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# Virulence-Associated Plasmids from *Yersinia enterocolitica* and *Yersinia pestis*

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A 44-megadalton plasmid associated with virulence and $Ca^{2+}$ dependence from *Yersinia enterocolitica* 8081 was compared at the molecular level with a 47-megadalton plasmid associated with $Ca^{2+}$ dependence from *Yersinia pestis* EV76. The plasmids were found to share 55% deoxyribonucleic acid sequence homology distributed over approximately 80% of the plasmid genomes. One region in which the plasmids differed was found to contain sequences concerned with essential plasmid functions. Forty-five mutants of *Y. pestis* were isolated which had spontaneously acquired the ability to grow on calcium-free medium ($Ca^{2+}$ independence). Of these mutants, 21 were cured of their 47-megadalton plasmid, whereas the remaining had either suffered a major deletion of the plasmid or had a 2.2-kilobase insertion located in one of two adjacent *Bam*HI restriction fragments encompassing approximately 9 kilobases. The inserted sequence was found at numerous sites on the *Y. pestis* chromosome and on all three plasmids in the strain and may represent a *Y. pestis* insertion sequence element.

*Yersinia pestis* and some strains of *Yersinia enterocolitica* are capable of causing an invasive disease in humans and other mammals (3, 6). Although these organisms are distinct species sharing approximately 50% DNA sequence homology (2, 5), they show similarities in their pathogenesis of disease. *Y. pestis* has been shown to survive and multiply within phagocytic cells (15), whereas invasive strains of *Y. enterocolitica* can penetrate and survive within epithelial cells (10, 17, 26). Both also exhibit $Ca^{2+}$-dependent growth in vitro, a property which is rare among procaryotes (23). Virulent strains of *Y. pestis* have an absolute growth requirement for calcium at 37°C, but not at 25°C ($Ca^{2+}$ dependent). In the absence of calcium cells cease to divide within a few generations (7). Some strains of *Y. enterocolitica* also exhibit $Ca^{2+}$ dependence, although the requirement for calcium is less stringent, resulting only in a reduced growth rate during growth in calcium-deficient medium at 37°C (9). Mutants of both organisms which no longer exhibit $Ca^{2+}$ dependence are avirulent (6, 9, 12). It has been speculated that $Ca^{2+}$ dependence may reflect the ability of yersiniae to respond to their intracellular environment, which is low in free calcium (7).

Recently, it has been demonstrated that virulent members of the yersiniae harbor plasmids of approximately 40 megadaltons (Mdal) that are associated with virulence and expression of $Ca^{2+}$ dependence (1, 11-13, 21, 28). Loss of this plasmid results in $Ca^{2+}$ independence and avirulence. This suggests a similarity among the plasmids from the yersiniae, but DNA homology among the plasmids has not been previously established. We have shown that, in pathogenic strains of *Y. enterocolitica* from around the world, there exists a family of related plasmids (21). In all of these strains, isolation of $Ca^{2+}$-independent variants was associated with avirulence and loss of the plasmid. However, the precise role of the plasmid in pathogenesis remains to be determined.

In the present study, we examined the relatedness between a plasmid from *Y. pestis* and one from *Y. enterocolitica*. We also characterized plasmid DNA from $Ca^{2+}$-independent mutants of *Y. pestis* and found that, whereas some had lost a 47-Mdal plasmid, many had suffered deletion of and insertions into the plasmid within a specific region of the plasmid genome.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains used in these studies are listed in Table 1. Bacteria were grown on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) at 25°C. Stock cultures were kept as suspensions of cells at −20°C in 50% glycerol.

**Plasmid DNA isolation.** Plasmid DNA was iso-

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TABLE 1. *Strains and plasmids*

| Strain | $Ca^{2+}$ dependence | Plasmid size (Mdal)[a] | Plasmid designation[b] | Source or reference |
|---|---|---|---|---|
| *Y. enterocolitica* 8081 (0:8) | + | *44* | pYV8081 | 21 |
| *Y. enterocolitica* 80811 | − | | | This study |
| *Y. pestis* 019[c] | + | *47* | pYV019 | |
| *Y. pestis* EV76[d] | + | *47*; 61; 6 | pYV76 | |
| *Y. pestis* EV766 | − | *61*; 6 | | This study |
| *Y. pestis* EV768 | − | *13*; 61; 6 | pYV76Δ8 | This study |
| *Y. pestis* EV7614 | − | *5*; 61; 6 | pYV76Δ14 | This study |
| *Y. pestis* EV7618 | − | *16*; 61; 6 | pYV76Δ18 | This study |
| *Y. pestis* EV7621 | − | *2.5*; 61; 6 | pYV76Δ21 | This study |
| *Y. pestis* EV7627 | − | *48.5*; 61; 6 | pYV76::IS100 | This study |
| *Y. pestis* EV7651 | − | *48.5*; 61; 6 | pYV76::IS100 | This study |

[a] Plasmid size was determined by restriction analysis of *Bam*HI-digested DNA.

[b] Plasmid designation refers to the italicized plasmid.

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lated as previously described (21), with the following modifications. For plasmid screening, approximately $5 \times 10^9$ cells were harvested from a brain heart infusion agar plate. For large-scale plasmid isolation, the cells were harvested from confluent lawns from two or three brain heart infusion agar plates.

**Solution hybridization.** Cellular DNA was isolated by the method of Brenner et al. (4). The purified DNA was dialyzed against 0.42 M NaCl–0.5 mM EDTA, pH 8.0, and stored at 4°C until use. The preparation of labeled plasmid DNA, hybridization conditions, and S1 nuclease analysis were performed as previously described (21).

**Restriction digests.** The restriction endonucleases *Bam*HI, *Hin*dIII, *Sal*I, and *Xba*I were used under conditions recommended by the supplier (Bethesda Research Laboratories, Rockville, Md.). Restricted DNA (0.2 to 1.0 μg) was subjected to electrophoresis in a horizontal 0.7% agarose gel (0.4 cm thick) in a Tris-acetate buffer (0.04 Tris–0.02 M sodium acetate–0.002 M EDTA, pH 8.0), or alternatively in a vertical 0.7% agarose gel (0.25 cm thick) in a Tris-borate buffer (19).

**Preparation of $^{32}$P-labeled probe DNA.** Specific DNA restriction fragments were isolated from Tris-borate buffered agarose gels and electroeluted through 3% acrylamide into dialysis tubing. The isolated DNA was phenol extracted and ethanol precipitated. The DNA (isolated fragment or plasmid DNA) was labeled in vitro with $^{32}$P-labeled deoxyribonucleotides (New England Nuclear Corp., Boston, Mass.) by nick translation (18) to a specific activity of $10^7$ to $10^8$ cpm/μg of DNA.

**DNA filter hybridization.** After gel electrophoresis, DNA was transferred to nitrocellulose filters (0.45 μm; Schleicher and Schuell, Inc., Keene, N.H.) by the method of Southern (24). To ensure equal transfer of all sized fragments, DNA was depurinated before alkali denaturation by rocking the gel in 0.25 M HCl for 15 min at room temperature (27). The treatment of the nitrocellulose filters and hybridizations with $^{32}$P-probe DNA were performed as previously described (21). Autoradiograms were prepared by exposing the X-ray film (X-Omat-R, Eastman Kodak, Rochester, N.Y.) to the nitrocellulose filter in the presence of one intensifying screen (Cronex Lightning-Plus; E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.) for various lengths of time at −70°C.

**Physical mapping of plasmid DNA.** Plasmid DNA from *Y. pestis* 019 was mapped by analysis of *Sal*I–*Xba*I, *Sal*I–*Bam*HI, and *Xba*I–*Bam*HI double digests. The fragments produced by *Sal*I–*Xba*I double digests were isolated from an agarose gel by electroelution and subsequently digested with *Bam*HI. Mapping was also facilitated by analysis of plasmid DNA from the deletion mutants of *Y. pestis* EV76. Plasmid DNA from deletion mutants was labeled with $^{32}$P as described above and used as a probe to locate homologous sequences in Southern blots of *Bam*HI-digested plasmid DNA from *Y. pestis* 019.

**Isolation of $Ca^{2+}$-independent mutants.** $Ca^{2+}$-independent mutants were selected by streaking cells from a single colony onto magnesium oxalate agar which consisted of blood agar base (BBL Microbiology Systems, Cockeysville, Md.), 20 mM $MgCl_2$, and 20 mM sodium oxalate (14). $Ca^{2+}$-independent clones of *Y. pestis* can form colonies on the medium at 37°C, whereas $Ca^{2+}$-dependent clones cannot. $Ca^{2+}$-independent clones of *Y. enterocolitica* form large colonies on this medium, whereas $Ca^{2+}$-dependent clones are clearly smaller in size after 24 h at 37°C. To ensure that each mutant represented an independent event, one colony was randomly chosen per plate. Colonies were further purified once on magnesium oxalate and twice on brain heart infusion agar.

## RESULTS

**Relatedness of plasmid DNA from *Y. enterocolitica* and *Y. pestis*.** We (21) and others have demonstrated that pathogenic strains of *Y. enterocolitica* harbor a plasmid associated with virulence and $Ca^{2+}$-dependence (12, 28). These plasmids comprise a family of related genetic elements ranging in DNA sequence homology from about 50 to 100% (21). Recently, it has been

reported that some strains of *Y. pestis* harbor three plasmids: a small plasmid associated with the production of pesticin, a large cryptic plasmid, and a plasmid associated with $Ca^{2+}$ dependence (1, 11) (Fig. 1, lanes A through D). We determined the molecular mass of these plasmids to be 6, 61, and 47 Mdal, respectively. Since both *Y. enterocolitica* and *Y. pestis* harbor a plasmid associated with $Ca^{2+}$ dependence, it seemed likely that the plasmids shared DNA sequence homology. Therefore, we sought to determine the extent of relatedness between plasmid DNA from representative strains, namely, *Y. pestis* EV76 and *Y. enterocolitica* 8081 (Table 1). Solution hybridization was performed between plasmid DNA from *Y. enterocolitica* 8081 (pYV8081) and cellular DNA from *Y. pestis* EV76 and a derivative, EV766, which was cured of its 47-Mdal plasmid. On the average, 55% homology existed between the plasmid from *Y. enterocolitica* and cellular DNA from *Y. pestis* EV76. There was less than 10% homology with cellular DNA from the $Ca^{2+}$-independent strain EV766. Since only strains containing the 47-Mdal plasmid showed significant homology to the plasmid from *Y. enterocolitica*, it appears that the plasmid in *Y. pestis* is related to the virulence-associated plasmid in *Y. enterocolitica*.

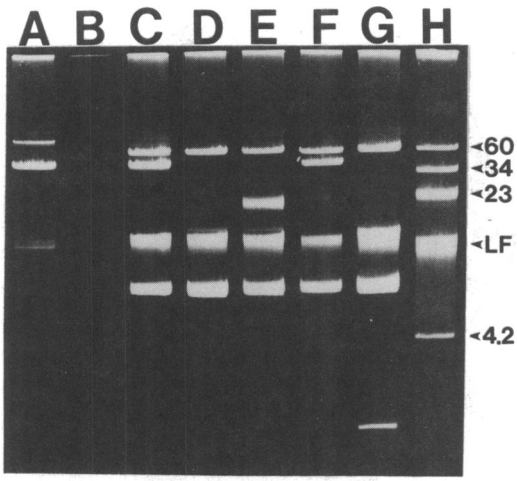


FIG. 1. *Agarose gel electrophoresis of plasmid DNA from $Ca^{2+}$-dependent and $Ca^{2+}$-independent strains of Y. enterocolitica and Y. pestis. Electrophoresis was for 2 h at 100 V. (A) Y. enterocolitica 8081; (B) Y. enterocolitica 80811; (C) Y. pestis EV76; (D) Y. pestis EV766; (E) Y. pestis EV7618; (F) Y. pestis EV7627; (G) Y. pestis EV7621; (H) molecular mass standards (ColE1, 4.2 Mdal; Sa, 23 Mdal; RP4, 34 Mdal; cryptic plasmid from Salmonella typhimurium LT2, 60 Mdal; R27, 112 Mdal; LF, linear DNA fragments).*

We examined the distribution of homology throughout the plasmid molecules by comparing the cleavage patterns of plasmid DNA after digestion with restriction endonucleases. The restriction endonuclease *Bam*HI was used because it cleaved the 47-Mdal plasmid from *Y. pestis* at least 15 times, whereas it cleaved both the 6- and the 61-Mdal plasmids only once. As expected, based on the DNA homology, the 47-Mdal plasmid from *Y. pestis* and the 44-Mdal plasmid from *Y. enterocolitica* shared some restriction bands (Fig. 2, lanes A and B). Nitrocellulose filter hybridization of radiolabeled plasmid DNA from *Y. enterocolitica* 8081 with *Bam*HI-restricted plasmid DNA from *Y. pestis* EV76 showed that DNA sequences from the *Y. enterocolitica* plasmid shared significant DNA homology with all but three (encompassing approximately 11 Mdal of DNA) of the *Bam*HI

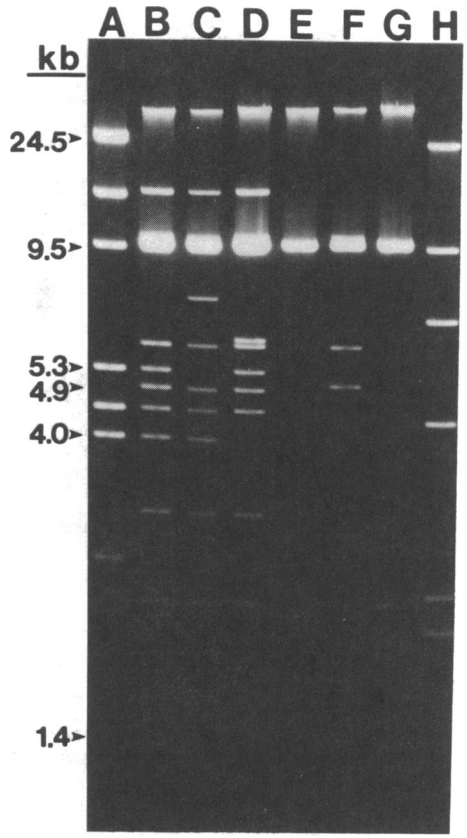


FIG. 2. *BamHI restriction endonuclease digestion patterns of plasmid DNA from Y. enterocolitica and Y. pestis. Electrophoresis was for 17 h at 50 V. (A) Y. enterocolitica 8081; (B) Y. pestis EV76; (C) EV7627; (D) EV7651; (E) EV766; (F) EV7618; (G) EV7621; (H) Lambda DNA digested with HindIII. Lengths are 23.72, 9.46, 6.67, 4.26, 2.25, and 1.96 kb.*

fragments from the *Y. pestis* plasmid (Fig. 3A). This demonstrated that the relatedness between the two plasmids spanned about 80% of the plasmid genome, which is similar to that seen for plasmids in *Y. enterocolitica* (21).

**Characterization of $Ca^{2+}$-independent mutants.** Previous studies have demonstrated that all $Ca^{2+}$-dependent *Yersinia* lose a plasmid after mutation to $Ca^{2+}$ independence (1, 11, 12, 21). However, only a few $Ca^{2+}$-independent isolates have been examined. Plasmid DNA from 40 $Ca^{2+}$-independent clones of *Y. enterocolitica* and from 45 clones of *Y. pestis* was examined by agarose gel electrophoresis. All of the *Y. enterocolitica* clones were cured of their 44-Mdal plasmid. However, although 23 of the $Ca^{2+}$-independent clones of *Y. pestis* had lost their 47-Mdal plasmid, most of the others showed either a smaller form of the plasmid or harbored plasmids of a slightly larger molecular mass (Fig. 1).

To further characterize the $Ca^{2+}$-independent mutants, plasmid DNA from representative mutants (Table 1) was digested with *Bam*HI and separated by agarose gel electrophoresis (Fig. 2). Analysis of the results demonstrated that, in most of the mutants, the plasmid had suffered either an insertion or a deletion. Plasmid DNA from $Ca^{2+}$-independent mutants could be classified into four categories: (i) the plasmid was lost (47%), (ii) plasmids with deletions (38%), (iii) plasmids with a 2.2-kilobase (kb) insertion (9%) or (iv) no detectable changes in plasmid DNA (7%).

A *Bam*HI restriction endonuclease fragment map of the *Y. pestis* plasmid was constructed, using plasmid DNA from *Y. pestis* 019 (Fig. 4). *Y. pestis* 019 was used because it contains only a single plasmid identical at the molecular level to the 47-Mdal plasmid from *Y. pestis* EV76. The $Ca^{2+}$-independent mutants represented by EV7627 and EV7651 showed a 2.2-kb insertion into one of two adjacent fragments, *Bam*-5 and *Bam*-8, encompassing approximately 9 kb. All of

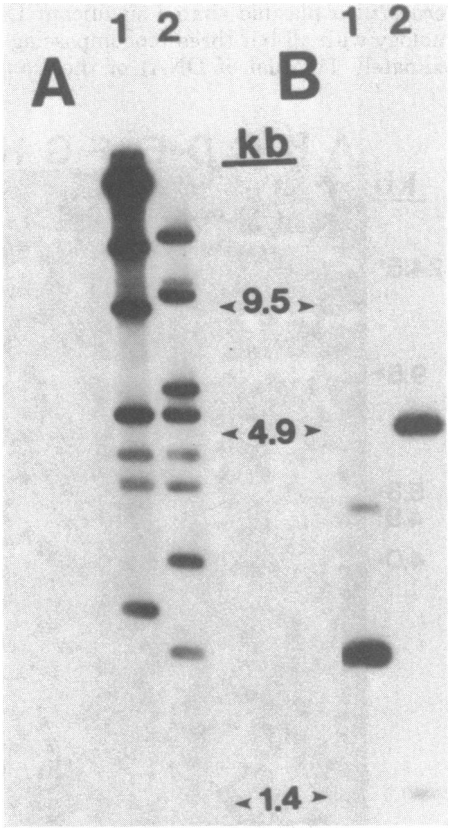


FIG. 3. *(A) Distribution of DNA sequence homology between plasmid DNA from Y. enterocolitica 8081 and Y. pestis EV76. $^{32}$P-plasmid DNA from Y. enterocolitica was hybridized to nitrocellulose filters which contained BamHI cleavage products of plasmid DNA from Y. enterocolitica 8081 (homologous control) (1) and Y. pestis EV76 (2). (B) Identification of DNA sequences homologous to $^{32}$P-labeled plasmid DNA from EV7621 (homologous control) (1). The upper band represents the open-circular form of the plasmid. (2) BamHI-digested plasmid DNA from Y. pestis EV76.*

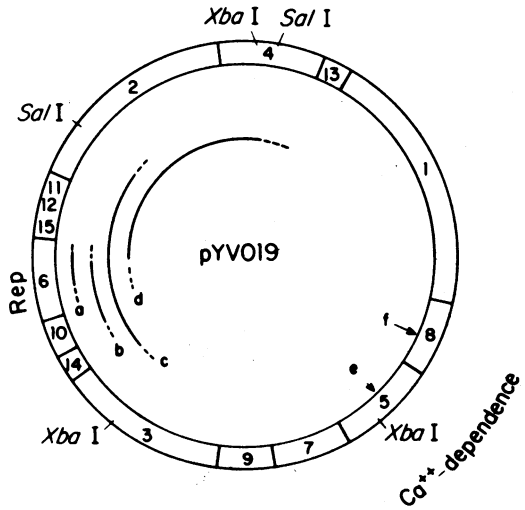


FIG. 4. *BamHI restriction map of plasmid DNA from Y. pestis 019 (pYV019). The plasmid sequences remaining from the deletion mutants are shown as solid lines; the dotted lines represent uncertainty in mapping of the junction fragments. (a) EV766; (b) EV7614; (c) EV768; (d) EV7618. The sites of insertion mutation are designated by arrows. (e) EV7627; (f) EV7651. Rep, Essential region of the plasmid. The size of BamHI restriction fragments 1 through 15, in kilobases, are: 14.5, 10.7, 9.7, 5.8, 5.3, 4.9, 4.4, 4.0, 2.9, 2.2, 1.7, 1.6, 1.4, 1.3, and 1.1, respectively.*

the remaining $Ca^{2+}$-independent deletion mutants were deleted for this region of the plasmid. We presume that this 9-kb region of the plasmid encodes determinants involved in the expression of $Ca^{2+}$ dependence. Examination of the plasmid DNA from *Y. enterocolitica* 8081 by restriction endonuclease analysis has shown that this is a conserved region of the plasmid genome in both *Yersinia* species.

**Characterization of the insertion mutants.** We sought to characterize the origin of the 2.2-kb DNA insertion found in some $Ca^{2+}$-independent strains. A 7.5-kb fragment was isolated from an agarose gel of *Bam*HI-digested plasmid DNA from a $Ca^{2+}$-independent strain of *Y. pestis* EV7627 and labeled with $^{32}P$ in vitro. This sequence of DNA included the entire *Bam* fragment 5 plus the 2.2-kb insertion. *Bam*-5 was also isolated and labeled with $^{32}P$. Therefore, any DNA sequence which hybridized with the $^{32}P$-labeled 7.5-kb fragment but failed to hybridize with $^{32}P$-labeled *Bam*-5 would contain a sequence homologous to the insertion. Homology existed with the insertion at approximately 10 sites in the bacterial chromosome as well as on the 6-Mdal plasmid, the 61-Mdal plasmid, and on *Bam*-2 of the 47-Mdal plasmid (Fig. 5). It was also found that all of the other $Ca^{2+}$-independent mutants caused by insertions showed homology with the 2.2-kb DNA sequence. The 2.2-kb sequence was hybridized with *Bam*HI-digested plasmid DNA from the deletion mutants, and it was found that no homology existed between the remaining plasmid sequences and the 2.2-kb sequence (data not shown). The 2.2-kb sequence was also hybridized with cellular DNA from *Y. enterocolitica* 8081, and no sequence homology was detected.

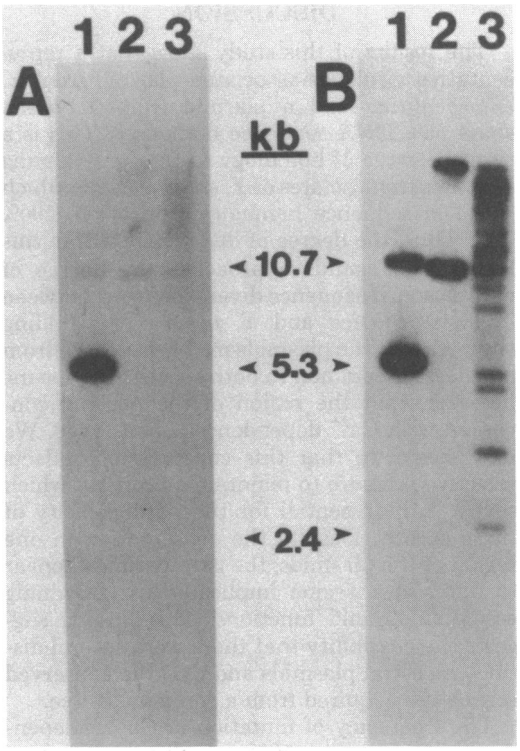


FIG. 5. *(A) Detection of DNA fragments containing sequences homologous to fragment Bam-5 (5.3 kb) of pYV019. (B) Detection of DNA fragments containing sequences homologous to Bam-5 plus the 2.2-kb insertion. (1) BamHI-digested plasmid DNA from Y. pestis 019 (pYV019); (2) BamHI digested plasmid DNA from Y. pestis EV766; (3) BamHI-digested cellular DNA from Y. pestis EV766.*

**Characterization of the deletion mutants.** The majority of the $Ca^{2+}$-independent deletion mutants of pYV76 had lost 95% of the plasmid, including all *Bam*HI restriction sites. One of these, pYV76Δ21, was chosen for further study. We wished to map the sequence of origin for this 2.5-Mdal (3.2-kb) miniplasmid on the parental plasmid pYV76. Therefore, the miniplasmid was isolated from an agarose gel, labeled with $^{32}P$, and hybridized to a *Bam*HI restriction digest of the parental plasmid. The results (Fig. 3B) showed that the miniplasmid shared homology with two fragments, *Bam*-6 (4.9 kb) and *Bam*-13 (1.4 kb). Since the miniplasmid contained no *Bam*HI sites and it was larger than the 1.1-kb fragment, we presume that the miniplasmid was derived from sequences internal to *Bam*-6. The miniplasmid was stably maintained, and, assuming that it shared its origin of replication with the parental plasmid, these data indicated that the origin of replication of the plasmids was located within *Bam*-6. This region of the *Y. pestis* plasmid shared no detectable DNA homology with the plasmid from *Y. enterocolitica* (Fig. 3A). This finding suggests that, whereas the two plasmids are related, a region in which they differ contains the origin of replication of the *Y. pestis* plasmid and other essential sequences for plasmid maintenance.

We cannot explain the homology of the miniplasmid with *Bam*-13 which maps far from *Bam*-6. However, this finding suggests either that the parental plasmid contains repeated sequences or that the miniplasmid represents a rearrangement of plasmid sequences. *Bam*-6 was isolated from an agarose gel, labeled with $^{32}P$, and hybridized to a *Bam*HI restriction digest of plasmid DNA from *Y. pestis* 019. *Bam*-6 was found to share homology with *Bam*-13 (data not shown), indicating that the plasmid from *Y. pestis* does contain at least one repeated sequence, although the significance of this observation is unclear.

## DISCUSSION

The results of this study show that a representative virulence-associated plasmid from *Y. enterocolitica* and a plasmid from *Y. pestis* share 55% DNA sequence homology. This is a similar degree of homology to that seen among plasmids from isolates of *Y. enterocolitica* which range in sequence homology from 50 to 100% (21). Thus, the degree of divergence within this family of plasmids approaches the degree of chromosomal sequence divergence seen between *Y. enterocolitica* and *Y. pestis*. This finding suggests that the plasmids may have arisen from an ancestor common to both species. It appears, however, that the region of the plasmid concerned with $Ca^{2+}$ dependence is conserved. We may speculate that this conservation reflects selective pressure to maintain a property which seems to be essential for the pathogenicity of these strains. Despite the conservation in one region of the plasmids, the two plasmids appear to differ in a region implicated as containing essential plasmid functions. This finding suggests the possibility that there were several distinct ancestral plasmids and that the conserved region was acquired from a common source.

The frequency of mutation of $Ca^{2+}$ independence for *Y. pestis* is $10^{-4}$ per cell per generation (6). We found that 9% of our $Ca^{2+}$-independent derivatives contained a unique 2.2-kb insertion. Thus, the frequency of insertion into the plasmid resulting in $Ca^{2+}$ independence is roughly $10^{-5}$. The insertion was found to share DNA homology with approximately 13 sites within the bacterial cell which is similar to IS*1* in *Escherichia coli* K-12, found at eight copies per chromosome (20). At this time, there are no phenotypes associated with the 2.2-kb insertion except for its genetic mobility. We believe this sequence of DNA represents a *Y. pestis* insertion sequence and have tentatively designated it IS*100*. Insertion sequences are known to cause deletions (16). Thus, it seems plausible that all of the deletions of the plasmid are linked to the insertion element. However, a current model for insertion sequence-mediated deletion formation dictates that a copy of the sequence remain on the replicon (22), and we have not found this to be true.

We found that two nonadjacent sites on the 47-Mdal plasmid from *Y. pestis* (*Bam*-6 and *Bam*-13) shared DNA sequence homology. Therefore, the deletions of the plasmid may have been generated by homologous recombination between repeated sequences on the plasmid. Further physical characterization of the plasmid may provide insight into the exact nature of the deletion formation.

The role of $Ca^{2+}$ dependence in pathogenesis is not clear. Both *Y. pestis* and *Y. enterocolitica* show changes in their cellular polypeptide profile after shifting from 25 to 37°C in calcium-deficient medium (21, 25), and these changes may play a role in pathogenesis. Brubaker has proposed that $Ca^{2+}$ dependence may reflect the ability of yersiniae to respond to the mammalian intracellular environment, which is low in calcium (7). One polypeptide that the yersiniae have been shown to express under calcium-limiting conditions is the V antigen. Most $Ca^{2+}$-independent mutants do not make the V antigen (6). This has led investigators to refer to the virulence-associated plasmid as the Vwa plasmid (13). We have found (unpublished data and R. R. Brubaker, personal communication), using V antisera to identify the V polypeptide, that most $Ca^{2+}$-independent mutants caused by insertions or deletions in the *Y. pestis* plasmid still express the V antigen. These data may be consistent with earlier work reported by Brubaker and Surgalla in which 20% of spontaneous streptomycin-resistant mutants of *Y. pestis* were $Ca^{2+}$ independent, V antigen positive, and avirulent (8). Therefore, $Ca^{2+}$ dependence and the expression of V antigen are separable phenomena, and the relationship of the plasmid to the V antigen requires further investigation.

We have localized the region of a plasmid in the yersiniae which is important for the expression of $Ca^{2+}$ dependence and virulence. Although the plasmid-encoded gene products and the mechanism of their action remains to be elucidated, our observations should facilitate further genetic analysis of the phenomenon of $Ca^{2+}$ dependence and the role it plays in the pathogenesis of yersinial infection.

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