Plasmid DNA Relatedness Among Different Serogroups of *Yersinia pseudotuberculosis*

NAOTAKA ISHIGURO,1* YUJI NAKAOKA,1 GIHEI SATO,1 AND MISAO TSUBOKURA2

Department of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080,1 and Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori-shi, Tottori 680,2 Japan

Received 2 November 1984/Accepted 18 December 1984

Thirteen different serogroup strains of *Yersinia pseudotuberculosis* and two strains of *Yersinia enterocolitica* O:3 were examined for the presence of plasmids and plasmid-mediated properties, calcium growth dependency, and autoagglutination. Two *Y. enterocolitica* strains and eight serogroup (IA, IIA, IIC, III, IVA, VB, VI, and VIII) strains, except for five serogroups (IB, IIB, IVB, VA, and VII), of *Y. pseudotuberculosis* harbored plasmids ranging in molecular size from 27 to 115 kilobases. Filter hybridization of restriction endonuclease-digested plasmid DNA from different serogroup strains indicated that all plasmid DNAs conferring calcium growth dependency and autoagglutination shared a high degree of DNA sequence homology, regardless of the different serogroups of *Y. pseudotuberculosis* and *Y. enterocolitica*.

The genus *Yersinia*, composed of the three species *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*, is known to be virulent for humans and animals (5). *Y. pestis*, the causative agent of bubonic plague, is highly infectious and lethal to humans, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* produce less severe infections, including enterocolitis, acute mesenteric lymphadenitis, and occasionally septicemia (5). In all three species, common virulence factors, such as tissue invasiveness, calcium growth dependency at 37°C, production of V and W antigens, and lethality to mice, have been demonstrated (1, 8, 16, 18). Recent studies have revealed that these probable virulence factors in the genus *Yersinia* have been associated with the presence of plasmids ranging in molecular size from 60 to 75 kilobases (kb) (3, 9, 19).

In many countries, including Japan, the most common serogroups of *Y. enterocolitica* isolated from human enteric infection are O:3, O:8, and O:9 (9, 26). On the other hand, infection with *Y. pseudotuberculosis* is less frequent than infection with *Y. enterocolitica*. Serogroup III, IVB, and VA strains of *Y. pseudotuberculosis* have rarely been isolated from humans (20, 22). However, *Y. pseudotuberculosis* has frequently been isolated from domestic animals, pets, and wild animals (7, 12, 23, 27, 28). Especially in Japan, it is reported that serogroup III strains of *Y. pseudotuberculosis* have been isolated from healthy pigs at abattoirs (24).

Little is known about the plasmid profile and pathogenicity of *Y. pseudotuberculosis* strains isolated from domestic and other animals. Recently, Tsubokura et al. (25) reported that many meliobio-fermenting serogroup III strains of *Y. pseudotuberculosis* isolated from animals in Japan showed virulence properties, such as autoagglutination and calcium growth dependency, and were virulent for mice. Furthermore, they proposed the new serogroup classification of *Y. pseudotuberculosis* on the basis of serological examinations of *Y. pseudotuberculosis* strains isolated from humans and other sources (24a). To further characterize the virulence factors in different serogroup strains of *Y. pseudotuberculosis*, plasmid profile analysis was performed. In this paper, we describe the isolation of plasmid DNA from different serogroup strains of *Y. pseudotuberculosis* and *Y. enterocolitica* O:3 strains and compare the sequence homology of plasmid DNAs by filter hybridization.

Thirteen different serogroup strains of *Y. pseudotuberculosis* employed in this study are shown in Table 1 (24a). Two *Y. enterocolitica* O:3 strains were also used in this study (Table 1). All *Yersinia* strains were grown on brain heart infusion (Eiken, Tokyo, Japan) agar medium at 25°C. To examine the calcium growth dependency, magnesium oxalate agar, as described by Heesemann and Laufs (10), was used. Autoagglutination tests for the strains were performed according to the method of Laird and Cavanaugh (14). For calcium growth dependency analysis and autoagglutination tests, incubation was done at 37 and 25°C. Plasmid DNA was isolated routinely by the method of Kado and Liu (11) and purified by cesium chloride-ethidium bromide density centrifugation (15). Plasmid DNA was digested with restriction endonucleases and subjected to electrophoresis in a horizontal Tris-borate 0.8% agarose gel (15). The restriction endonucleases BamHI, EcoRI, and HindIII were used under conditions recommended by the supplier (Takara Shuzo, Kyoto, Japan). To examine the sequence homology of plasmid DNA from different serogroup strains, nitrocellulose filter hybridization tests were done. Restriction endonuclease-digested DNA fragments were separated by electrophoresis and were transferred from the gel to a nitrocellulose filter as described by Southern (21). Plasmid DNA from *Y. pseudotuberculosis* 208 or *Y. enterocolitica* 2672 was labeled in vitro with [α-32P]dCTP by nick translation (6). After hybridization, the filters were autoradiographed with Fuji RX film for 24 h at ~80°C.

Results obtained in calcium growth dependency and autoagglutination tests with 13 *Y. pseudotuberculosis* and 2 *Y. enterocolitica* strains are given in Table 1. Of 13 strains of *Y. pseudotuberculosis*, 6 serogroup strains were calcium-dependent, and of them all except 1 strain (212; serogroup...
TABLE 1. Relationship of plasmids and phenotypic properties of different serogroup strains of *Y. pseudotuberculosis* and *Y. enterocolitica* 0:3 strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Serogroup</th>
<th>Source</th>
<th>CADa</th>
<th>AA'</th>
<th>Plasmid size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. pseudotuberculosis</em></td>
<td>2883</td>
<td>IA (1a)</td>
<td>Duck</td>
<td>–</td>
<td>–</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>354</td>
<td>IB (1b)</td>
<td>Human</td>
<td>–</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>208</td>
<td>IIA (2a)</td>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>266</td>
<td>IIB (2b)</td>
<td>Cow</td>
<td>–</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>274</td>
<td>IIC (2c)</td>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>III (3)</td>
<td>?d</td>
<td>+</td>
<td>+</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>IVA (4a)</td>
<td>?</td>
<td>–</td>
<td>–</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>212</td>
<td>IVB (4b)</td>
<td>Human</td>
<td>+</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>VA (5a)</td>
<td>Human</td>
<td>–</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>197</td>
<td>VB (5b)</td>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>#1</td>
<td>VI (6)</td>
<td>Guinea pig</td>
<td>–</td>
<td>–</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>257</td>
<td>VII (7)</td>
<td>Dog</td>
<td>–</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>151</td>
<td>VIII (8)</td>
<td>Rat</td>
<td>+</td>
<td>+</td>
<td>67</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>2672</td>
<td>O:3</td>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>9529</td>
<td>O:3</td>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>68</td>
</tr>
</tbody>
</table>

a Values in parentheses represent new serogroups proposed by Tsubokura et al. (24a).
b CAD, Calcium growth dependency.
c AA, Autoagglutination.
d ?, No information on source.

IVB) also autoagglutinated. For two *Y. enterocolitica* strains, calcium growth dependency was always accompanied by positive autoagglutination tests (Table 1).

To assess the relationship between these virulence markers and plasmids in *Yersinia* strains, the plasmid contents were examined. As shown in Table 1, 8 of 13 *Y. pseudotuberculosis* strains carried plasmids, and their molecular sizes ranged from 27 to 115 kb. No plasmid DNA was detected in five strains (354, 266, 212, 210, and 257) in this study. On the other hand, two *Y. enterocolitica* strains harbored plasmids of the same molecular size (68 kb).

Plasmid DNA digested with the restriction endonuclease *BamHI, EcoRI*, and *HindIII* were analyzed by agarose gel electrophoresis. The representative pattern of DNA digested

![FIG. 1. Detection of sequence homology among plasmid DNAs from different serogroup strains of *Y. pseudotuberculosis* and from *Y. enterocolitica* O:3 strains. (A) HindIII-digested plasmid DNA was separated by agarose gel electrophoresis. (B) The same DNA was transferred to a nitrocellulose filter and hybridized with 32P-labeled plasmid DNA from *Y. pseudotuberculosis* 208. Lambda DNA digested with HindIII was used as the molecular marker. Lengths are 23, 9.4, 6.6, 4.2, 2.2, 1.9, and 0.5 kb (panel A, lane 1) (15). Shown is HindIII-digested plasmid DNA from *Y. pseudotuberculosis* 2883 (lanes 2), 208 (lanes 3), 274 (lanes 4), 83 (lanes 5), 51 (lanes 6), 197 (lanes 7), 1 (lanes 8), and 151 (lanes 9) and from *Y. enterocolitica* 2672 (lanes 10) and 9529 (lanes 11).
with HindIII is shown in Fig. 1A. The plasmids from Y. pseudotuberculosis 208, 274, 83, 197, and 151 shared many fragments of identical size (Fig. 1A, lanes 3, 4, 5, 7, and 9). The cleaved plasmids from two Y. enterocolitica O:3 strains showed identical numbers and size distributions of DNA fragments. When these plasmid DNAs were digested with BamHI or EcoRI, the same results were obtained (data not shown). To characterize the distribution of DNA homology, we performed nitrocellulose filter blot hybridization of radiolabeled plasmid DNA from strain 208 with HindIII-digested DNA fragments of the other plasmids (Fig. 1B). The results (Fig. 1B) showed that DNA relatedness among the plasmids was distributed in the plasmid molecules from five strains (208, 274, 83, 197, and 151) of Y. pseudotuberculosis and two Y. enterocolitica strains. Plasmid DNA fragments from strain 208 displayed no homology with the plasmids from strains 2883 and #1, and plasmid DNA from strain 51 showed a little homology with the probe plasmid (from strain 208). The same results were obtained when 32P-labeled plasmid DNA from Y. enterocolitica 2672 was used as the hybridization probe (data not shown). Also, in the case of BamHI- or EcoRI-digested fragments, the results of the filter hybridization tests (data not shown) were similar. From these results, it is demonstrated that plasmids conferring calcium growth dependency and autoagglutination have sequence homology among their DNA fragments, regardless of different serogroups or sources.

Plasmids ranging from 60 to 75 kb in molecular size are known to carry genetic information essential for virulence of Yersinia species (1, 3, 19). Avirulent derivatives from these virulent Yersinia strains lose the common plasmids (8, 16). In contrast to these results, Kay et al. (13) reported that 82-megadalton (ca. 120-kb) plasmids isolated in Y. enterocolitica strains might be associated with the virulence. Molecular sizes of plasmids detected in eight Y. pseudotuberculosis strains in this study are variable from 27 to 115 kb, compared with the 68-kb plasmids from Y. enterocolitica (Table 1). In recent reports by Portnoy et al. (19) and by Bolin and Wolf-Watz (4), the virulence plasmids from Y. pestis, Y. enterocolitica, and Y. pseudotuberculosis were characterized and identified as carrying the encoding determinants that confer calcium growth dependency and outer membrane protein. High DNA relatedness among plasmids detected in Y. pseudotuberculosis and Y. enterocolitica in this study is probably due to both strains carrying common virulence determinants. The plasmid from Y. pseudotuberculosis 51 was found to have a little homology with the labeled plasmid from strain 208; these related plasmids may encode the same replication system (2, 19).

Although Y. pseudotuberculosis 212 (serogroup IVB) showed calcium growth dependency, no plasmid DNA was detected in this study. As an insertion sequence (IS100) was identified in Y. pestis 195-P (17), the plasmid conferring calcium growth dependency in strain 212 may integrate to the chromosome by the transposable elements, such as the insertion sequence.

We have not isolated plasmids from five Y. pseudotuberculosis strains. However, these strains might carry common virulence plasmids at the time of the primary isolation step, because the virulence plasmids in Yersinia strains are unstable and therefore can be deleted during the long stock maintenance period. As reported by Tsubokura et al. (25), 54 (83%) serogroup III strains of Y. pseudotuberculosis strains isolated from humans and animals were shown to be calcium dependent. The results obtained in their studies and in this study reveal that virulence plasmids are widely distributed in the different serogroup strains of Y. pseudotuberculosis from humans and animals.

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