pRK340 delivers Tn5 into the *Legionella pneumophila* chromosome. *J. Bacteriol.* **162,** 1332–1335.

MINTZ, C. S., AND SHUMAN, H. A. (1987). Transposition of bacteriophage Mu in the Legionnaires' disease bacterium. *Proc. Natl. Acad. Sci. USA* **84,** 4645–4649.

SATO, M., STASKAWISZ, B. J., PANOPULOS, N. J., PETERS, S., AND HONMA, M. (1981). A host-dependent hybrid plasmid suitable as a suicidal carrier for transposable elements. *Plasmid* **6,** 325–331.

SEUFERT, W., AND MESSER, W. (1987). DnaA protein binding to the plasmid origin region can substitute for primosome assembly during replication of pBR322 in vitro. *Cell* **48,** 73–78.

SKOGMAN, S. G., AND NILSSON, J. (1984). Temperature-dependent retention of a tryptophan-operon-bearing plasmid in *Escherichia coli. Gene* **31,** 117–122.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–517.

SUMMERS, D. K., AND SHERRATT, D. J. (1984). Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability. *Cell* **36,** 1097–1103.

YAKOBSON, E. A., AND GUINEY, D. G. (1984). Conjugal transfer of bacterial chromosomes mediated by the RK2 plasmid transfer origin cloned into transposon Tn5. *J. Bacteriol.* **160,** 451–453.

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