Isolation of Avirulent Mutants of *Erwinia stewartii* Using Bacteriophage Mu pf7701

By S. L. McCAMMON,† D. L. COPLIN* AND R. G. ROWAN‡

Department of Plant Pathology, The Ohio State University, Wooster, Ohio 44691, USA

(Received 3 April 1985; revised 12 June 1985)

Bacteriophage Mu pf7701, a KmR derivative of Mu cts62, was inserted into the conjugative *Erwinia stewartii* plasmid pDC251, which carries Tn10. When pDC251::Mu pf7701 plasmids were conjugated from *Escherichia coli* into derivatives of *E. stewartii* strain SS104 they were unstable; loss of either or both drug resistance markers occurred. Stable transconjugants resulted from deletion of Mu sequences, integration of the plasmid into the chromosome, or loss of an indigenous 34 kb cryptic plasmid. Among transconjugants selected for KmR, the largest colonies arose from transconjugants in which Mu pf7701 had transposed to the chromosome and the pDC251::Mu pf7701 plasmid had been lost; 1300 transconjugants of this type were screened for pathogenicity to corn (*Zea mays*) seedlings and eight mutants were obtained that did not cause watersoaking symptoms. The insertions of Mu pf7701 in these mutants were in the chromosome.

INTRODUCTION

*Erwinia stewartii* causes a vascular wilt and leaf blight of corn (*Zea mays*) known as Stewart's wilt. The genetics of *E. stewartii* appear similar to other enterics; for example, it exchanges plasmids readily with *Escherichia coli* and supports replication of bacteriophage Mu if it is introduced into the cell on a vector plasmid (Coplin, 1979). *E. stewartii* is unusual, however, in that it harbours a large number of cryptic plasmids comprising 20% or more of its genome (Coplin et al., 1981). Strain SS104, used in this study, contains 11 plasmids of the following sizes: 318, 103, 74, 65, 63, 45, 34, 25, 13, 4-1 and 4-1 kb. The 34 kb plasmid, pDC140, is closely related to a 52 kb derepressed conjugative plasmid, pDC250, frequently found in other *E. stewartii* strains (Coplin et al., 1985).

Transposon mutagenesis has proved to be a valuable tool for the isolation of mutations in genes for nitrogen fixation in *Rhizobium*, tumorigenicity in *Agrobacterium*, and pathogenicity in *Pseudomonas* and *Erwinia*. This technique requires the use of a vector that will carry the transposon into the cell but will not survive as an autonomous replicon. A ‘suicide’ plasmid, pJB4J1, has been developed for *Rhizobium* which exploits the property of Mu to interfere with replication of IncP plasmids in some species of bacteria (Beringer et al., 1978). Transposon Tn5, carried by plasmid pJB4J1, has been used as a mutagen in *E. herbicola* (Gantotti et al., 1981), and in some strains of *E. chrysanthemi* (Chatterjee et al., 1983) and *E. carotovora* subsp. *carotovora* (Zink et al., 1984). pUT13, a Tn5-containing temperature sensitive IncP plasmid, has also been used as a suicide plasmid in *E. chrysanthemi* (Chatterjee et al., 1981). Strains of *E. chrysanthemi* that are sensitive to Mu have been mutagenized by direct infection with mini-Mu (Thierry et al., 1984) and Mu S::Tn9 (Van Gijsegm et al., 1985). Unfortunately pJB4J1 is quite stable in *E.*
2994

S. L. McCAMMON, D. L. COPLIN AND R. G. ROWAN

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant markers</th>
<th>Derivation or source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. stewartii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS104</td>
<td>Wild-type</td>
<td>ICPPB, M. P. Starr</td>
</tr>
<tr>
<td>SW2</td>
<td>Wild-type</td>
<td>Coplin &amp; Rowan (1979)</td>
</tr>
<tr>
<td>SW211</td>
<td>Wild-type</td>
<td>D. L. Coplin</td>
</tr>
<tr>
<td>DC283</td>
<td>NalR</td>
<td>Spontaneous mutant of SS104</td>
</tr>
<tr>
<td>DC336</td>
<td>Mu cts62</td>
<td>From DC283, Coplin (1979)</td>
</tr>
<tr>
<td>Esch. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C600mr</td>
<td>thr-l thi-l leuB6 lacY1 hsdR hsdM</td>
<td>S. N. Cohen</td>
</tr>
<tr>
<td>DC921</td>
<td>C600mr NalR</td>
<td>Spontaneous mutant of C600mr</td>
</tr>
<tr>
<td>MH2855</td>
<td>Spc8 (A pro-lac) pΔ(leu::Mu AL-SUara)</td>
<td>M. Howe</td>
</tr>
<tr>
<td>Q1</td>
<td>SuII Thr- Leu- Lac- tonA rpsL</td>
<td>Howe (1973)</td>
</tr>
<tr>
<td>MH5247</td>
<td>F+ Mel- Pro- SuIII (Mu pf7701 hPl#1)</td>
<td>M. Howe</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDC251</td>
<td>pDC250::ΔTn10</td>
<td>Coplin et al. (1985)</td>
</tr>
<tr>
<td>pDC2501, pDC2502, pDC2504 to pDC2507, pDC2509 to pDC2512, pDC2514, pDC2516 to pDC2519</td>
<td>This paper</td>
<td></td>
</tr>
<tr>
<td>pDC25106</td>
<td>Te8 ΔMu</td>
<td>Deletion mutant of pDC2510</td>
</tr>
<tr>
<td>pKN54</td>
<td>Left-end PstI fragment of Mu cloned in pBR322</td>
<td>M. Howe (Schumann, 1979)</td>
</tr>
</tbody>
</table>

* Addresses: M. P. Starr, University of California, Davis, Calif., USA; S. N. Cohen, Stanford University, Calif., USA; M. Howe, University of Wisconsin, Madison, USA.

E. stewartii and this species does not grow in temperatures nonpermissive for pUT13 and is not sensitive to Mu(G-) phage (D. L. Coplin, unpublished). Thus, a new means of isolating transposon-induced mutations was needed for locating virulence genes in E. stewartii. This paper describes the behaviour of pDC251 : : Mu pf7701 hybrid plasmids in E. stewartii and their use in isolating avirulent mutants. pDC251 is a Tn10-containing derivative of E. stewartii plasmid pDC250 (Coplin et al., 1985), and Mu pf7701 is a derivative of Mu cts62 Δ445-3 that contains the KmR gene from Tn5 (Thompson & Howe, 1979).

METHODS

Bacterial strains, plasmids and bacteriophages. Those used in this study are listed in Table 1. Bacteriophage Mu pf7701 hPl#1 (hereafter referred to as simply Mu pf7701) was a gift from M. Howe and was constructed as follows (M. Howe, personal communication): Tn5 was inserted into Mu cts62 A445-3, which has a 2.26 kb deletion removing the right end of the G and left end of the B regions (Chow et al., 1977), at 4.4 kb from the immunity end of Mu between genes B and C. A plaque-forming derivative was then isolated that contained a deletion extending rightward from within the Tn5 insertion and removing 2.8 kb of Mu and 3.3 kb of Tn5. This made Mu pf7701 Kii- (Waggoner et al., 1984) and Tn5 nontransposable. The hPl#1 derivative of Mu pf7701 has the host range of P1. Lysates of Mu pf7701 were prepared by heat induction of a lysogen of QD5003 and titrated on Q1.

Media and mating conditions. Culture media (Coplin et al., 1985), mating conditions (Coplin, 1978) and procedures for propagation and titration of Mu (Coplin, 1979) have been described previously.

Mutants resistant to 2-deoxygalactose were selected on a medium containing 0.1% (w/v) NH₄H₂PO₄, 0.02% (w/v) KCl, 0.02% (w/v) MgSO₄, 0.2% (w/v) lactic acid, 0.1% (w/v) yeast extract, 0.1% (w/v) 2-deoxygalactose and 1.5% (w/v) agar.

Construction of pDC251 : : Mu pf7701 plasmids. A culture of Esch. coli DC921(pDC251) at 6·4 × 10⁸ cells ml⁻¹ was infected with Mu pf7701 at a m.o.i. of 0.4. After 20 min, the culture was diluted 1 : 50 in L broth and incubated overnight at 30 °C. The bacteria were then subcultured, grown to 10⁸ cells ml⁻¹ and mated with MH2855 at a donor to recipient ratio of 1 : 5; transconjugants were then selected for Te8 KmR Spc8. After purification on selective medium, clones were further tested for stability and the ability to donate Te8 and Km8.
**Mu pf7701 in Erwinia stewartii**

Fig. 1. Restriction map of pDC251 (preceding paper, Coplin et al., 1985) showing the locations of Mu pf7701 insertions in plasmids pDC2501 through pDC2519 (designated 01 to 19, respectively). The insertions in pDC2501 and pDC2509 are in opposite orientations. We did not determine which end of the 9-2 kb HindIII was the site for the insertions of pDC2505 and pDC2519. They are arbitrarily shown on the right end. E, EcoRI; H, HindIII; K, KpnI; S, SmaI.

**Stability of transconjugants.** To check the stability of plasmids after transfer from Esch. coli to E. stewartii, transconjugants were grown overnight in L broth without antibiotics and then dilution plated on L agar. After 2 d, 25 to 50 single colonies were transferred with toothpicks to antibiotic-containing media.

**DNA isolation and gel electrophoresis.** Plasmid DNA was isolated by the procedure of Birnboim & Doly (1979) and electrophoresed in 0-7% (w/v) agarose (Seakem ME) in Tris/acetate buffer as previously described (Coplin et al., 1981). For restriction analysis, the DNA was further purified by precipitation in 20 m m-spermine (Hoopes & McClure, 1981). Procedures for digestion with restriction endonucleases and for gel electrophoresis are described in the preceding paper (Coplin et al., 1985). Genomic DNA was isolated using the growth and lysis conditions of Currier & Nester (1976). Following lysis, DNA was extracted with phenol, precipitated with ethanol and purified by caesium chloride/ethidium bromide gradient centrifugation.

**Nick translation and Southern blot hybridization.** Procedures for nick translation of pKN54 and pBR322 DNA with [32P]dCTP and for Southern blot hybridizations are described in the preceding paper (Coplin et al., 1985). Zetabind (AMF Cuno, Meriden, Conn., USA) was used as the blotting matrix rather than nitrocellulose in these experiments. The Zetabind blot of genomic DNA from Mu-induced avirulent mutants was stripped for hybridization with additional probes according to the manufacturer's directions.

**Pathogenicity tests.** Plant growth conditions, toothpick inoculations and disease rating have been previously described (Bradshaw-Rouse et al., 1981). Whorl inoculations of 7- to 8-d-old sweet corn (Zea mays cv. Earliking) seedlings were done by suspending an overnight culture of bacteria in 0-01 M-potassium phosphate buffer (pH 7-0), containing 0-2% (v/v) Tween 40, at 107 cells ml-1 and pipetting a 0-1 ml sample into the whorl of each seedling. Symptoms were recorded after 3 d. Unlike inoculation methods which involve wounding, this technique resulted in the formation of watersoaked lesions without any wilt.

**RESULTS**

**Construction and characterization of Mu pf7701 plasmids in Esch. coli**

Lysogenization of *Esch. coli* DC921(pDC251) with Mu pf7701 and subsequent transfer of the KmR and TcR markers to MH2855 resulted in 15 clones containing pDC251::Mu pf7701 plasmids. These were designated pDC2501 through pDC2519 (exclusive of pDC2503, pDC2508, pDC2513, and pDC2515). Agarose gel electrophoresis (AGE) revealed that each plasmid was 95 kb in size, which is the equivalent of an insertion of Mu pf7701 (36 kb) into pDC251 (59 kb). The plasmids were stable in *Esch. coli* and could transfer between *Esch. coli* strains at about 100% per donor cell in 3 h broth matings. TcR, KmR, and Mu pf7701 production were always linked in the transconjugants.

Restriction analysis of the hybrid plasmids with single and double digests of HindIII and EcoRI revealed that the Tn10 and Mu pf7701 insertions were grouped in two regions of pDC251 (Fig. 1). In pDC251, the site of Tn10 insertion was located at 7-5 kb from the left end of the 18-3 kb EcoRI fragment of pDC250. Ten Mu pf7701 insertions were in the 12-5 kb HindIII fragment and the other five were on either side of, or within, Tn10. All of the Mu pf7701 insertions appeared to be independent as determined by their map position and orientation.

**Characterization of E. stewartii pDC251::Mu pf7701 transconjugants**

All 15 pDC251::Mu pf7701 plasmids were conjugated from *Esch. coli* MH2855 into *E.
Table 2. Conjugal transfer of pDC251::Mu pf7701 plasmids from Esch. coli MH2855 to E. stewartii DC283 and DC336 and linkage of plasmid and phage markers

Filter matings (2 h) were done as described in Methods. DC336 is DC283(Mu cts62).

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Plasmid</th>
<th>Transfer Frequency*</th>
<th>Percentage Km R</th>
<th>Transfer Frequency*</th>
<th>Percentage Tc R</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC283</td>
<td>pDC2504</td>
<td>$3.5 \times 10^{-2}$</td>
<td>95</td>
<td>$3.0 \times 10^{-4}$</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>pDC2505</td>
<td>$6.0 \times 10^{-3}$</td>
<td>20</td>
<td>$1.7 \times 10^{-6}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pDC2510</td>
<td>$2.3 \times 10^{-3}$</td>
<td>79</td>
<td>$3.7 \times 10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pDC2511</td>
<td>$5.7 \times 10^{-3}$</td>
<td>90</td>
<td>$3.6 \times 10^{-4}$</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>pDC2514</td>
<td>$2.4 \times 10^{-3}$</td>
<td>70</td>
<td>$3.6 \times 10^{-4}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pDC251</td>
<td>-</td>
<td>-</td>
<td>$1.6 \times 10^{-2}$</td>
<td>-</td>
</tr>
<tr>
<td>DC336</td>
<td>pDC2504</td>
<td>$4.1 \times 10^{-1}$</td>
<td>99</td>
<td>$5.3 \times 10^{-3}$</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>pDC2505</td>
<td>$7.7 \times 10^{-1}$</td>
<td>0</td>
<td>$2.7 \times 10^{-5}$</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>pDC2510</td>
<td>$3.5 \times 10^{-1}$</td>
<td>90</td>
<td>$3.0 \times 10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pDC2511</td>
<td>$4.0 \times 10^{-1}$</td>
<td>100</td>
<td>$1.3 \times 10^{-4}$</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>pDC2514</td>
<td>$3.4 \times 10^{-1}$</td>
<td>94</td>
<td>$7.3 \times 10^{-4}$</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>pDC251</td>
<td>-</td>
<td>-</td>
<td>$1.8 \times 10^{-2}$</td>
<td>-</td>
</tr>
</tbody>
</table>

* Transconjugants per input donor cell.

Fig. 2. Agarose gel electrophoresis of DNA extracted from E. stewartii strains (b) DC283, (c) DC283-443(pDC2504), (d) DC336(pDC25106), (e) DC336(pDC2501, Mu), (f) DC336, (h) DC283(pDC2512AMu), (i) DC283(pDC2516AMu), and (j) DC283 and from Esch. coli strains (a) MH2855(pDC2504) and (g) DC921(pDC251).
Mu pf7701 in Erwinia stewartii

stewartii DC283 and DC336 (a Mu cts62 lysogen of DC283); representative transfer frequencies and linkages between the selected and unselected markers for five strains are presented in Table 2. All of the Mu-hybrid plasmids transferred into E. stewartii at much lower frequencies than did their parent plasmid pDC251. In general, KmR and TcR were linked even though the frequency of KmR transfer was higher than that for TcR. We believe that this discrepancy was due to initially poor expression of TcR in the transconjugants. The lysogenic strain DC336 was a better recipient indicating that zygotic induction occurred in the DC283 transconjugants. Similar transfer frequencies for KmR and TcR were obtained using E. stewartii strains SW211 NalR and SW2 NaI.

In the absence of antibiotic selection, DC283(pDC251::Mu pf7701) strains from all of the matings were unstable and readily lost one or both of the drug resistances. Maintenance on selective media eventually produced stable strains expressing one or both markers. Stable transconjugants of each phenotype (i.e. KmR TcR, KmS TcR and KmR TcS) were further examined to determine if pDC251::Mu pf7701 had integrated into the chromosome or another plasmid, or if either Tn10 or Mu pf7701 had transposed to another replicon.

Thirty stable KmR TcR DC283 strains were obtained from matings with nine of the pDC251::Mu pf7701 plasmids by selecting transconjugants for KmR followed by maintenance on both antibiotics. AGE of plasmid DNA showed that 20 of the stable strains harboured an autonomous Mu-hybrid plasmid but had lost the 34 kb cryptic plasmid, pDC140 (e.g. DC283-443(pDC2504), Fig. 2). To distinguish whether the stability of such strains had arisen from a change in the plasmid or in the host cell, we transferred pDC2504 from DC283-443 to Esch. coli MH2855, and then reintroduced it into DC283. The plasmid was still unstable indicating that a change in the host cell was probably responsible for the stability of the plasmid in DC283-443. In six other TcR KmR stable strains, the pDC251::Mu pf7701 plasmid had apparently integrated into the chromosome, since these strains did not exhibit any new plasmids on agarose gels and still had pDC140. Furthermore, they were still able to transfer KmR, and in some cases TcR, to DC283 RifR and MH2855 at frequencies of \(10^{-6}\) per donor cell for KmR and \(<10^{-7}\) for TcR; they lost drug resistance markers after storage in glycerol at -4 °C, which suggests excision of an integrated plasmid.

Another four of the KmR TcR stable strains contained plasmids in which all or part of the Mu pf7701 prophage had been deleted. After transfer to MH2855, the plasmids were compared with pDC251 by digestion with EcoRI and HindIII. In three of these four strains, Mu pf7701 appeared to have excised almost precisely from pDC251 (e.g. pDC2501AMu, Fig. 2) and pDC2511. The restriction fragments of pDC251 that Mu pf7701 had originally inserted into were again present in these deletion plasmids and were their original size. In these three strains Mu pf7701 apparently both excised from the plasmid and transposed to the chromosome. The plasmid in the fourth strain, designated pDC25106, had a 26 kb deletion (Fig. 2). The deletion spanned the 20-7 and 12-6 kb HindIII fragments of pDC2510, which are comprised of 29 kb from the variable end of Mu pf7701, 2-0 kb of pDC250 and 2-3 kb of Tn10. pDC25106 transferred at normal frequencies in Esch. coli (0.7 per donor cell for both markers) and was stable.

To determine if the pDC251::Mu pf7701 plasmids might be useful vectors for Tn10 mutagenesis, we examined the TcR KmR class of stable DC283 transconjugants to determine if they could still donate TcR. Ability to donate TcR would indicate that Tn10 was still located on a plasmid and that transposition of Tn10 to the chromosome followed by loss of the plasmid had not occurred. TcR KmS transconjugants were obtained from matings with pDC2501, pDC2504, pDC2506, pDC2507, pDC2509, pDC2511, pDC2512, pDC2514, pDC2516 and pDC2518. (TcR KmS transconjugants were not obtained with pDC2502, pDC2505, pDC2510 and pDC2519.) The transconjugants were patch mated with E. stewartii DC356 and 32 out of 346 were Tra-. These came from matings with pDC2506 (14/27), pDC2516 (9/43), pDC2512 (3/17), pDC2518 (1/33), pDC2514 (4/25) and pDC2504 (1/44). When analysed by AGE 29 of 32 Tra- strains contained a plasmid about the same size as pDC251 (e.g. pDC2512AMu and pDC2516AMu, Fig. 2) and were missing pDC140. Only three strains (one each from matings with pDC2506, pDC2504 and pDC2518) did not have any new plasmids and retained pDC140.
The latter may have been formed by transposition of Tn10 to the chromosome followed by loss of pDC251::Mu pf7701.

Km\(^R\) Tc\(^S\) transconjugants were readily obtained by selection on Km. When MH2855(pDC2504) was mated with DC283, most of the transconjugants formed small, pale yellow colonies on L agar with Km, but 3 to 12\% had the normal, large, gold colony morphology. A highly variable proportion of the small colonies were Tc\(^R\) (14 to 100\%) but consistently greater than 96\% of the colonies that appeared normal were Tc\(^S\). No changes in the plasmid content of the Km\(^R\) Tc\(^S\) transconjugants were detected by AGE. Most of these transconjugants arose by transposition of Mu pf7701 into the chromosome with subsequent loss of pDC2504. The frequency of this transposition event was apparently increased by zygotic induction since only 0.5\% of the transconjugants had normal colony type when a Mu lysogen, DC336, was used as the recipient.

Isolation of avirulent mutants

The finding that pDC2504 transconjugants selected for Km\(^R\) and normal colony type usually receive only Mu pf7701 from the donor suggested that this would be a good strategy for isolating transposon-induced mutants in E. stewartii. In a preliminary experiment, we measured the frequency of 2-deoxygalactose-resistant mutants among the transconjugants from a mating between MH2855(pDC2504) and DC283. The frequency of spontaneous 2-deoxygalactose-resistant mutants in a control culture of DC283 was 1.5 \( \times \) 10\(^{-6}\) and among Km\(^R\) transconjugants the frequency was 2.1 \( \times \) 10\(^{-4}\), an increase of 140-fold.

To isolate avirulent mutants, MH2855(pDC2504) was mated with DC283 and transconjugants were selected for Km\(^R\) Nal\(^R\). Normal sized colonies (4-8\% of the transconjugants) were inoculated into sweet corn seedlings by wounding with toothpicks. Initial inoculations indicated that 14 of the 1318 transconjugants tested (about 1\%) were weakly virulent and these were subsequently retested by the whorl inoculation technique. Eight mutants that could not cause watersoaking were found; two of these were impaired in the synthesis of extracellular polysaccharide and formed butyrous colonies on L agar with 1\% (w/v) glucose added. Examination of the plasmid profiles of the mutants did not reveal any differences when compared to their parent, DC283. The 318 kb plasmid, however, was not extracted by the plasmid isolation method used. In a virulent transconjugant which was also screened, Mu pf7701 had inserted into the 103 kb cryptic plasmid (pDC192).

To determine whether the mutagenesis procedure was producing single or multiple insertion events, we hybridized pKN54 (the left-end PstI fragment of Mu cloned into pBR322) to blots of HindIII-digested genomic DNA from nine weakly virulent mutants. For each copy of Mu pf7701 we expected only one fragment, representing the left junction, to hybridize. Instead, pBR322 by itself hybridized strongly to two fragments in every strain and weakly to several other fragments. By comparing the same blot probed first with pBR322 and then with pKN54 we were able to indentify junction fragments in eight strains. All strains contained only one Mu pf7701 insertion except for MU141, which had two. The missing junction fragment in MU5136 was probably masked by the pBR322 background since we have confirmed that this strain is a Mu pf7701 lysogen.

DISCUSSION

In our attempts to develop a Mu-mediated system for gene exchange in E. stewartii, we discovered that pDC251::Mu pf7701 plasmids were unstable when reintroduced into E. stewartii and therefore, could be used to generate Mu pf7701 insertion mutants. The results of this study indicate that zygotic induction of the Mu pf7701 prophage occurred following plasmid transfer and frequently resulted in single transpositions of the phage, and subsequent loss of the plasmid. Suspected insertion mutants were identified by colony size and subsequently screened for virulence. We found that about 1\% of these were unable to cause watersoaking in young corn seedlings. The effectiveness of this system for mutagenesis is probably enhanced by the kil mutation of Mu pf7701, which reduces host cell killing following infection or induction.
Mu pf7701 in Erwinia stewartii

The Km\(^R\) gene of Mu pf7701 should allow future conjugational mapping of the insertions in E. stewartii. Since the host range of pDC251 includes many genera in the Enterobacteriaceae (Coplin et al., 1985), this system may be useful for introducing Mu pf7701 into other enteric bacteria which are not Mu-sensitive. In contrast, pDC251 ::Mu pf7701 plasmids will probably not function as vectors for Tn10 mutagenesis.

The reason for the instability of pDC251 ::Mu pf7701 plasmids in E. stewartii is unclear because pDC251 is an indigenous plasmid that is stable in DC283, and Mu-hybrid plasmids per se are not unstable in E. stewartii (Coplin, 1979). Following introduction of pDC251 ::Mu pf7701, stable strains arose by either of two events: integration of pDC251 ::Mu pf7701 into the chromosome, or loss of all or part of Mu pf7701 from the hybrid plasmid. Casey et al. (1983) concluded that it is the presence of Mu on IncP plasmids which makes them unstable in Rhizobium. They observed, as we did, that deletion of Mu sequences occurred when the plasmids became successfully established in transconjugants. As cited above, Mu also makes IncP plasmids unstable in some Erwinia sp. Thus, an explanation for the instability of pDC250 ::Mu pf7701 plasmids may be that a Mu function interferes with pDC250 replication in a manner that is independent of the site of insertion.

Although pDC251 ::Mu pf7701 plasmids were unstable in E. stewartii strain SW211, which does not harbour pDC250 or pDC140 (Coplin et al., 1985), incompatibility with pDC140 may have further increased their instability in SS104. We have shown that pDC140 is closely related to pDC250 and have suggested that pDC250 may have two origins of replication, since incompatibility between the two plasmids is exhibited by instability of pDC140 but not of pDC250 (Coplin et al., 1985). Addition of Mu to pDC251 may have affected replication from one origin so that it became completely incompatible with pDC140.

The high frequency of excision of Mu pf7701 that we observed is unusual. Excision has only been observed for X mutants of Mu which have been shown to have polar insertions in the B gene (Bukhari & Taylor, 1975). The Tn5 insertion in Mu pf7701 is at 4.4 kb and just outside of the B gene, but it could be close enough to exert a partial anti-polar effect on the activity of B so that Mu pf7701 is phenotypically an X mutant. This trait may permit reversion studies on Mu with pDC251-induced mutants. When the Mu excises it occasionally causes adjacent deletions and inversions. This could explain the Tra\(^{−}\) plasmids produced by loss of Mu pf7701 from pDC2506, pDC2516, pDC2512 and pDC2514. The Mu pf7701 insertions in these plasmids mapped together, so that excision could result in loss of transfer ability if the insertions happened to be located next to a Tra gene(s).

All strains of E. stewartii harbour many large cryptic plasmids. Strain DC283, used in this study, contains 11 plasmids; together they constitute 20 to 25% of the genome. Due to the abundance of plasmids in this species, we have speculated that some of them may be determinants of pathogenicity. With the possible exception of the 318 kb plasmid, insertions of Mu pf7701 into any of the cryptic plasmids would have been easily detected in gels had they occurred. We, therefore, conclude that many of the genes affecting virulence are probably chromosomal rather than plasmid-borne.

We are grateful to Dr Martha Howe for cultures and helpful advice and to Reid Frederick and Laura Mihuta for technical assistance. Salaries and research support were provided by the Science and Education Administration of the US Department of Agriculture under Grant No. 59-2392-1-1-694-0 from the Competitive Research Grants Office and by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal Article No. 65-85.

REFERENCES


insertions on packaging of host sequences covalently linked to bacteriophage Mu DNA. *Proceedings of the National Academy of Sciences of the United States of America* 72, 4399-4403.


