Integration of the Vwa Plasmid into the Chromosome of *Yersinia pestis* Strains Harboring F′ Plasmids of *Escherichia coli*

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Conditional virulent strains of *Yersinia pestis* were used as recipients of an F-lac plasmid from either *Escherichia coli* CSH23. The transconjugants of *Y. pestis* were calcium independent; however, calcium dependence was restored after the loss of the *E. coli* plasmid. The plasmid contents of several *Y. pestis* F-lac clones were compared with those of the parent strains and several lac segregants. Although the 6- and 61-megadalton plasmids were detected in all strains tested, the Vwa plasmid was absent in the F-containing strains. The plasmid was evident, however, in the lac segregants derived from the F-lac clones. Results of Southern transfers indicated that the Vwa plasmid had integrated into the chromosome of *Y. pestis* strains harboring F-lac.

The virulence of *Yersinia pestis* depends on at least five distinct determinants (4). These determinants include the biosynthesis of endogenous purines; the production of V and W antigens, pesticin, and fraction I (a capsular antigen); and the adsorption of hemin to form pigmented colonies.

Virulent strains of *Y. pestis* also require calcium for growth at 37°C (11). Calcium dependence has been shown to be encoded in one of three indigenous plasmids of *Y. pestis* (2, 10, 18). The need for calcium is linked with a plasmid of molecular weight 45 to 47 megadaltons (Mdal) (10, 18), and the plasmid has been designated Vwa since it contains the genetic information necessary for the synthesis of the V and W antigens (2, 18). Specific alterations in the Vwa plasmid are known to affect the calcium requirement of *Y. pestis*, but such alterations do not necessarily prevent the expression of the V and W antigens (18). Thus, calcium dependence and V antigen production are separable traits (5, 17, 18, 21).

The virulence of *Y. pestis* for mice is obliterated in strains of *Y. pestis* that harbor an F′lac from *Escherichia coli* CSH23 (21). Although the lactose-fermenting strains of *Y. pestis* were shown to retain all of their virulence traits, virulence was attributed to calcium independence. Virulence was restored, however, after the presumed loss of the *E. coli* plasmid.

In this study, we compared the plasmid contents of several *Y. pestis* F′lac strains with those of the parent strains and the spontaneous lac segregants derived from the F′lac clones. Although the 6- and 61-Mdal plasmids were detected in all strains tested, the Vwa plasmid was not evident in any of the lactose-fermenting strains of *Y. pestis*. The Vwa plasmid was detected in such strains, however, after the loss of the *E. coli* plasmid. Results of Southern blots (20) indicated that the Vwa plasmid had integrated into the chromosome of *Y. pestis* strains harboring F′lac.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. *Y. pestis* EV76 and EV76 51F were employed as recipients of an F-lac plasmid from either *E. coli* CSH23. The mating conditions and the medium used for the isolation of lactose-fermenting transconjugants of EV76 51F were as described previously (21). The medium used for the selection of F-lac-containing strains of EV76 was essentially M9SFM6 (6), consisting of the following ingredients (per liter): K₂HPO₄, 10.5 g; KH₂PO₄, 4.5 g; (NH₄)₂SO₄, 1.0 g; sodium citrate, 0.5 g; MgSO₄ · 7H₂O, 0.1 g; Na₂S₂O₃, 0.1 g; glycine, 1.0 mM; DL-isoleucine, 0.5 mM; DL-valine, 0.2 mM; L-cysteine, 0.2 mM; DL-methionine, 0.2 mM; DL-phenylalanine, 0.2 mM; lactose, 2.0 g; agar, 15.0 g (pH 7.2). All strains were grown in heart infusion broth (Difco Laboratories, Detroit, Mich.) with aeration at 26°C (*Y. pestis*) or 37°C (*E. coli*) overnight.

**Plasmid isolation and agarose gel electrophoresis.** Plasmid DNA was isolated for agarose gel electrophoresis, using the lysozyme-sodium dodecyl sulfate procedure described by Crosa and Falkow (8). Plasmid DNA was loaded onto a vertical 0.8% agarose gel (12 cm by 14 cm by 3 mm), and electrophoresis was performed either for 2 or 3 h at 26°C in a Tris-acetate buffer (0.04 M Tris, 0.02 M sodium citrate, 2 mM EDTA (pH 7.8)). Gels were stained with ethidium bromide (1 µg/ml) and viewed with a transilluminator.

**Isolation and 32P labeling of probe DNA.** Plasmid DNA was isolated from *Y. pestis* 019 which contained only the Vwa plasmid. A modification of the procedure described by Birnboim and Doly (3) was employed and consisted of the following. Heart infusion broth (500 ml) in a 2.5-liter Fernbach flask was heavily seeded, and the flask was incubated overnight at 26°C with aeration. After centrifugation at 8,000 × g for 20 min at 5°C, the cells were suspended in 10 ml of buffer containing 50 mM glucose, 25 mM Tris, and 10 mM EDTA (pH 8.0) and were lysed by the addition of 80 ml of freshly prepared 0.2 M NaOH containing 1% sodium dodecyl sulfate. The mixture was placed on ice for 10 min, and 40 ml of precooled (4°C) 5 M potassium acetate (pH 4.8) was slowly added with gentle mixing. The lysate was then centrifuged at 4,000 × g for 20 min at 4°C. The supernatant fluid was filtered through cheesecloth, and 0.6 volume of isopropanol was added. The solution was mixed gently and then remained undisturbed at room temperature for 15 min. The plasmid DNA was pelleted at 9,000 × g for 20 min at room temperature. The supernatant fluid was discarded, and the pellet was washed once by layering with a solution of 75
TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>F&lt;sup&gt;-&lt;/sup&gt; plasmid content</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. pestis EV 76</td>
<td>+</td>
<td>None</td>
<td>R. Brubaker</td>
</tr>
<tr>
<td>Y. pestis EV 76/2</td>
<td>-</td>
<td>pCSH23</td>
<td>This study</td>
</tr>
<tr>
<td>Y. pestis EV 76/3</td>
<td>-</td>
<td>p23.10S</td>
<td>This study</td>
</tr>
<tr>
<td>Y. pestis EV 76 51F</td>
<td>+</td>
<td>None</td>
<td>D. Cavanaugh</td>
</tr>
<tr>
<td>Y. pestis EV 76 52F</td>
<td>-</td>
<td>pCSH23</td>
<td>This study</td>
</tr>
<tr>
<td>Y. pestis EV 76 53F</td>
<td>-</td>
<td>p23.10S</td>
<td>This study</td>
</tr>
<tr>
<td>Y. pestis 019</td>
<td>+</td>
<td>None</td>
<td>S. Straley</td>
</tr>
<tr>
<td>E. coli 23 10S</td>
<td>+</td>
<td>p23.10S</td>
<td>W. Lawton</td>
</tr>
<tr>
<td>E. coli CSH 23</td>
<td>-</td>
<td>pCSH23</td>
<td>C. Thorne</td>
</tr>
</tbody>
</table>

* Cad, Calcium dependence; Lac, lactose utilization.

ml of 95% ethanol plus 25 ml of Tris buffer (100 mM Tris, 1 mM EDTA [pH 8.0]). The ethanol wash was discarded, and the pelleted DNA was dried under vacuum. The pellet was redissolved in 7 ml of TE buffer (8) for 24 h at room temperature.

The solution of plasmid DNA was added to a tube containing 9 g of CsCl in 2 ml of distilled water, and the tube was gently inverted to dissolve the CsCl. Upon dissolution, 0.96 ml of ethidium bromide (10 mg/ml) was added. The mixture was centrifuged at 183,000 × g in a Beckman 50 Ti rotor for 40 h at 20°C. The lower plasmid band was recovered, and the fraction was extracted several times with an equal volume of isoamyl alcohol. The plasmid fraction was dialyzed extensively against standard saline-citrate (pH 8.0).

The purity of the Vwa plasmid was tested by restriction endonuclease analysis. The plasmid DNA was treated with BamHI according to the manufacturer’s specifications (Bethesda Research Laboratories, Bethesda, Md.), and the restriction segments were analyzed by electrophoresis (50 V, 5 h, Tris-acetate buffer, pH 7.8) in 0.8% agarose gels. The size of each fragment generated with BamHI was identical to that reported by Portnoy and Falkow (18), except that two bands, rather than one as reported by Portnoy and Falkow, existed in the molecular size region of 9.5 kilobases (kb). These DNA fragments were evident, however, in a subsequent publication by Portnoy et al. (19).

The DNA was labeled in vitro with [α-32P]dCTP (New England Nuclear Corp., Boston, Mass.) by nick translation (16) to a specific activity of 7.4 × 106 cpm/μg of DNA.

Hybridization studies. Southern filter hybridization (20) and solution hybridization (7) were used to detect homologous sequences between the Vwa plasmid and plasmid extracts, and between the Vwa plasmid and cellular DNA extracted from F<sup>lac</sup> strains of Y. pestis, respectively. Before DNA transfer, the agarose gel was treated with 0.25 M HCl to ensure transfer of high-molecular-weight DNA to Trans-Blot membranes (Bio-Rad Laboratories, Richmond, Calif.). The methods employed in Southern transfers, DNA hybridizations, and visualization were as described by Maniatis et al. (15).

RESULTS

Calcium independence of Y. pestis strains harboring F<sup>lac</sup>. Transconjugants of Y. pestis were isolated and purified by several passages on complete indicator medium (21). The clones were initially unstable, and lac segregants appeared at a high frequency; however, the stability of each clone was increased with each successive passage.

The parent strains of Y. pestis (EV76 and EV76 51F) were calcium dependent (Table 2). The plating efficiency of these strains on magnesium oxalate medium (12), on the average, was less than 8.3 × 10<sup>-4</sup> under restrictive conditions (37°C) of growth. On the other hand, the plating efficiency of strains EV76 and EV76 51F that harbored an F<sup>lac</sup> plasmid from either strain of E. coli was on the order of 0.8. Thus, most of the F<sup>lac</sup> cells were calcium independent when tested on magnesium oxalate medium.

Within every lac<sup>+</sup> clone of Y. pestis, a number (0.1 to 40%) of progeny arose that appeared to have lost the E. coli plasmid, as judged by their phenotype on complete indicator medium and the restoration of their calcium requirement (Table 2). The loss of F from these cells was confirmed when we showed that the lac segregants supported the replication of phage T7, whereas the F-containing strains of Y. pestis inhibited completely the formation of plaques (data not shown). The inhibition of growth of T7 by F-containing strains of E. coli has been described previously (14).

Absence of the Vwa plasmid in lac<sup>-</sup> Y. pestis. The gene(s) governing calcium dependence are plasmid encoded (2, 10, 18). Calcium-independent clones of Y. pestis have been described in which the Vwa plasmid was altered either by an insertion of an IS element or by deletion of bases (18). We tested for similar alterations in the Vwa plasmid of F<sup>lac</sup> clones by subjecting plasmid extracts to agarose gel electrophoresis. The Vwa plasmid associated with calcium dependence was not apparent in the Y. pestis strains harboring F<sup>lac</sup> but was readily apparent in both the parental strain and the lac segregants derived from the F<sup>lac</sup> clones (Fig. 1). Although the F<sup>+</sup> plasmids were not detected in this preparation, the E. coli plasmids were seen in most preparations used in subsequent experiments after electrophoresis for 3 h (Fig. 2).

The only alterations detected in the plasmid content of the F<sup>lac</sup> clones were noted with EV 76/3 (Fig. 2). An insertion in the 61-Mdal plasmid and a deletion in the plasmid of E. coli 23.10S was observed. The nature of these alterations is unknown at this time.

DNA hybridizations. The agarose gel shown in Fig. 2 served for the transfer of plasmid DNA and chromosomal DNA to nitrocellulose filters by the Southern method (20). The Vwa probe was then used for detection of homologous sequences in the DNA bound to the nitrocellulose filter. The Vwa probe with the E. coli plasmids showed no homology (Fig. 2). Likewise, little or no homology was

TABLE 2. Loss of calcium dependence and segregation frequency of Y. pestis strains harboring an F<sup>+</sup> lac

<table>
<thead>
<tr>
<th>Y. pestis strain</th>
<th>Growth on MgOx medium (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio of lac segregants</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV 76</td>
<td>&lt;0.09</td>
<td>0.20</td>
</tr>
<tr>
<td>EV 76/2</td>
<td>33</td>
<td>0.20</td>
</tr>
<tr>
<td>EV 76/2 segregant</td>
<td>&lt;0.08</td>
<td>0.41</td>
</tr>
<tr>
<td>EV 76/3</td>
<td>81</td>
<td>0.41</td>
</tr>
<tr>
<td>EV 76/3 segregant</td>
<td>&lt;0.03</td>
<td>0.41</td>
</tr>
<tr>
<td>EV 76 51F</td>
<td>&lt;0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>EV 76 52F</td>
<td>100</td>
<td>0.06</td>
</tr>
<tr>
<td>EV 76 52F segregant</td>
<td>&lt;0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>EV 76 53F</td>
<td>90</td>
<td>0.04</td>
</tr>
<tr>
<td>EV 76 53F segregant</td>
<td>&lt;0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent growth = colony-forming units at 37°C/colony-forming units at 26°C. The range of colony-forming units at 26°C was 1.1 × 10<sup>9</sup> to 4.7 × 10<sup>9</sup> per ml. MgOx, Magnesium oxalate.
evident between the probe and the 61-Mdal plasmid. Considerable homology existed, however, between the probe and the Vwa plasmid, the 6-Mdal plasmid, and the chromosomal fraction of Y. pestis, indicating that the Vwa plasmid had integrated into the Y. pestis chromosome. The presence of homologous sequences in these strains was confirmed by liquid hybridization studies with DNA extracts of whole cells.

The nature of the integration site of the Vwa plasmid was investigated by restriction endonuclease analysis. DNA extracts of whole cells were treated with a 10-fold excess of Sall to ensure adequate digestion, and the restriction fragments were separated on an 0.8% agarose gel at 40 V for 8 h. In this experiment, the agarose gel was not pretreated with HCl before transfer of the DNA to nitrocellulose filters, since many fragments were less than 10 kb.

A minimum of seven Sall restriction fragments were found to share homology with the Vwa plasmid in every DNA extract examined (Fig. 3). Fragments ranging in size from 5.2 to 10.8 kb were common to both the F-containing strains of Y. pestis and EV 76 51F. A novel 11.2-kb fragment was observed, however, with the F-lac strains and most likely represented one of the junctions between chromosome and the Vwa plasmid. The 9-kb fragment was associated with the 6-Mdal plasmid after digestion of plasmid extracts with Sall.

DISCUSSION

The results of this study show that the loss of calcium dependence in strains of Y. pestis harboring an F' plasmid from either E. coli 23 10S or E. coli CSH 23 accompanied the physical integration of the Vwa plasmid into the bacterial chromosome. We do not know whether these cellular alterations were caused by separate, independent events or by a common mechanism.

The F' lac of E. coli 23 10S is small relative to other F' lac plasmids, and the only chromosomal markers presumably associated with F are the genes governing lactose utilization. This information, coupled with previous findings with Folac (21), argues that a gene(s) contained in F is influencing the autonomous nature of the Vwa plasmid.

The virulence (Vwa) plasmid of Yersinia enterocolitica has been reported to be incompatible with F (R. Bakour and G. Cornelis, Abstr. Int. Congr. Microbiol. 13th, Boston, Mass., abstr. no. P267, p. 97, 1982). When alone, the F' lac plasmid segregated at a probability of 0.02 per cell generation; however, in the presence of the Vwa plasmid, the segregation probability increased to 0.95. Recently, Bakour et al. (1) have shown that the incompatibility observed between F and the Vwa plasmids of Y. enterocolitica and Y. pseudotuberculosis is mediated by the incD locus of F.

Similarly, in our experiments, the frequency with which lac segregants initially arose from the lac+ transconjugants of Y. pestis suggested that the Vwa plasmid was either partially or completely incompatible with F. After several passages on complete indicator medium, however, relatively stable clones were selected that most likely contained the Vwa plasmid in the integrated state and thus permitted the F' plasmid to coexist in cells containing the Vwa plasmid.

DeVries and Maas (9) have shown that an F' plasmid originating in a mutant Hfr strain of E. coli expresses incompatibility in the autonomous but not in the integrated state. Hillenbrand et al. (13) characterized the altered plasmid and suggested that the Inc'' mutation resulted from an insertional inactivation of the incC locus by a transposon carried by F. The resulting plasmid was postulated to have increased copy numbers when in the autonomous state and

![FIG. 1. Plasmid content of Y. pestis EV 76 51F, EV 76 52F, and several lac segregants derived from EV 76 52F. Lanes: 1 and 3, EV 76 51F; 2 and 4, EV 76 52F; 5 to 8, lac segregants; 9, E. coli plasmids (standards) of 62, 34, 25, and 5.5 Mdal. Arrow indicates the position of the Vwa plasmid.](image)

![FIG. 2. (A) Plasmid profiles of Y. pestis EV 76 and EV 76 51F containing F-lac plasmids from E. coli 23 10S and E. coli CSH23. Lanes: 1, EV 76 2; and 3, EV 76 52F; 4, EV 76 53F; 5, EV 76 2; 6, EV 76 3. (B) Distribution of DNA homology between Vwa plasmid DNA and the DNA associated with plasmid extracts of Y. pestis F'lac. 32P-plasmid DNA from Y. pestis 019 was hybridized to nitrocellulose filters which contained the DNA bands shown in A. The position of the Vwa plasmid is indicated by arrows.](image)
displayed an Inc<sup>+</sup> due to a gene dosage effect of the inc<sub>B</sub> function. Thus, it is possible that fewer copies of the Vwa plasmid existed in the integrated state as compared with the autonomous state.

The initial hybridization studies described here showed that the Vwa plasmid had integrated into the chromosome of <i>Y. pestis</i> strains containing F<sub>lac</sub>, since homologous sequences were widely distributed in the chromosomal fraction of these strains. The presence of homologous sequences in the chromosomal fraction from the wild-type strain was attributed to breakage of some of the Vwa plasmids on extraction, resulting in their conigration with chromosomal DNA.

Southern hybridizations of <i>Sall</i>-cleaved, whole <i>Yersinia</i> DNA indicated that a specific integration site for the Vwa plasmid existed in the <i>Y. pestis</i> chromosome, since a novel 11.2-Kb fragment was common to every F<sub>lac</sub>-isolate tested. Undoubtedly, this fragment represented one of the junctions between plasmid and chromosome. We were unable to resolve the second junction between plasmid and chromosome due to its large estimated size of 50 kb or more.

Treatment of the Vwa plasmid with <i>Sall</i> produces two fragments (18). We estimated these fragments to be 10.8 and 61.2 kb. Since a fragment of ca. 10.8 kb was observed in Southern blots, we assume that the integration of the Vwa plasmid occurred outside the 10.8-kb region.

The observation that the 6-Mdal plasmid shared homologous sequences with the Vwa plasmid but not with the 61-Mdal plasmid suggested either that the two plasmids originated from a common progenitor or that one of the plasmids was derived from the other.

Portnoy and Falkow (18) have found an insertion sequence (IS100) responsible for deletions and insertions in the Vwa plasmid of <i>Y. pestis</i> which affects calcium dependence. IS100 was found to occupy ca. 10 sites on the bacterial chromosome. Although the mechanism by which the F<sub>lac</sub> plasmid relieves the calcium requirement remains unknown, insertion sequence-mediated replicon fusion, in conjunction with plasmid incompatibility, remains a plausible explanation and might indicate a degree of relatedness between plasmids of the two genera.

ACKNOWLEDGMENTS

We thank Alfred Donroe and Neil Malone for providing the procedure for the large-scale isolation of plasmid DNA, and Barbara Bachmann for information regarding the F<sup>+</sup> plasmid of <i>E. coli</i> 23.105. This work was supported in part by grant 5320 from the Central University Research Fund of the University of New Hampshire to K.Z.

LITERATURE CITED