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Molecular Typing of Yersinia pseudotuberculosis by Using an IS200-Like Element

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The IS200-like insertion sequence (IS) is a 708-bp element recently found in Yersinia pestis. Its nucleotide sequence is 85% identical to that of IS200 recovered in most Salmonella enterica isolates. It is also present in multiple copies in Y. pseudotuberculosis. In contrast, this IS is found in some (biotype 1B strains) but not other Y. enterocolitica strains and is absent in the nonpathogenic yersiniae: Y. frederiksenii, Y. kristensenii, Y. intermedia, Y. bercovieri, and Y. mollaretii. The number and locations of the ISs in the Y. pseudotuberculosis genome vary among strains, resulting in a high degree of polymorphism, but IS fingerprints are stable after multiple subcultures of clinical isolates. The discriminative power of IS typing is better than that of ribotyping and almost as good as that of the time-consuming method of pulsedtyping. Overall, IS200-like is a useful molecular marker in determining the epidemiology of Y. pseudotuberculosis infections.

The genus Yersinia includes three pathogenic species: Y. pestis (the causative agent of bubonic plague), Y. enterocolitica, and Y. pseudotuberculosis, the last two being responsible mainly for enteritis and mesenteric lymphadenitis, which are sometimes complicated with septicemia. Seven environmental, nonpathogenic species, Y. frederiksenii, Y. kristensenii, Y. intermedia, Y. bercovieri, Y. mollaretii, Y. aldovae, and Y. rohdei, have been described (37). Y. pestis causes epidemic or pandemic infections, while Y. pseudotuberculosis and Y. enterocolitica are mostly involved in sporadic infections. However, several outbreaks involving the two enteropathogenic species, Y. enterocolitica (especially in North America) and Y. pseudotuberculosis (mostly in Japan), have been reported (3, 15, 21, 27–29, 32–35). In addition to serotyping, biotyping, and phage typing, a variety of DNA-based methods have been developed for Yersinia typing, including analysis of restriction fragment polymorphism in chromosomal DNA or in the plasmid pYV (1, 4, 10, 12, 13, 17, 22, 24), a 70-kb plasmid harbored by all pathogenic strains and responsible for bacterial virulence (for a review, see reference 5).

We recently discovered a novel insertion sequence (IS) in Y. pestis (31). This 708-bp IS, integrated within the inv gene from Y. pestis, has 85% nucleotide identity with IS200, an element which was first described in Salmonella enterica (14). This IS, designated the IS200-like element, was found in multiple copies (at least 15 to 20) within the genomes of several Y. pestis strains. Y. pseudotuberculosis DNA hybridizes with a probe internal to the IS200-like element, but the insertion element was present in fewer copies than in Y. pestis in the limited number of strains tested (31). The degree of similarity between the Y. pestis and Y. pseudotuberculosis IS200-like elements is unknown, but restriction fragment length polymorphism results with the PCR products of the IS elements from both species were identical (unpublished results).

ISs can be used as molecular tools for typing pathogenic microorganisms, including Salmonella enterica, Staphylococcus aureus, and Mycobacterium tuberculosis (2, 19, 20, 23, 36). This conclusion appears to be paradoxical at first sight because ISs are mobile genetic elements; however, transposition events occur at a very low frequency. IS100 and IS285 have been identified in pathogenic yersiniae (9, 25, 26), but these insertion elements have not previously been used as molecular markers in epidemiological analyses of plague and other forms of yersiniosis. The aim of this work was to study the usefulness of IS200-like elements for discriminating among strains associated with known serotypes of enteropathogenic yersiniae. IS fingerprinting was performed with 20 epidemiologically unrelated strains of Y. pseudotuberculosis, and the results allowed good discrimination of the strains. In contrast, this molecular typing method is not useful for epidemiological studies of Y. enterocolitica infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The Yersinia strains used in this study are listed in Table 1. They were obtained from the Centre National de Référence des Yersiniæ, Institut Pasteur (Paris, France). Plasmid pYV was detected in bacteria by colony-blot hybridization with a probe consisting of a 5.3-kb BamHI fragment from the Ca2+ dependence locus of virulence plasmid pPB1 as previously described (30). Bacteria were grown at 30°C with aeration in Luria-Bertani broth or on agar.

Preparation of DNA probes. The IS200-like gene probe (421 bp) was obtained by PCR amplification of pYV-cured Y. pestis 6/69Mc with the primers 5′-TTCT TGATATCCGCGGT-3′ and 5′-TGGCGTCTGGCAACCT-3′ as previously described (31). The ribosomal 16S RNA (rrn) probe (about 1.5 kb) was obtained by PCR amplification of Y. pseudotuberculosis with the universal primer pair 5′-G GTTACCTTGTTACGACTT-3′ and 5′-AAGAGTTTGTATCATGGC-3′. Probes were labeled by random priming (Redprime kit; Amersham, Buckingham, France) with [γ-32P]dCTP (Amersham), and labeled DNA was separated from unincorporated nucleotides by Sephadex G-50 column (Pharmacia, Uppsala, Sweden) chromatography.

Southern hybridization. DNA extracted from bacterial cells as previously described (31) was digested with appropriate restriction endonucleases according to the manufacturer’s instructions (New England Biolabs, Beverly, Mass.). Restricted DNA fragments were separated by electrophoresis through a 0.8% agarose gel in Tris-borate or Tris-aceate buffer and then transferred to a nylon membrane (Boehringer GmbH, Mannheim, Germany). Probes were hybridized at 65 or 68°C in buffer containing 0.1% sodium dodecyl sulfate (SDS), 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), and 0.05% nonfat milk. After hybridization, filters were washed twice at room temperature for 30 min in 1× SSC and 0.1% SDS and then twice at 65 or 68°C for 45 min in 0.2× SSC and 0.1% SDS. Nylon membranes were autoradiographed by exposure to X-Omat film (Eastman Kodak, Rochester, N.Y.) at –80°C.

Pulsed-field gel electrophoresis. Bacterial DNA was embedded in agarose plugs as previously described (7). DNA fragments generated by restriction endonuclease digestion were separated in a 1% agarose–Tris-borate buffer gel by
electrophoresis with a CHEF apparatus (Bio-Rad, Paris, France) at 14°C and at 6 V/cm with alternating pulses at a 120° angle in a 1- to 31-s pulse-time gradient for 40 h. The gels were stained with ethidium bromide. DNAs from bacteriophage lambda concatemers were used as size markers.

RESULTS AND DISCUSSION

The distribution of IS200-like elements in the genomes of the pathogenic yersiniae Y. pseudotuberculosis and Y. enterocolitica and in the nonpathogenic species Y. frederiksenii, Y. kristensenii, Y. intermedia, Y. bercovieri, and Y. mollaretii was investigated by Southern blot analysis. Total bacterial DNA was digested with HincII, a restriction endonuclease that does not cut within the IS element from either Y. pestis or Y. pseudotuberculosis, and DNA fragments were hybridized with a 421-bp probe internal to the IS200-like element.

Twenty strains of Y. pseudotuberculosis isolated from human and animal infections in several different countries (Table 1) were studied: 16 strains belonged to serotypes 1, 2, 3, 4, or 6, and 4 were not typeable with specific antisera. All strains tested contained the IS, and the number of copies and locations of the

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype</th>
<th>Origin</th>
<th>Country</th>
<th>No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. pseudotuberculosis</td>
<td>1</td>
<td>Human, pig, and goat</td>
<td>France and Italy</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Human and hare</td>
<td>France</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Human and cow</td>
<td>Spain and Argentina</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Human and sheep</td>
<td>United Kingdom</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Guinea pig</td>
<td>Japan</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Nontypeable</td>
<td>Human</td>
<td>France</td>
<td>4</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>American strains (biotype 1B)</td>
<td>8</td>
<td>Human</td>
<td>Norway and United States</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>13 and 18</td>
<td>Unknown</td>
<td>United States</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>13a and 13b</td>
<td>Unknown</td>
<td>United States</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Unknown</td>
<td>United States</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Unknown</td>
<td>United States</td>
<td>2</td>
</tr>
<tr>
<td>European strains (biotype non-1B)</td>
<td>3</td>
<td>Human</td>
<td>France and Belgium</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Human</td>
<td>France and Belgium</td>
<td>3</td>
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<tr>
<td>Y. frederiksenii</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Y. kristensenii</td>
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<tr>
<td>Y. intermedia</td>
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<td>Y. bercovieri</td>
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<tr>
<td>Y. mollaretii</td>
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</tbody>
</table>

TABLE 1. Yersinia strains used in this study

FIG. 1. IS200-like element profiles of Y. pseudotuberculosis. Southern blot of HincII-digested DNA with an internal fragment of the IS200-like element labeled with [32P]dCTP. Lanes 1 to 5, serotype 1 strains; lanes 6 to 9, serotype 2 strains; lanes 10 to 12, serotype 3 strains; lanes 13 and 14, serotype 4 strains; lanes 15 and 16, serotype 6 strains; lanes 17 to 20, nontypeable strains. The positions and sizes (in kilobases) of HindIII fragments of bacteriophage lambda DNA are indicated on the left.

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element in the genomes varied among strains, resulting in a high degree of polymorphism (Fig. 1). Comparisons of the IS200-like profile of two parental strains harboring the virulence plasmid pYV and their respective isogenic pYV-cured derivatives indicated that most IS copies, if not all, were inserted into the bacterial chromosome (data not shown). Eighteen different genomic DNA restriction patterns for the IS200-like element were found among the 20 isolates. Some IS bands were shared by several strains. Three of the four untypeable strains (Fig. 1, lanes 18, 19, and 20) contained 13 copies of the IS, with similar distributions occurring in the genomes. In the fourth untypeable strain (Fig. 1, lane 17), there was one copy in an ~2.8-kb fragment instead of the 2.5-kb fragment observed in strains 18, 19, and 20 (Fig. 1). By using restriction endonuclease BamHI, EcoRI, or EcoRV, enzymes that do not cut within the IS element, we were still unable to distinguish the four untypeable strains (data not shown).

The stability of restriction patterns of \textit{Y. pseudotuberculosis} DNA for the IS200-like element was studied to assess the value of this insertion element as a marker for typing. Five strains containing 7 to 13 copies of the insertion element were subcultured at 30°C on agar plates, and after 20 subcultures (i.e., about 600 generations), DNA of each strain was extracted and digested with \textit{Hin}cII and DNA fragments were hybridized with the IS200-like probe. For all of the strains tested, we were unable to detect any difference in IS patterns after these multiple passages (data not shown); the frequency of IS200-like transposition is under investigation. Consequently, IS200-like fingerprinting appears to be useful for typing \textit{Y. pseudotuberculosis}.

We then compared the discriminative capacity of IS typing with two other molecular typing methods, pulsotyping and ribotyping. \textit{Y. pseudotuberculosis} was typed by pulsed-field gel electrophoresis of \textit{Not}I-restricted DNA; \textit{Not}I is a rare-cutting enzyme (one site per 100 kb of \textit{Yersinia} DNA) (16). This method was previously reported to be very efficient for \textit{Y. pseudotuberculosis} typing (12). The resulting macrorestriction patterns of the 20 \textit{Y. pseudotuberculosis} strains each contained about 20 fragments ranging in size from ~40 to ~300 kb (Fig. 2). The 20 strains could be classified into 19 pulsotypes according to these electrophoretic patterns. Interestingly, the four untypeable strains that exhibited identical IS200-like profiles displayed different macrorestriction patterns (Fig. 2, lanes 17, 18, 19, and 20). However, the two serotype 6 strains (Fig. 2, lanes 15 and 16) had the same pulsotype but different IS types (Fig. 1, lanes 15 and 16). Thus, the analytical performance of IS typing was similar to that of pulsotyping, which is a time-consuming method; however, the two methods were complementary since they allowed discrimination of all 20 strains used in the study.

Finally, restriction fragment length polymorphism patterns of the rDNA regions of \textit{Y. pseudotuberculosis} were studied by HindIII digestion and probing with the 16S rRNA probe. This restriction endonuclease does not cut within the 1.5-kb probe and was previously shown by Picard-Pasquier et al. (24) to discriminate \textit{Y. pseudotuberculosis} strains well (five ribotypes were individualized among nine unrelated strains). Bands containing the \textit{rrn} gene were 7 to >12 kb (Fig. 3), and 14 ribotypes were obtained among the 20 strains. One strain of serotype 1 (Fig. 3, lane 4) and one of serotype 2 (lane 7) exhibited the

![FIG. 2. NotI restriction profiles of \textit{Y. pseudotuberculosis}. DNA was digested with \textit{Not}I, and fragments were separated by pulsed-field gel electrophoresis as described in Materials and Methods. Lanes 1 to 5, serotype 1 strains; lanes 6 to 9, serotype 2 strains; lanes 10 to 12, serotype 3 strains; lanes 13 and 14, serotype 4 strains; lanes 15 and 16, serotype 6 strains; lanes 17 to 20, untypeable strains. The positions and sizes (in kilobases) of bacteriophage lambda DNA concatemers are indicated on the right.](http://jcm.asm.org/)

![FIG. 3. 16S rRNA profiles of \textit{Y. pseudotuberculosis}. Southern blot of HindIII-digested DNA with a 16S rRNA probe labeled with [32P]dCTP. Lanes 1 to 5, serotype 1 strains; lanes 6 to 9, serotype 2 strains; lanes 10 to 12, serotype 3 strains; lanes 13 and 14, serotype 4 strains; lanes 15 and 16, serotype 6 strains; lanes 17 to 20, untypeable strains. Size markers (1-kb DNA ladder) are indicated on the left.](http://jcm.asm.org/)
same 16S rRNA profile, and one strain of serotype 2 (lane 6) and two strains of serotype 3 (lanes 11 and 12) similarly fell into the same riboclass. Moreover, the two serotype 6 strains, which were not discriminated by pulsortyping, displayed the same rRNA pattern (lanes 15 and 16). Finally, the four untypeable strains (lanes 17 to 20) were separated into two groups (lanes 17 and 20 and lanes 18 and 19) on the basis of their 16S rRNA pattern. Consequently, the discriminatory power of IS typing is better than that of ribotyping. In addition, analysis of rRNA pattern is not easy because of the fact that fragments containing the \( mn \) genes were mostly of large size and thus poorly separated by agarose gel electrophoresis.

We also looked for the presence of the IS in the other enteropathogenic *Yersinia* sp., *Y. enterocolitica*. Several biotypes of this species have been defined by Wauters, Kandolo, and Janssens (38). Biotype 1B strains, mainly from North America, are highly pathogenic and responsible for major outbreaks (especially of serotype O:8), whereas strains belonging to other biotypes are less pathogenic or not pathogenic. We studied 15 strains, of which 8 were from biotype 1B (American strains). Unlike for *Y. pseudotuberculosis*, the IS200-like probe hybridized with only some of the *Y. enterocolitica* strains (Fig. 4): only four biotype 1B strains (serotypes O:8, O:13,18, and O:13a,13b) gave strong hybridization signals (one or two bands). However, several faint background bands were detected for most of the American strains. These faint bands were still detected when DNA-DNA hybridization was carried out under higher stringency conditions (68°C); thus, the weak intensities of most of the bands suggest the presence of another repetitive element in biotype 1B strains, similar but not identical to the IS200-like element; cloning and sequencing of this element from these strains is in progress. None of the seven non-biotype 1B strains (four of serotype O:3 and three of serotype O:9) hybridized with the IS200-like probe. These data are an additional argument indicating that the two groups of *Y. enterocolitica* correspond to two divergent clusters, as previously suggested by Caugant et al. (6). Production of iron-repressible proteins and the siderophore yersiniabactin under iron starvation conditions are characteristic traits of the highly pathogenic biotype 1B strains (8, 11), and the IS200-like element could be an additional marker of *Y. enterocolitica* pathogenicity. This proposition is reinforced by the absence of this element in the nonpathogenic *Yersinia* species *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. bercovieri*, and *Y. mollaretii* (three strains were tested for each species [data not shown]).

**ACKNOWLEDGMENTS**

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**REFERENCES**


**FIG. 4.** IS200-like profiles of *Y. enterocolitica*. Southern blot of HinCII-digested DNA with an internal fragment of the IS200-like element labeled with \(^{32}P\)dCTP. Lanes 1 to 8, biotype 1B strains; lanes 9 to 15, biotype non-1B strains. Size markers (1-kb DNA ladder) are indicated on the left.