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Site-specific mutagenesis in *Legionella pneumophila* by allelic exchange using counterselectable ColE1 vectors

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1. SUMMARY

To study the molecular pathogenesis of infection by *Legionella pneumophila,* a technique of site-specific mutagenesis by allelic exchange was evaluated. To develop this system, we optimized conjugal DNA transfer by isolating a mutant that functions 1000-fold more efficiently as a recipient than the wild type strain, identified two counterselectable markers, *rpsL* and *sacB,* that function in *L. pneumophila,* and constructed a counterselectable ColE1 vector. Allelic exchange of a *L. pneumophila* chromosomal gene was achieved at a frequency of 10^{-5} per transconjugant. The allelic exchange procedure itself did not alter the ability of *L. pneumophila* to infect macrophages, indicating that the system can be used to study this aspect of virulence.

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2. INTRODUCTION

To identify factors with definitive roles in intracellular infection, we sought to develop a system for site-specific mutagenesis in *Legionella pneumophila,* but we recognized two potential obstacles to this goal. First, there are no methods for efficient gene transfer and recombination in *L. pneumophila.* Conjugal DNA transfer to *L. pneumophila* has been limited to the transfer of broad-host range plasmids [1,2], and although conjugative plasmids have been used to deliver transposons [3,4], their large size makes them unsuitable as vectors for site-specific mutagenesis. Second, since strains of *L. pneumophila* loss infectivity with passage on artificial media [5], it is possible that the genetic manipulations used to achieve site-specific mutagenesis might reduce severely the ability of *L. pneumophila* to infect macrophages. We report a method of allelic exchange in *L. pneumophila* in which mutated gene sequences are introduced from a small, mobilizable and counterselectable ColE1 vector to the bacterial chromosome without a significant loss of bacterial infectivity.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids, and growth conditions

All *L. pneumophila* strains are derivatives of a clinical isolate, strain 130b (Los Angeles), serogroup 1 [6]. Strain AA101 is a spontaneous nalidixic acid resistant derivative of 130b [7]. *E. coli* strain HB101 served both as host for recombinant plasmids and as the donor strain in conjugation experiments.

For our plasmid constructions, we used pEYDG1 as the source of the kanamycin resistance gene (Km^r) and the origin of transfer from the IncP group of conjugative plasmids (*oriT*) [8]. The plasmids pRTP1 [9] and pUCD800 [10] were the sources of the counterselective markers *rpsL* and *sacB*, respectively. Plasmid pTLP1 is an *oriT*-containing derivative of pBR322 that was transferred to *L. pneumophila* and maintained under antibiotic selection (i.e., Km^r) [7]. Plasmid pSMJ90.9 contains an *L. pneumophila* locus, designated *efa*, which confers upon *E. coli* reactivity with an anti-flagellin monoclonal antibody, Mab 1D5 (see 3.3).

E. coli clones were grown on Luria-Bertani agar containing 50 μg/ml kanamycin and/or 50 μg/ml ampicillin. *L. pneumophila* strains were grown on buffer charcoal yeast extract (BCYE) agar for 48 h at 37°C [6]. Antimicrobial agents were added to the following concentrations: 25 μg/ml nalidixic acid (Nal), 25 μg/ml kanamycin (Kan), and 300 μg/ml streptomycin (Str). The methods for intracellular infection of transformed U937 cells, a macrophage-like cell line, by *L. pneumophila* were presented previously [12]. To compare the infectivity of strains of *L. pneumophila*, we infected monolayers of transformed U937 cells with serial dilutions of each strain, and then determined the inoculum size (ID_{50}) which yielded bacterial growth in 50% of the inoculated monolayers ($N = 8$) [12,14].

3.2. Bacterial conjugations and molecular genetic techniques

ColE1 replicons containing *oriT* sites were introduced into *L. pneumophila* by triparental conjugal matings using a helper strain containing conjugative plasmid pRK212.1 as previously described [7,8]. Whole cell DNA and plasmid DNAs were isolated from *E. coli* and *L. pneumophila* as previously outlined [6,11]. DNA hybridization analysis and molecular cloning procedures were previously described [6,7].

3.3. Immunoassays

To test for the expression of *L. pneumophila* flagellin, bacterial colonies were transferred from agar to nitrocellulose membranes, air-dried, and washed in 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.05% Tween-20 for 1 h. Membranes were then incubated overnight in undiluted hybridoma supernatants containing anti-flagellin Mabs 1D5 or 2A5 (E. Pearlman et al., unpublished observations). Bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse antibody and HRP Color Development Reagent (Bio-Rad, Richmond, CA) according to manufacturer's specifications.

4. RESULTS AND DISCUSSION

4.1. Conjugative matings with plasmid-cured L. pneumophila

Although an *oriT*-containing ColE1 replicon, pTLP1, was transferred by conjugation to *L. pneumophila* strain AA101, the transfer frequency was only 10^{-6} [7]. However, when matings were performed using an AA101 transconjugant that had been cured of pTLP1 as the recipient (strain AA102), transfer of pTLP1 to AA102 occurred at a frequency of 10^{-3}–10^{-2}, (viz., more than 10^3-fold increase compared to AA101). Although the reason for the increased plasmid transfer is not known, we infer that a mutation had occurred in the *L. pneumophila* host which facilitated plasmid transfer or replication. Due to this property, strain AA102 was chosen as the recipient strain in all our subsequent experiments.

4.2. Effect of the rpsL *and* sacB *genes on* L. pneumophila

To identify a counterselectable marker for allelic exchange mutagenesis, we evaluated the expression of the *rpsL* allele of *E. coli* and the *sacB*

gene of *Bacillus subtilis* in *L. pneumophila.* The *rpsL* allele encodes the ribosomal protein S12 and, when provided in *trans,* renders a streptomycin-resistant (Str^r) strain streptomycin-sensitive (Sm^s) [13]. When introduced on plasmid pTLP3 (Fig. 1) into a Str^r derivative of AA102 (strain AA103), the *rpsL* allele rendered *L. pneumophila* Sm^s, so that the efficiency of plating on streptomycin-containing medium was reduced 10^3-fold. The *sacB* gene encodes an enzyme which is localized to the periplasmic space, when cloned in certain Gram-negative bacteria; there it converts sucrose, when present in high concentrations (i.e., 5%), into lethal amounts of insoluble polysaccharides [10]. When the *sacB* gene from pUCD800 was introduced into strain AA102, there was a 10^5-fold reduction in the plating efficiency of that strain on sucrose-containing medium. These data indicate that both the *rpsL* and *sacB* genes are suitable counterselectable markers in *L. pneumophila.*

4.3. Demonstration of allelic exchange in L. pneumophila

As a target for allelic exchange, we chose *efa* because we thought it likely that an interruption of this gene would be nonlethal, would not alter the infectivity of *L. pneumophila,* and might yield an easily-scored phenotype, i.e., altered flagella expression. For the mutagenesis of this gene, we constructed pNC90.2 (Fig. 1), which carries the *rpsL* (Sm^s) allele and *efa* inactivated by the insertion of a Km^r marker (viz., *E. coli* carrying pSMJ90.9 or pNC90.1 reacted with Mab 1D5, whereas a strain carrying pNC90.2 did not). We transferred pNC90.2 by conjugation into AA103, and then plated the transconjugant on BCYE/Nal/Kan/Str agar. We screened 100 Str^r, Km^r colonies by dot-blot hybridization using the ampicillin resistance gene of pNC90.2 as a probe; five were negative, indicating that they had been cured of pNC90.2.

To determine if these five had undergone allelic exchange, genomic DNA was examined by Southern hybridization analysis (Fig. 2). The model of recombination predicts that the 4 kb fragment (solid arrow, lane b) containing the *Hin*dIII site within the *efa* gene should be lost during allelic exchange, and a 4.3 kb fragment (open arrow) should be present. In addition, three of the nine *Ava*I fragments of pNC90.2 (ca. 5.4, 3.1, and 1.3 kb in size) would also be absent in a strain that had undergone exchange (lane c). The predicted rearrangement occurred in all five Str^r, Km^r isolates that lost pNC90.2 (see representative isolate, designated strain AA104, in lane e). The overall frequency of allelic exchange among Km^r transconjugants was 10^{-5}, since 1 in 10^3 Km^r isolates were Str^r, and ca. 1 in 10^2 of these had undergone the predicted DNA rearrangement.

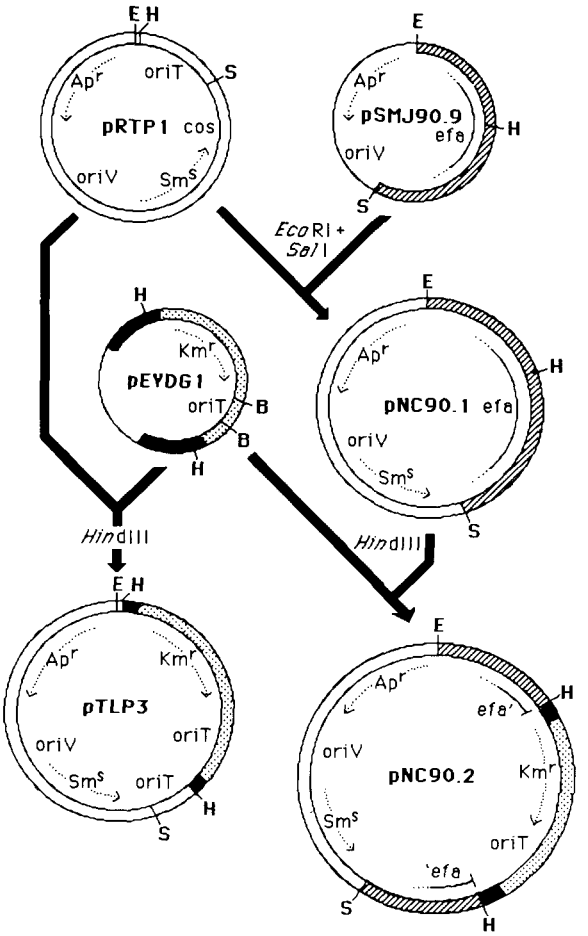

Fig. 1. Construction of plasmids pTLP3 and pNC90.2. Fragments contributed by pRTP1 (□), pEYDG1 (▤), and pSMJ90.9 (▨) are as indicated. The transpositionally defective remnants of the inverted repeats of Tn*5* (*IS50*) from pEYDG1 are also indicated (■). Restriction enzyme sites for *Eco*RI (E), *Hin*dIII (H) and *Sal*I (S) are also noted.

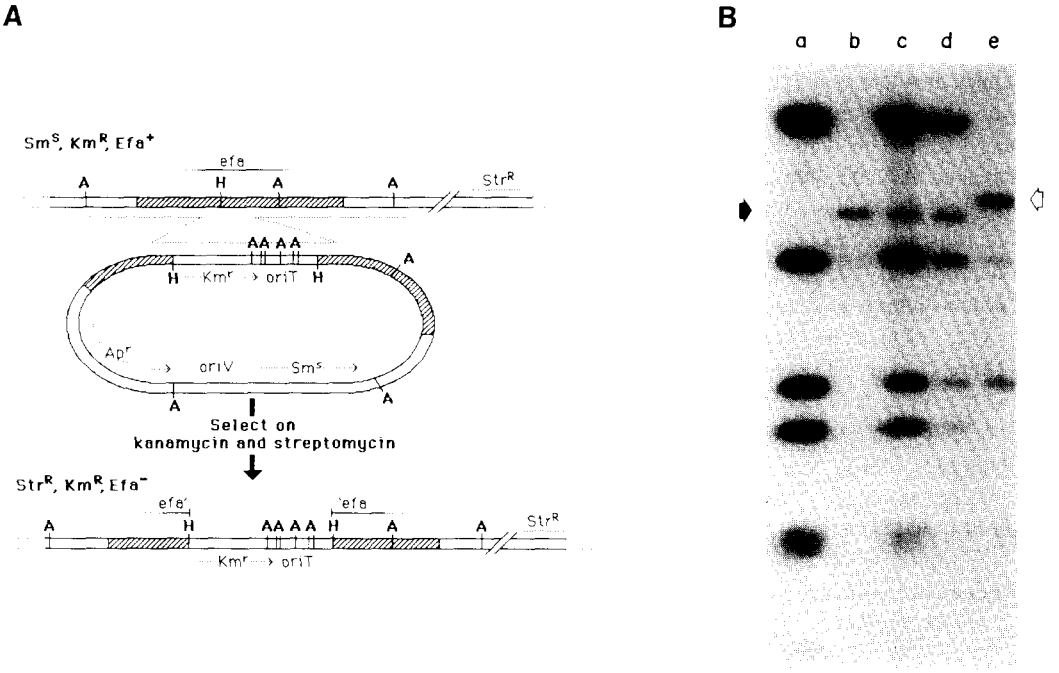

Fig. 2. A. Diagram of the allelic exchange mutagenesis procedure in *L. pneumophila.* The upper portion of the diagram depicts schematically a pNC90.2-containing *L. pneumophila* transconjugant. A restriction map of the chromosomal region carrying the *efa* target gene is shown as a double line. A circular map of pNC90.2 is also shown, with dotted lines indicating a possible double crossover event between the plasmid and the chromosome. The predicted result of this recombination, with the mutated *efa* gene replacing the wild type gene, is shown at the bottom of the diagram. The location of *Ava*I (A) sites are indicated and all other notations are the same as in Fig. 1. B. Southern hybridization analysis of *L. pneumophila* strains probed with pNC90.2. All DNA samples were digested with *Ava*I. Lane a, pNC90.2; lane b, AA103; lane c, AA103(pNC90.2); lane d, AA103 containing pNC90.2 integrated into the chromosome; and lane e, AA104 which has undergone allelic exchange at the *efa* locus. Note that the reduction in copy number associated with chromosomal integration makes the hybridization with the smaller *Ava*I fragments internal to Tn*5* too weak to be visualized at this level of exposure (lanes d and e).

4.4. Phenotypic analysis of the efa *mutant, strain AA104*

Strain AA104 reacted with the Mabs 2A5 and 1D5 and was motile by wet mount microscopy (data not shown), suggesting that *efa* is required neither for flagella antigen expression nor motility. Since our intent was to test if the genetic manipulations themselves would alter the infectivity of *L. pneumophila,* the demonstration of an additional phenotype for strain AA104 was not pursued. Rather, we compared strains 130b, AA103 and AA104 for their capacity to infect macrophage-like cells in tissue culture. Strain AA103 was somewhat less infective than 130b (ID_{50}, 10^3 vs. 10^2), perhaps because of changes related to multiple passage on artificial media or those associated with enhanced conjugal transfer or antibiotic resistance. Nevertheless, this background attenuation of strain AA103 will not confound the future evaluation of directed mutations in virulence-associated genes. Strains AA104 and AA103 had similar ID_{50}s, indicating that the allelic exchange procedure and the presence of foreign DNA in the chromosome did not significantly alter infectivity. We conclude that this method of site-specific mutagenesis can be used to study individual determinants of intracellular infection in *L. pneumophila* strain AA103.

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