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Transposon Mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn10 Derivative

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A transposon mutagenesis procedure functional in the gram-negative swine pathogen *Actinobacillus pleuropneumoniae* was developed for the first time. The technique involved the use of a suicide conjugative plasmid, pLOF/Km, carrying a mini-Tn10 with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible transposase located outside the mobile element (M. Herrero, V. de Lorenzo, and K. N. Timmis, J. Bacteriol. 172:6557–6567, 1990). The plasmid was mobilized from *Escherichia coli* to *A. pleuropneumoniae* through the RP4-mediated broad-host-range conjugal transfer functions provided by the chromosome of the donor strain. When IPTG was present in the mating medium, *A. pleuropneumoniae* CM5 transposon mutants were obtained at a frequency of 10⁻⁵, while no mutants were detected in the absence of IPTG. Since the frequency of conjugal transfer of the RP4 plasmid from *E. coli* to *A. pleuropneumoniae* CM5 was found to be as low as 10⁻⁴, the above result indicated that the expression level of the transposase was a critical factor for obtaining a workable efficiency of transposon mutagenesis. The transposon insertions occurred at random, as determined by Southern blotting of chromosomal DNA of randomly selected mutants and by the ability to generate mutants defective for the selected phenotypes. Almost all the mutants analyzed resulted from a single insertion of the Tn10 element. About 1.2% of the mutants resulted from the cointegration of pLOF/Km into the *A. pleuropneumoniae* chromosome. The applicability of this transposon mutagenesis system was verified on other *A. pleuropneumoniae* strains of different serotypes. The usefulness of this transposon mutagenesis system in genetic studies of *A. pleuropneumoniae* is discussed.

The gram-negative, facultatively anaerobic, encapsulated bacterium *Actinobacillus pleuropneumoniae* (formerly classified in the genus *Haemophilus*) is an important veterinary pathogen of the *Pasteurellaceae* family (23, 32). It is the etiologic agent of porcine pleuropneumonia, a highly contagious respiratory infection causing heavy economic losses to the swine industry (30, 38). Clinically, *A. pleuropneumoniae* infection occurs as acute outbreaks of high mortality in 24 to 48 h or as chronic persistent infections. The latter are of special concern because of their high prevalence and because they follow an insidious course, manifested by impairment of the growth rates of pigs in the fattening units (30, 38).

Currently available vaccines to *A. pleuropneumoniae* are empirical and are not satisfactory enough in controlling the disease in porcine populations. The major drawbacks of these vaccines include a lack of cross-protection between serotypes (12 have been described to date [20, 31]) and failure to prevent the spread of the chronic infection from carriers (18, 36). As a first step in the development of new efficient vaccines, considerable efforts and resources have been invested in research on the pathogenic and immunogenic mechanisms of *A. pleuropneumoniae*. Several putative virulence factors have been investigated (19), such as the serotype-specific capsular material (8, 19, 36), the lipopolysaccharide (2, 8), the surface proteins (33, 45), and the cytolysic protein toxins responsible for the hemolytic phenotype of *A. pleuropneumoniae* (9, 11, 12). The study of these putative virulence factors has been approached by means of purification and characterization techniques (2, 9, 11, 12, 45) and, in recent years, by gene cloning and sequencing (5, 10, 13, 16, 28, 41). Although some experimental evidence indicating that these factors are involved in virulence has been obtained (19, 21, 42), their exact role in the pathogenicity of *A. pleuropneumoniae* has not been assessed so far.

The analysis of mutants affected in the expression of specific genes is perhaps the most powerful approach to investigate the role and the mechanism of action of molecules possibly involved in pathogenicity and virulence. To date, only spontaneously occurring or chemically induced *A. pleuropneumoniae* mutants, defective in capsule or hemolysin production, have been studied (1, 21, 22, 35). However, since these mutants are not well characterized genetically (in the sense that the kind and number of genetic determinants affected remain unknown), virulence studies based on them did not provide meaningful information. When genetic systems are not sufficiently developed for a certain bacterial species, as is the case for *A. pleuropneumoniae*, transposon mutagenesis could be a useful tool to generate well-characterized insertion mutants. Although this approach has been widely used in studies on the virulence of a number of gram-negative and gram-positive pathogenic bacteria (14, 47), to our knowledge, transposon mutagenesis has not been achieved in *A. pleuropneumoniae*. In this article, we describe a transposon mutagenesis system that allows the generation, with suitable frequencies, of single, stable, and

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random insertional disruptions in the genome of this bacterium.

**Bacterial strains and plasmids.** The bacteria and plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* CMS (serotype 1) (34), a strain that has been used in several investigations on the virulence and immunogenic properties of *A. pleuropneumoniae* (35, 36), was used to develop the transposon mutagenesis system. This strain has no detectable plasmids, as determined by us (not shown) and others (35). For mutagenesis experiments, spontaneously occurring nalidixic acid-resistant mutants of the *A. pleuropneumoniae* strains were obtained.

**Culture media and growth conditions.** Tryptic soy (TS) broth and TS agar were used for routine cultures. For *A. pleuropneumoniae*, these media were supplemented with 6 mg of yeast extract and 10 µg of NAD (Sigma Chemical Co.) per ml. Antibiotics were added to TS media as required at the following concentrations: ampicillin, 150 µg/ml; kanamycin, 50 µg/ml; tetracycline, 50 µg/ml; and nalidixic acid, 60 µg/ml. For tryptophan auxotrophy experiments, the chemically defined medium of Maudsley and Kadis (27) solidified with 1.5% agar was used. This medium was modified to contain the same concentration of NAD as described above and was supplemented when appropriate with tryptophan (50 µg/ml).

**DNA techniques.** Plasmid DNA was prepared by using a kit from Stratagene (La Jolla, Calif.). Chromosomal DNA was obtained from *A. pleuropneumoniae* by a simplified lysis procedure with proteinase K and sodium dodecyl sulfate (SDS) (48). For Southern blots (43), chromosomal DNA was digested with *XhoI* as indicated by the manufacturer (New England Biolabs, Inc., Beverly, Mass.), electrophoretically separated on 0.8% agarose gels, and transferred to a nitrocellulose filter (Schleicher & Schuell Inc., Dassel, Germany) as previously described (37). The probe was pLOF3/Km radiolabelled by random priming (kit from Amersham Corp., Buckinghamshire, England) with [α-32P]dCTP (Amersham).

Hybridization was done under stringent conditions, as follows: prehybridization at 65°C for 4 h with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% SDS-0.05% skim milk; hybridization overnight at 65°C in the same solution but with only 0.1% SDS; two 30-min washes at room temperature with 2× SSC-0.1% SDS; and two additional 45-min washes at 68°C in 0.2× SSC-0.1% SDS. Filters were then exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.) for 24 h at -70°C with an amplifying screen.

**Conjugation and transposition in *A. pleuropneumoniae*.** Transposon mutagenesis in gram-negative bacteria other than *Escherichia coli* is usually achieved with plasmid vectors mobilizable by the conjugal properties of the broad-host-range plasmid RP4 (39). Since there was scarce information about the conjugal properties of *A. pleuropneumoniae*, a first step in our investigation was the determination of the ability of the RP4 plasmid to transfer from *E. coli* LE392 to *A. pleuropneumoniae* CMS. We observed that *A. pleuropneumoniae* CMS and *E. coli* conjugate, with a frequency of RP4 mobilization in the range of 10⁻⁴ (Table 2). RP4-mediated tetracycline resistance was not expressed in *A. pleuropneumoniae* CMS, so conjugal transfer events were selected with ampicillin and kanamycin.

The results obtained prompted us to approach transposon mutagenesis in *A. pleuropneumoniae* by the use of plasmids mobilizable through RP4-based conjugal transfer (29). We first used the suicide plasmid pJM703.1::Tn5, obtained by M. Simonet and S. Falkow (40) from pJM703.1 (29), with *E. coli* SM10(xpir) as the donor strain. pJM703.1 and its derivatives replicate only in specific bacterial strains, used as donors, producing the π protein determined by the pir gene from the plasmid R6K (29). Although this insertional mutagenesis system has proven to be efficient for other bacteria, such as *Vibrio pseudotuberculosis* (40) and *Vibrio cholerae* (29), we have been unable to detect Tn5 transposition in *A. pleuropneumoniae* CMS.
TABLE 2. Conjugation and transposition experiments in *A. pleuropneumoniae* CM5

<table>
<thead>
<tr>
<th>Donor strain (plasmid)</th>
<th>Selection</th>
<th>Resistance frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;5 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>LES292 (RP4)</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;1 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SM10 pJM703.1::Tn5</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;5 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>S17-1 (pLOF/Km), with IPTG</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>S17-1 (pLOF/Km), without IPTG</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>&lt;5 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> Plasmids were mobilized from *E. coli* donor strains to *A. pleuropneumoniae* by conjugal transfer by means of a filter mating technique, as described previously (7). In brief, 100 µl each of overnight cultures of donor and recipient strains were mixed in 5 ml of sterile 10 mM MgSO<sub>4</sub> and then passed through a Millipore type HA 0.45-µm filter. Mating filters containing donor and recipient bacteria were placed onto a TS agar plate (containing 100 µM IPTG when needed for tac induction in the case of the Tn10 derivative). After incubation at 37°C for 8 h, the bacterial cells were suspended in sterile 0.05 M phosphate buffer (pH 7.4) containing 0.85% NaCl, and appropriate dilutions were plated on the counterselecting medium. Frequencies were determined as the ratio of recipient cells expressing plasmid or transposon antibiotic markers to the total number of recipients.

*<sup>b</sup> Unmated recipient. Resistance frequency corresponds to spontaneous Km<sup>+</sup> mutants.

Without prejudice that the frequency at which an element transposes is dependent on a great number of factors acting on the system, according to previously reported Tn5 transposition rates ranging from 10<sup>-5</sup> to 10<sup>-6</sup> (6), we can speculate that our negative results are due to the loss of efficiency derived from the low frequency of RP4-mediated conjugal transfer from *E. coli* to *A. pleuropneumoniae* CMS (10<sup>-4</sup>) (Table 2). Following this interpretation, the final efficiency rate of mutagenesis (mobilization plus transposition) was so low that Tn5 insertion mutants were undetectable in practice. In favor of this hypothesis is the fact that, in an identical system involving donor strain *E. coli* SM10 and plasmid pSUP2011, from which pJ703.1 is derived (29), the transfer frequency of Tn5 into the *Rhizobium meliloti* genome was 5 × 10<sup>-6</sup> per recipient, being 10<sup>-2</sup> per recipient the rate of RP4 mobilization between the same bacteria (39). An additional implication that might account for the lack of detection of Tn5 insertion mutants is the fact that the kanamycin resistance (Km<sup>+</sup>) gene of Tn5 has been reported not to be expressed in *A. pleuropneumoniae* (49).

In view of these problems, we decided to try anotherconjugal suicide vector, pLOF/Km, constructed by Herrero et al. (17). This is a derivative of pGP704 (pJ703.1 with a polylinker) (29), containing an insert with a mini-Tn10 (which carries a Km<sup>+</sup> cassette derived from Tn903) initially designed by Way et al. (46). In the pLOF/Km Tn10-based delivery system, the transposase gene of IS10<sub>9</sub> is located outside of, and provided in cis to, the mobile element. In addition, the transposase gene is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter (17), a strategy which could improve transposition efficiency (Fig. 1). When this vector was conjugally transferred from *E. coli* S17-1(p<sub>pir</sub>) to *A. pleuropneumoniae* CMS in the presence of IPTG, we found transposition mutants with an efficiency of 10<sup>-3</sup> (Table 2). In contrast, when IPTG was absent from the mating medium, no mutants were detected, indicating that the transposase expression level is a limiting factor for transposition of the Tn10 element in the system under study. This result is consistent with the fact that in natural conditions (i.e., without overexpression of the transposase) the IS10 transposition rate is reported to be about 10<sup>-4</sup> per element per generation (24), and supports the aforementioned argument explaining our failure in detecting Tn5 mutants with the pJM703.1::Tn5 delivery system.

**Mutagenesis of *A. pleuropneumoniae* CMS with the Tn10 derivative.** The transposition properties of the mini-Tn10/Km<sup>+</sup> in *A. pleuropneumoniae* were investigated further in order to determine the usefulness of the system for insertional mutagenesis in this bacteria. Cointegration of pLOF/Km was determined by selecting for kanamycin resistance (mini-Tn10) and ampicillin resistance (determined by the suicide plasmid). We found cointegration rates of about 1%, e.g., 13 of 1,100 colonies were resistant to both antibiotics (1.2% of cointegrates). This frequency is lower than that reported by Herrero et al. (17), who found 5% cointegrates with pLOF/Km in *Pseudomonas putida*, and is much lower than the 35% found by other investigators (3) in *Proteus mirabilis* with a mini-Tn5 delivery system also derived from pGP704 (7, 29).

A critical parameter in evaluating a transposon mutagenesis system is the ability to produce single, random insertions. To analyze these characteristics, chromosomal DNA from 25 exconjugants from 25 independent mutagenesis experiments was restricted with *XhoI* (which cuts at a unique site in the Tn10 derivative) (Fig. 1) (17) and analyzed by Southern blotting with pLOF/Km as the probe. Figure 2, in which 19 of these mutants are shown, illustrates that almost all the insertions were single and occurred at different locations. Of the 25 mutants, the insertions in only 2 mapped identically, and in another, a multiple insertion likely took place (Fig. 2). These findings allow to assume that no significant mini-Tn10 hot spots occur in the *A. pleuropneumoniae* chromosome. This might be explained by the fact that the Tn10 derivative bears a mutant IS10 transposase.
with a much lower degree of target site specificity than the wild type (25). Since four of the six consensus base pairs of the target sites of Tn10 insertion are G-C pairs (25), the relatively low G+C content of the A. pleuropneumoniae genome (42%) (23, 32) may also contribute to explaining the absence of significant hot spots.

The insertion randomness of the mini-Tn10 was also demonstrated by indirect evidence, as provided by the ability to isolate mutants of selected phenotypes. For this purpose, we assessed the frequency of auxotrophic dependence on the amino acid tryptophan in a bank of mini-Tn10-induced A. pleuropneumoniae mutants. In a prototypic experiment carried out with 1,136 insertional mutants (randomly selected from 10 different mutagenesis experiments), 4 of them were tryptophan auxotrophs. This represents a frequency of about 0.35%, a result in agreement with data on transposon-induced auxotrophic mutations in other bacteria (3, 26). The production of mutants deficient in the expression of other phenotypes was also tested. For instance, we isolated seven nonhemolytic mutants of A. pleuropneumoniae (44), indicating that not only metabolic but also virulence-associated genes could be insertional disrupted by the use of pLOF/Km.

Finally, we carried out experiments to determine the stability of the transposon insertions. By a previously published protocol (3), we subcultured 550 Kmr colonies, selected randomly from five different transposition experiments, three times on TS agar without antibiotics at 37°C for 24 h. After these three passages without selective pressure, the colonies were cultured again on a medium containing kanamycin. None of the 550 clones failed to grow, indicating that the mini-Tn10 insertions were stably maintained. This result is consistent with the fact that the transposase gene is absent from the mobile element (see above), providing highly stable insertions even without antibiotic selection (17, 25).

Applicability of the mini-Tn10 transposon mutagenesis system to other A. pleuropneumoniae strains. In order to determine whether the transposon mutagenesis system described in this article is of general applicability to the species A. pleuropneumoniae, we carried out experiments with 12 additional strains representing different serotypes (Table 1).

We were able to obtain nalidixic acid-resistant spontaneous mutants of all these A. pleuropneumoniae strains. With the abovementioned E. coli donor strain, KmR exconjugants were found with frequencies ranging from 10⁻⁵ to 10⁻⁷ (Table 3). We failed to detect KmR colonies for only 2 of the 12 strains we tested.

Concluding remarks. We have successfully developed, for the first time, a transposon mutagenesis system that is functional in A. pleuropneumoniae. This was achieved with a suicide conjugative vector, pLOF/Km, designed by Herrero et al. (17), carrying a mini-Tn10 delivery system. In our hands, this vector has proven to be a useful tool for generating single, random, and stable transposon insertions, at a workable frequency, in the A. pleuropneumoniae genome. Since the transposable element in transposon-induced mutants is physically linked to the mutated gene, the transposon mutagenesis technique reported here will allow easy identification of the genetic determinants involved in the investigated phenotypes of A. pleuropneumoniae. For this purpose, the drug resistance marker of the transposon could also be used for the selection of recombinant clones carrying the target region. Besides these general advantages of transposon mutagenesis, specific benefits derived from the particular features of the vector we used have to be considered.

First, pLOF vectors carry a minitransposon which has important advantages over classical transposable elements (25). Since the transposase gene is located outside the minitransposon, the mobile element does not undergo secondary transposition events, and thus the insertions generated are highly stable. On the other hand, a smaller minitransposon is more manageable, it transposes with an increased efficiency, and, once inserted, it is less subject to rearrangements with adjacent regions (25). Second, the presence of unique NotI or SfiI sites internal to the mobile element (17) allows, conjointly with the use of the auxiliary cloning plasmids pUC18Not and pUC18Sfi (specifically constructed for this purpose) (17), the introduction of modified or foreign genes into the genome of the mutated A. pleuropneumoniae strain. Third, the modular conception of the mobile element in pLOF vectors (17) enables the replacement of the selection marker. This constitutes an important application which allows the use of nonantibiotic markers (e.g., resistance cassettes to the herbicide bialaphos, to arsenite or mercuric salts, or to organomercurial compounds [17]), opening the possibility of environmental release of the genetically engineered strain, e.g., in the case of a live.
vaccine. Finally, the possibility of replacing the selection marker and the fact that recipient cells are not immune to new transposition runs because of the lack of the transposase permit multiple transposon insertions in the same strain (17).

In conclusion, because of the enormous utility of insertional mutagenesis with transposons in genetic studies of bacteria (4), the availability of such a system for *A. pleuropneumoniae* will contribute greatly to the development of research on molecular aspects of the structure, metabolism, physiology, and virulence of this pathogenic bacterium.

We are greatly indebted to V. de Lorenzo (Centro de Investigaciones Biológicas, Velázquez 144, 28006 Madrid, Spain) for providing us with the pLOF/Km vector and *E. coli* donor strains, for useful comments, and for critical reading of the manuscript. We thank R. Nielsen and S. Rosendal for sending us the *A. pleuropneumoniae* strains, M. Simonet for the gift of pJM703.1: Tn5 and *E. coli* SM10, J. Blázquez for advice, and Joana López for editorial assistance.

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**REFERENCES**


8. Fenwick, B. W., and B. Y. Osburn. 1986. Immunoreactive responses to the lipopolysaccharides and capsular polysaccharides of *Hae-
mophilus pleuropneumoniae* in convalescent and immunized pigs. Infect. Immun. 54:575-582.


