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Transposon Insertion and Subsequent Donor Formation Promoted by Tn*501* in *Bordetella pertussis*

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The mercuric chloride resistance transposon, Tn*501*, was introduced into *Bordetella pertussis* by using the chimeric plasmid pUW942, which is unable to replicate in this species. Tn*501* insertions which conferred a thiamine requirement were the predominant insertion class. In many cases, the mercuric chloride-resistant transconjugants were also resistant to the other plasmid markers, but failure to detect plasmid DNA in these isolates indicated that integration of the entire plasmid into the chromosome had occurred. One such insertion was further characterized. Southern hybridization with a Tn*501*-specific probe indicated that chromosomal DNA from one strain containing the integrated plasmid had two copies of Tn*501* and an intervening copy of the plasmid associated with the chromosome. The presence of the plasmid was unstable, and derivatives which had lost all of the plasmid markers except mercuric chloride resistance were obtained. These strains had a single copy of Tn*501* and had lost all of the rest of the plasmid-specific sequences. Strains containing the plasmid in the integrated state could act as genetic donors and mobilize chromosomal genes.

Bordetella pertussis is a fastidious microorganism that causes whooping cough. The organism synthesizes several determinants of pathogenicity including an extracellular adenylate cyclase (26), fimbriae (21), dermonecrotic toxin (3), and pertussis toxin (lymphocytosis-promoting factor, islet-activating protein) (9), which is believed to be responsible for the systemic effects accompanying *B. pertussis* infection. Although some of these determinants of pathogenicity have been characterized biochemically, genetic analysis of *B. pertussis* has not received much attention, perhaps because it is difficult to cultivate in vitro and it tends to spontaneously lose en bloc its determinants of pathogenicity after in vitro passage (18). It would be useful, if possible, to develop a reliable means of gene transfer to understand better the genetic and molecular basis of virulence in *B. pertussis*.

Sato et al. (20) have described a chimeric plasmid pAS8Rep-1, which contains a ColE1 replicon and RP4 (P incompatibility group) conjugation genes. The advantage of this system is that cells containing plasmid RP4 will mate with a wide variety of bacterial species (5, 7), but ColE1 plasmids will replicate only in a limited number of species (5). We recently showed that *B. pertussis* does not support replication of plasmid ColE1 but can successfully mate with strains containing plasmid RP4 (25).

In this study, we used plasmid pAS8Rep-1 and a derivative, plasmid pUW942 containing transposon Tn*501*, to introduce transposons into *B. pertussis*. One of these transposons, Tn*501*, is able to promote integration of the plasmid into the *B. pertussis* chromosome and to create genetic donors.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. *Escherichia coli* and *B. pertussis* strains and plasmids used in this study are listed in Table 1. *B. pertussis* strains were cultivated on Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.), Stainer-Scholte broth (11), or Stainer-Scholte agarose plates (0.6% agarose). *E. coli* strains were grown in L-broth or L-agar plates (4).

Conjugation experiments. Plate matings were performed by the method of Bradley et al. (2) on media suitable for *B. pertussis*. After mating, the cells were harvested in Stainer-Scholte salts without supplements and plated on selective media. Antibiotics used for selection of *B. pertussis* were incorporated into Bordet-Gengou (15) plates at the following concentrations: kanamycin, 25 μg/ml; trimethoprim lactate (a gift from Lynn Elwell, Wellcome Research Laboratories, Research Triangle Park, N.C.), 25 μg/ml; mercuric chloride, 200 μg/ml; ampicillin, 100 μg/ml; streptomycin, 400 μg/ml; rifampin, 25 μg/ml; and erythromycin, 0.15 μg/ml. The high level of mercuric chloride was necessary for selection on Bordet-Gengou plates, a medium supplemented with 15% sheep blood. Similar concentrations of mercuric chloride were necessary to select for resistant *E. coli* on Bordet-Gengou plates.

Construction of plasmid pUW942. A derivative of plasmid pAS8Rep-1 containing the transposon Tn*501* was obtained by mating pAS8Rep-1 into *E. coli*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source or reference
E. coli		
AB2463	*thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 sup-37 recA13*	20
UW941	*pro-22 metF63 gyrA zxx*::Tn*501*	G. Dougan, Trinity College
UW937	*thr-1 leu-6 thi-1 lacY-1 supE44 tonA21* λ^- *gyrA*	This laboratory
B. pertussis		
Tohama I	Phase I, Ery^s	C. Manclark, NIH (21)
Tohama III	Phase III, Ery^r	C. Manclark
BP304	Str^r	Spontaneous mutation, Tohama I
BP321	Str^r Rif^r	Spontaneous mutation, BP304
BP330-1, BP330-2, and BP330-3	*thi*::Tn*501* and pUW942 inserted in BP321	This study
BP331-2	*thi*::Tn*501*, from BP_{330-2}	This study
BP333	*trp-2*	*trp-2* from reference 11
Plasmids		
pAS8Rep-1	RP4-ColE1, Tc^s, rep(RP4)::Tn*7*	20
pUW942	pAS8Rep-1 *zxx*::Tn*501*	This study
R388	Tra^+ Tp Su	6

[a] *E. coli* nomenclature according to Bachmann and Low (1). Ery^s, erythromycin sensitive; Ery^r, erythromycin resistant; Str^r, streptomycin resistant; Rif^r, rifampin resistant; Tp, trimethoprim resistant; Su, sulfonamide resistant.

UW941, which contains Tn*501* in the chromosome, and selecting for kanamycin-resistant transconjugants. Plasmid derivatives with Tn*501* transposed onto pAS8Rep-1 were obtained by mating the plasmid into strain UW937 and selecting for kanamycin and mercuric chloride resistance (25 μg/ml each) on minimal plates containing threonine and leucine. One plasmid containing Tn*501* (designated pUW942) was chosen, and the site of the Tn*501* insertion was mapped by restriction analysis.

Isolation of DNA. Plasmid DNA was isolated by the method previously described (19) and was either used as a crude preparation or purified by cesium chloride density centrifugation. Large fragments of chromosomal DNA were prepared by the method of Hull et al. (8).

Restriction digests and filter hybridization. Restriction endonucleases were used under conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of cleaved DNA was performed in 0.7% agarose gels buffered with either Tris-borate (14) or Tris-acetate (19). Transfer of DNA to nitrocellulose and hybridization with ^{32}P-labeled DNA probes was performed as previously described (19).

Preparation of Tn*501*-specific DNA probe. The 5.9-kilobase *Eco*RI fragment specific to Tn*501* was cut from a Tris-acetate agarose gel, electroeluted, ethanol precipitated, and nick translated by the method of Maniatis et al. (12).

RESULTS

Generation of transposon insertions. We decided to use plasmid pAS8Rep-1, a chimeric plasmid containing a ColE1 replication region and incompatibility group P conjugation genes, to introduce a ColE1 replicon into *B. pertussis*. The plasmid pAS8Rep-1 contains two transposons, Tn*7* (trimethoprim resistance) and Tn*1* (ampicillin resistance). This plasmid also contains a gene encoding resistance to kanamycin, but this marker does not transpose. By monitoring the acquisition of kanamycin resistance, we could determine whether the plasmid was maintained in the transconjugants. Plate matings were performed, and we attempted to select for kanamycin-, ampicillin- or trimethoprim-resistant transconjugants. No kanamycin-resistant *B. pertussis* transconjugants were obtained, indicating that pAS8Rep-1, unlike plasmid RP4 (25), cannot be maintained in *B. pertussis*. We were unable to select for ampicillin-resistant transconjugants because the *E. coli* donors produced enough β-lactamase to protect sensitive *B. pertussis* strains. Trimethoprim-resistant transconjugants, however, were obtained at a frequency of 10^{-9} per *B. pertussis* recipient. Two trimethoprim-resistant *B. pertussis* transconjugants from a mating with *E. coli* were kanamycin- and ampicillin-sensitive and did not contain plasmid DNA, indicating that Tn*7* had transposed into the chromosome and the plasmid was lost.

Next, we used a derivative of pAS8Rep-1, plasmid pUW942 (Fig. 1), to introduce the transposon Tn*501*, encoding mercuric ion resistance, into *B. pertussis*. Mercuric ion-resistant transconjugants were obtained at a frequency of 10^{-7} per *B. pertussis* recipient. One hundred mercu-

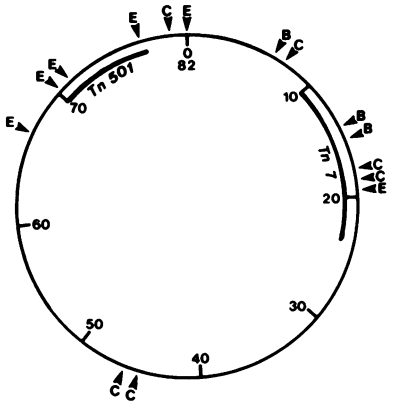

FIG. 1. Restriction map of plasmid pUW942. The numbers inside the circle are plasmid coordinates in kilobases, starting from one of the *Eco*RI sites joining RP4 with ColE1. Sequences of the transposons Tn*7* and Tn*501* are marked by the inside arcs. E marks an *Eco*RI cleavage site; B, a *Bam*HI site; and C, a *Cla*I site. The coordinates from 0 to the *Eco*RI site at approximately 67 kilobases are derived from RP4 (except Tn*7*); those for the rest of the plasmid (excluding Tn*501*) are from ColE1.

ric ion-resistant insertion mutants were screened for nutritional requirements by monitoring growth in Stainer-Scholte broth, a completely synthetic medium. The most frequent class of Tn*501* insertion mutants (65 of 100) required thiamine. The rest of the mercuric ion-resistant transconjugants were prototrophic (34 of 100), except for 1 which had an uncharacterized nutritional requirement.

Most mercuric ion-resistant transconjugants that required thiamine for growth (50 of 65) were also resistant to kanamycin, ampicillin, and trimethoprim, indicating that the plasmid genes were not lost from these strains. However, all except one of the prototrophic mercuric ion-resistant transconjugants were sensitive to all of the other resistance markers on plasmid pUW942. DNA was prepared from three thiamine-requiring transconjugants, but no plasmid DNA was detected in these isolates (Fig. 2). Since plasmid DNA was isolated from a *B. pertussis* strain containing the low-copy-number plasmid R388 (Fig. 2E), we believe that these transconjugants do not maintain plasmid pUW942 as a replicating plasmid.

To test for the stability of the drug resistance markers, strain BP330-2, which contained the plasmid, was streaked for single colonies on nonselective plates, and 20 individual colonies were scored for their resistance patterns by patching the colonies onto Bordet-Gengou plates containing the appropriate antibiotic. Two classes of resistance patterns were observed. One class, comprising 25% of the isolates (5 of 20), was the same as the parent; that is, the organisms contained all of the resistance markers of the plasmid. The other 75% (15 of 20) were resistant only to mercuric ions. The fact that mercuric ion resistance genes were stable but the other resistance genes were not suggested that Tn*501* could have promoted the integration of the plasmid, probably as a replicon fusion intermediate. DNA was isolated from the *B. pertussis* strain containing all of the plasmid markers (BP330-2) and from a derivative of strain BP330-2 which is resistant only to mercury (BP331-2). Strain BP330-2 was grown on trimethoprim to insure that the plasmid would not be lost from the cells. DNA was digested with *Cla*I, which does not cut within transposon Tn*501* (Fig. 1). The DNA fragments were separated by electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P nick-translated probe derived from the 5.9-kilobase *Eco*RI fragment internal to Tn*501*.

The pattern of the bands homologous to transposon Tn*501* (Fig. 3) is consistent with a Tn*501*-mediated replicon fusion model (23). *E. coli* containing the plasmid had a single band which comigrated with purified plasmid DNA. Strain BP330-2 had two major restriction fragments homologous to Tn*501*, one larger and one smaller than the Tn*501* fragment from the plasmid (Fig. 3C). The DNA from the strain which had lost all of the plasmid markers except mercury

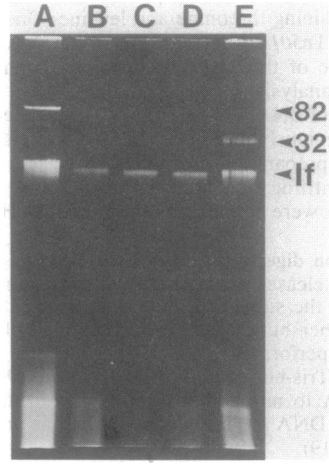

FIG. 2. Plasmid DNA in pUW942 transconjugants. Crude plasmid preparations were separated by electrophoresis on a 0.7% agarose gel buffered with Tris-borate. (A) *E. coli* UW937(pUW942); (B through D) *B. pertussis* transconjugants BP330-1, BP330-2, and BP330-3, respectively; (E) BP304(R388). Arrows indicate the molecular masses of the plasmids in kilobases; lf denotes linear fragments of DNA generated by random shear during the isolation procedure. The material at the bottom of the gel is probably RNA.

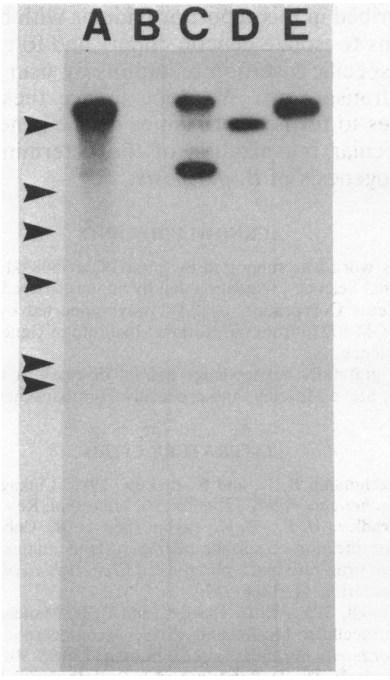

FIG. 3. Detection of transposon Tn*501*-specific sequences. Chromosomal or plasmid DNA was digested with *Cla*I, separated by electrophoresis on a 0.75% agarose slab gel buffered with Tris-acetate, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled probe specific to Tn*501*. (A) Total (plasmid and chromosomal) DNA from *E. coli* UW937(pUW942); (B through D) total DNA from *B. pertussis* BP321, BP330-2, and BP331-2, respectively; (E) purified plasmid pUW942 from *E. coli*. The arrows on the left indicate the mobility of lambda *Hin*dIII size standards run on the same gel: 23.72, 9.46, 6.67, 4.26, 2.25, and 1.96 kilobases, from top to bottom.

(BP331-2) had only one fragment homologous to Tn*501* (Fig. 3D), and this fragment did not migrate with either of the fragments seen in BP330-2, the strain from which it originated. Similar results were obtained with *Sma*I and *Bam*HI restriction digests (data not shown). The minor bands seen for strain BP330-2 and total DNA from strain UW937(pUW942) probably represent a subpopulation of cells in which secondary transposition events have occurred.

With other Tn*501* plasmid-containing insertions in the thiamine gene, other rearrangements of plasmid bands were observed to have occurred (data not shown), including deletions removing ampicillin resistance. A simple cointegrate formation seemed to have occurred in the case of strain BP330-2, but this was not the only possible outcome from a Tn*501* transposition at this site.

Transfer of chromosomal markers. Previous studies by Pemberton and Bowen (17) have shown that Tn*501* insertions on the autonomously replicating plasmid R388 can mobilize chromosomal genes in *Rhodopseudomonas sphaeroides*, whereas matings with R388 alone are not fertile. To determine whether a strain containing the entire integrated plasmid could mobilize chromosomal markers in *B. pertussis* matings, strain BP330-2 was used as a donor in a mating with Tohama III, a phase III *B. pertussis* strain which is resistant to erythromycin. Streptomycin- and erythromycin-resistant transconjugants were obtained after a mating at 20 times the spontaneous mutation frequency (Table 2). Rifampin resistance, an unselected marker, was cotransferred 7% of the time, indicating that these markers are linked in *B. pertussis* as in other gram-negative bacteria (1, 13, 24). In another mating, the ability to synthesize the amino acid tryptophan was transferred to a mutant strain at a frequency some thousand times the spontaneous reversion frequency, and all transconjugants were thiamine independent. In both mating experiments, the transconjugants did not express any of the plasmid resistance markers, indicating that the plasmid DNA was not transferred. We were unable to construct a linkage map in *B. pertussis* because of the lack of other chromosomal mutants.

DISCUSSION

The chimeric plasmids pAS8Rep-1 and pUW942, which possess a ColE1 origin of replication, can be used to introduce transposons into *B. pertussis* and do not seem to be main-

TABLE 2. Transfer of chromosomal markers in *B. pertussis*

Strain		Selection	Frequency[a]
Recipient	Donor		
Tohama III	BP330-2	Streptomycin resistance[b]	1.2×10^{-7}
	None	Streptomycin resistance	5.9×10^{-9}
BP333	BP330-2	Tryptophan[c]	1.1×10^{-5}
	None	Tryptophan	$<5 \times 10^{-9}$

[a] Ratio of recombinant *B. pertussis* to recipient *B. pertussis*.

[b] Strain BP330-2 was mated with Tohama III for 23 h to allow phenotypic expression of streptomycin resistance (400 μg/ml). Erythromycin (0.15 μg/ml) was used to counterselect the donor. Transconjugants were scored for ability to grow without thiamine to avoid counting spontaneous erythromycin-resistant BP330-2 mutants.

[c] Strain BP330-2 was mated with BP333 on Bordet-Gengou plates for 3 h and then was plated on Stainer-Scholte plates supplemented with 0.15% bovine serum albumin. There was insufficient tryptophan to support BP333, and insufficient thiamine to support BP330-2.

tained as extrachromosomal elements within this species. We have been able to introduce both transposon Tn*7* and transposon Tn*501* into the *B. pertussis* chromosome. Tn*501* has a preferred insertion site which confers a thiamine requirement. Tn*501* can also insert into at least two other chromosomal sites.

In *B. pertussis*, our data show that transposon Tn*501* promotes the integration of plasmid pUW942 into the chromosome during the transposition process. The *B. pertussis* isolates selected for mercuric ion resistance appear to contain the entire plasmid flanked by two copies of Tn*501* (Fig. 3). *B. pertussis* cells in which the plasmid had been excised from the chromosome had only a single copy of Tn*501* at the insertion site. This would account for the instability of all of the markers except mercuric ion resistance. The two copies of Tn*501* in the cointegrate support the hypothesis that integration of the plasmid occurs by a transposon-promoted replicon fusion.

The integration of plasmid pUW942 into the *B. pertussis* chromosome was mediated by the transposon Tn*501*, and not by Tn*7* or Tn*3*, since plasmid cointegrates were not seen with plasmid pAS8Rep-1, which is identical to plasmid pUW942 except for the lack of Tn*501*. Others have shown that the transposase function and resolvase function of Tn*501* are induced by mercuric ions (10, 22), and they have observed a 3- to a 100-fold increase in transposition if mercuric chloride was added to the cells. It was something of a surprise when our transconjugants, selected and purified on mercuric chloride, retained the other plasmid markers as well. It has been shown that copy number does not influence the level of mercuric ion resistance (16), so it is unlikely that selection maintained two copies of the plasmid in the cointegrate state. It is interesting to note that 77% (50 of 65) of the insertions in the thiamine gene, but only 3% (1 of 34) of the prototrophic insertions, contained the integrated plasmid; this indicates that there might be some effect specific to the site of insertion within the thiamine gene that prevents resolution of the plasmid cointegrate.

Conjugative plasmids which have been integrated into the bacterial chromosome have been one of the basic tools for promoting exchange of chromosomal markers between bacterial strains. We have now been able to achieve this result in *B. pertussis*, a microorganism which has previously been without a means of chromosomal transfer. Very few genetic loci have been defined by mutation in *B. pertussis* (11), so our ability to generate a linkage map, or even determine if transfer of markers occurs from a single origin of transfer in a single direction, is limited. Nevertheless, the development of the methods described in this report provide us with both the means to isolate genetic donors and to generate site-specific insertion mutations by using bacterial transposons. We hope to use these techniques to further our studies on the genetic and molecular organization of the determinants of pathogenesis of *B. pertussis*.

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