NOTES

Isolation of an R⁻ M⁺ Mutant of Yersinia enterocolitica Serotype O:8 and Its Application in Construction of Rough Mutants Utilizing Mini-Tn5 Derivatives and Lipopolysaccharide-Specific Phage

LIUJAN ZHANG* AND MIKAEL SKURNIK
Department of Medical Microbiology, Turku Centre for Biotechnology, University of Turku, SF-20520 Turku, Finland

Received 21 June 1993/Accepted 12 January 1994

Yersinia enterocolitica is an enteric gram-negative bacterium which mainly causes enteritis and mesenteric lymphadenitis. As sequelae to infection by Y. enterocolitica, patients may develop reactive arthritis or erythema nodosum. Interestingly, lipopolysaccharide (LPS) of Y. enterocolitica was found in synovial fluid cells of patients suffering from reactive arthritis (14). LPS is a complex molecule; it is one of the virulence factors of gram-negative bacteria, and it plays an important role in the structure of the outer membrane as well as in the interaction between cells and their environment. It is composed of three parts: lipid A, core, and O side chain (or O antigen). Since there is great interest in the role of LPS in the virulence of Yersinia spp. and in triggering reactive arthritis, we have focused our efforts on genetic studies of LPS of Y. enterocolitica serotypes O:3 and O:8. We cloned and sequenced the rfb region of Y. enterocolitica O:3 (1, 42), and we cloned the rfa region of Y. enterocolitica O:3 (2). Since transposon mutagenesis is one of the most efficient approaches to the identification of gene clusters of interest, our intention was, in the case of Y. enterocolitica O:8, to isolate rough transposon insertion mutants using an LPS-specific bacteriophage. However, the major difficulty which we encountered with Y. enterocolitica O:8 was the restriction barrier.

Restriction-modification (R-M) systems are known to protect bacterial cells against invasion by bacteriophages (6). The main role of R-M systems is in the defense against foreign DNA, although it has been proposed that an alternative role could be promotion of recombination with exogenous DNA, i.e., a means to enhance genetic diversity within a population (34). Several kinds of R-M systems have been discovered. They seem to do equivalent biological jobs, but in different ways (7, 25, 36). The systems generally comprise pairs of opposing intracellular enzyme activities: a restriction endonuclease and a DNA-methyltransferase. Restriction endonucleases interact with specific nucleotide sequences in DNA and cut both DNA strands at that site; in addition, a few also recognize single-stranded DNA (9, 41). Thus, restriction enzymes destroy foreign DNA segments before they can establish themselves; for example, by recombination into the chromosomal DNA. The restriction endonucleases are not able to cleave the bacterium's own DNA because it is modified by specific DNA methylases, which add methyl groups to adenine or cytosine residues within the recognition sequences (8, 13, 18, 22, 28, 35).

Y. enterocolitica serotype O:8 strains of different origins have been reported to produce the chromosomally encoded restriction endonuclease Yen1, an isoschizomer of PstI, but Yen1 is not produced by other serotypes of Y. enterocolitica, by Y. pseudotuberculosis, or by Y. pestis (30). Apparently because of this restriction barrier, the transposition frequency was under the detection limit in our initial experiments, which used a suicide vector delivery system in wild-type Y. enterocolitica O:8. This hampered our attempts to isolate rough mutants, since we were not able to construct a representative transposon library into the wild-type strain. Thus, a restriction-deficient derivative was needed. Cloning the gene of the restriction enzyme and subsequently inactivating it could have been one approach. But since it would have been time consuming and since one useful selective antibiotic resistance marker would have been wasted, we decided to isolate a spontaneous restriction-deficient mutant of Y. enterocolitica O:8. We used a quick and generally applicable procedure to isolate a restriction-negative strain.

Isolation of restriction-deficient mutant. One could anticipate that a plasmid carrying a Yen1 (or PstI) recognition site could not easily get through the restriction barrier of Y. enterocolitica O:8. The host restriction enzyme would digest the plasmid already in the periplasmic space; most of the restriction endonucleases are periplasmic (15). The best method for the introduction of foreign DNA into Y. enterocolitica O:8 has been mobilization; however, the mobilization

* Corresponding author. Mailing address: Turku Centre for Biotechnology, Biocity Tykistokatu 6, SF-20520 Turku, Finland. Phone: 358-21-6338010. Fax: 358-21-6338000. Electronic mail address: lzhang@finabo.abo.fi.
frequency has been very low. One could speculate that any of those few bacteria in a population which accepted the mobilized plasmid had spontaneously lost the ability to produce the restriction enzyme. Therefore, a restriction-deficient mutant should be found among those bacteria. To facilitate the curing of the mobilized indicator plasmid from the putative restriction-deficient mutants, the thermoresistant plasmid pMMB70, a Tn5 derivative of pMMB19 (38), was used in this study. pMMB70 can be mobilized from E. coli S17-1 (39), and it can replicate only at low temperatures (<30°C). Replication does not occur at 42°C because of a temperature-sensitive mutation in the oriV gene of replication origin. Thus, curing of pMMB70 from a strain takes place rapidly at 42°C.

E. coli S17-1/pMMB70 and Y. enterocolitica 8081-c were grown overnight at 30°C in Luria-Bertani (LB) medium (10 g of Bacto Tryptone, 10 g of yeast extract, 5 g of NaCl per liter), and 200 and 400 μl, respectively, of the cultures were mixed together. The mixture was filtered with a nitrocellulose filter (pore size, 0.45 μm); the filter with the bacteria was then incubated at 30°C on an LB agar plate for 3 to 5 h. The bacterial cells were resuspended in 2 ml of phosphate-buffered saline buffer and plated on CIN (Yersinia selective agar base; Oxoid) plates containing kanamycin (25 μg/ml). Transconjugants were allowed to grow at 30°C for 48 h. Ten large colonies were pooled and inoculated into LB medium without kanamycin and allowed to grow at 42°C for 2 days. After the first day, the culture was diluted 1:100 into new LB medium. The bacteria were then spread on Luria agar (LA) plates and grown at 30°C for 36 to 48 h. One hundred separate colonies were patched on LA plates with or without kanamycin to determine the number of bacteria which had lost plasmid pMMB70. More than 90% of the bacteria had lost pMMB70 during the 2 days of growth in LB without kanamycin. One of the plasmid-cured colonies was picked up for further identification and tentatively named 8081-res.

8081-res is a spontaneous restriction-deficient mutant. Remobilization of pMMB70 into 8081-res and 8081-c was done as described above. Mobilization frequency into 8081-res was 7 × 10⁻⁴ per recipient, and that into 8081-c was 4.2 × 10⁻⁶ per recipient (Table 1). To confirm this observation, we tested whether the transposition frequency in 8081-res had increased by using a suicide vector delivery system. This was assessed using a mini-Tn5-phoA transposon. The donor strain was E. coli Sm10(xpir)/pUT::mini-Tn5-phoA (27). pUT is a derivative of the suicide vector pGPT704 (29). For the determination of transposition frequencies, the recipient strains 8081-c and 8081-res were grown in LB overnight at 30°C, and the donor strain was grown overnight at 37°C in the presence of kanamycin (25 μg/ml). For matings, 100-μl samples of the approximate overnight cultures were mixed and filtered with Millipore type HA filter (pore size, 0.45 μm), and the matings were performed as described above. Transposition frequencies were scored by counting the number of colonies growing on CIN-kanamycin plates after 48 h at 30°C. Meanwhile, the numbers of recipient and donor bacteria in the mating mixtures were determined by a dilution plating method using CIN and LA-kanamycin plates, respectively. The transposition frequency in 8081-res was approximately 6.7 × 10⁻⁶ per recipient (Table 1). The transposition frequency in 8081-c was below the threshold of detection. Similar transposition frequencies were obtained when other Tn5 derivatives were used, including mini-Tn5-Cm and Tn5-Tc1 (data not shown). The transposants obtained from transposition experiments were pooled to generate a Tn5 transposon library of Y. enterocolitica O:8.

We wanted to find out whether electroporation efficiency was also affected. A 40-μl volume of electroporation-compotent cells (about 3 × 10¹⁰ cells per ml, prepared for electroporation as described previously [4]) was mixed with 1 to 2 μl of DNA (about 0.5 μg) in a 0.2-cm cuvette that was prechilled on ice. Electroporation was carried out with a Bio-Rad Gene Pulser apparatus at a capacitance of 25 μF. A resistance of 200 Ω and a voltage of 12.5 kV/cm were employed. These settings gave a time constant of about 5 ms. Immediately after the single discharge, the cells were suspended in 1 ml of SOC medium (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose) and grown at 30°C for 2 h before being plated. Electroporation frequencies of plasmid pJAC4 and pACYC184 are given in Table 1. pJAC4 is a promoter cloning vector which contains four PstI sites. pJAC4 was electroporated into 8081-res 10⁻²-fold more efficiently than it was electroporated into the wild-type strain 8081-c under the same electroporation conditions. pJAC4 isolated from 8081-res had been modified, since it could not be digested by PstI (data not shown). pACYC184 does not contain any PstI sites; consequently, it was electroporated into 8081-res and 8081-c with equal efficiencies.

8081-res does not produce the restriction endonuclease YenL. 8081-c and 8081-res were grown overnight at 37°C in 50 ml of LB broth. Cells were collected by centrifugation and washed once with S buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM 2-mercaptoethanol) (31) after which the pellets were resuspended in 10 ml of S buffer. The bacteria were broken by sonication, which was performed with samples immersed in an ice-water bath. Sonication was performed for periods of 30 to 60 s, allowing 2 to 3 min between bursts for the samples to cool down until the suspensions became clear, indicating that the cells had been broken down. A 0.5-ml sample of the sonic extract was transferred into an Eppendorf tube, and the cell debris was pelleted by centrifugation (16,000 g × 4°C for 20 min). The supernatant was transferred to a new Eppendorf tube, and serial 1:2 dilutions were made with TA buffer (31). Fifty microliters of dilutions were then tested for restriction enzyme activity by using 0.5 μg of pTE7 (Table 2) as the indicator plasmid at 37°C for 1 h. Yeast tRNA was added to the digestion reactions to a final concentration of 200 μg/ml to inhibit nonspecific endonuclease activity. YenL could be detected in the wild-type strain 8081-c dilutions from 1:8 to 1:128, whereas none of the dilutions of the mutant strain 8081-res were able to digest the plasmid pTE7. Figure 1 shows the digestions from 1:64 dilutions of lysates of both 8081-res and 8081-c.

In summary, the above experiments strongly indicate that the increased transformation, mobilization, and transposition efficiencies of 8081-res were due to a spontaneous mutation.
TABLE 2. Strains and plasmid used

<table>
<thead>
<tr>
<th>Strain, plasmid, or bacteriophage</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y. enterocolitica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8081-c</td>
<td>pYV̂ derivative of 8081, serotype O:8</td>
<td>32</td>
</tr>
<tr>
<td>8081-res</td>
<td>R'M' derivative of 8081-c</td>
<td>This study</td>
</tr>
<tr>
<td>8081-R6</td>
<td>8081-res::Tn5</td>
<td>This study</td>
</tr>
<tr>
<td>8081-R7</td>
<td>8081-res::Tn5</td>
<td>This study</td>
</tr>
<tr>
<td>8081-R14</td>
<td>8081-res::Tn5</td>
<td>This study</td>
</tr>
<tr>
<td>8081-R15</td>
<td>8081-res::Tn5</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>thi pro hsd R lsdM· recAclipse::RP4-2-Tc::Mu· Km::Tn7</td>
<td>39</td>
</tr>
<tr>
<td>Sm10 λpir</td>
<td>thi thr leu tonA lacY supE pir RK6 recAclipse::RP4-2-Tc::Mu· Km·</td>
<td>29</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMMB70</td>
<td>pMMB19::Tn5</td>
<td>38</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm· Tc· P15A</td>
<td>10</td>
</tr>
<tr>
<td>pJAC4</td>
<td>Promoter cloning vector</td>
<td></td>
</tr>
<tr>
<td>pTE7</td>
<td>6.5-kb EcoRI fragment of rfb gene cluster of Y. enterocolitica O:3 inserted into the EcoRI site of pGEM3Z</td>
<td>This study</td>
</tr>
<tr>
<td>pUT::Mini-Tn5-phoA</td>
<td>Mini-Tn5-phoA delivery system</td>
<td>27</td>
</tr>
<tr>
<td>pUT::Mini-Tn5-Cm</td>
<td>Mini-Tn5-Cm delivery system</td>
<td>27</td>
</tr>
<tr>
<td>pUT::Mini-Tn5-Tc1</td>
<td>Mini-Tn5-Tc1 delivery system</td>
<td>27</td>
</tr>
<tr>
<td><strong>Bacteriophage φ80-18</strong></td>
<td>Bacteriophage reacting with core of 8081-c</td>
<td>This study</td>
</tr>
</tbody>
</table>

that made 8081-res unable to express the YenI restriction endonuclease.

**Selection of rough Tn5 insertion mutants using LPS-specific bacteriophage.** Y. enterocolitica serotype O:8 strain 8081-specific phages were isolated from raw sewage obtained from the Turku City sewage treatment plant by using the method described previously (1). Briefly, Y. enterocolitica-specific bacteriophages in the sewage were enriched by adding 0.1 ml of an overnight culture of Y. enterocolitica O:8 strain 8081 (32) in 9 ml of tryptic soya broth to 3 ml of sewage and incubating the mixtures for 6 h at room temperature. A 3-ml sample was removed from the mixture, and 300 μl of chloroform was mixed with it and the mixture was incubated for at least 1 h at 4°C. The chloroform-treated sample was diluted to obtain individual plaques on strain 8081 in soft agar. After an additional purification, step phage stocks were prepared from individual plaques as described previously (5). The specificity of the phages was tested by an inhibition experiment using purified LPS of Y. enterocolitica O:8. One bacteriophage from the 8081-enriched sewage, designated φ80-18, was inhibited by the purified LPS preparation, and a few of the spontaneous rough mutants selected from 8081-c by using this phage were rough strains with a defective core (data not shown), indicating that the phage receptor of φ80-18 is the LPS core. φ80-18 was subsequently used for screening rough mutants from the Tn5 (Cm') library constructed in 8081-res (see above).

About 100,000 bacteria of the library were plated on 10 CIN-chloramphenicol plates and allowed to grow at room temperature overnight until visible colonies appeared on the plates. A set of tryptic soy agar plates was flooded with φ80-18 stock (4.2 × 10⁸ PFU/ml) and allowed to dry before being used. The colonies grown on the CIN-chloramphenicol plates were transferred onto the tryptic soy agar-φ80-18 plates by replica plating using a nitrocellulose filter. The plates were incubated at room temperature overnight. The bacteria expressing the wild-type LPS on the cell surface should be sensitive to the phage and be lysed; only phage receptor-deficient strains, i.e., LPS mutants should be able to grow. φ80-18 resistant colonies were analyzed for their LPS profiles. LPS isolation was performed by using the phenol-water extraction method as described previously (19), with minor modifications. Briefly, bacterial cells from 3 ml of overnight cultures were collected by centrifugation and resuspended into 500 μl of distilled water. The bacterial suspensions were incubated at 70°C for 1 h and then mixed with an equal volume of water-saturated phenol (pH 4.0) preheated to 70°C. The mixture was incubated at 70°C for another 10 min and then transferred to ice to cool it below 10°C. After centrifugation at 2,000 × g for 20 min, the aqueous layer was carefully transferred to a new Eppendorf tube and 2 volumes of acetone were added to precipitate LPS. The final pellet was dissolved into 50

![Figure 1](https://via.placeholder.com/150)

**FIG. 1.** Cleavage patterns of plasmid pTE7 after digestion with crude lysates of 8081-c or 8081-res. Lane 1, plasmid pTE7; lane 2, pTE7 with 8081-res lysate; lane 3, pTE7 with 8081-c lysate; lane 4, PstI-digested pTE7.
μl of water; 1 μl of solubilized LPS was sufficient for subsequent analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, which were performed by using the Pharmacia Phast System as described previously (2). As shown in Fig. 2, the ϕ80-18-resistant clones were all rough mutants. 8081-R15, -R6, -R14, and -R7 produced no O-side chain and possessed apparently defective core profiles compared to the wild type. Many of the genes determining the core biosynthesis in other enterobacteria are located in the rfa region, and thus, most probably, the mutants isolated here carry the transposons inserted in the rfa region of 8081. This is currently under investigation. For further studies we also wanted to isolate transposon insertion mutants in the rfb region responsible for the O-antigen biosynthesis. Unfortunately, we did not obtain any O-antigen-specific bacteriophages from the phage isolation procedure. Therefore, we have to screen the transposon library using a monoclonal antibody specific for the O antigen of Y. enterocolitica O:8 and select for further characterization those mutants not expressing the O antigen.

The R-M systems are found in every known major group of prokaryote (11, 12, 16, 17, 36). The R-M system often is an impenetrable barrier to the application of recombinant DNA technology to wild-type bacteria, such as Y. enterocolitica O:8. In order to facilitate the study of Y. enterocolitica O:8 LPS genetics, a restriction-deficient mutant was a prerequisite. Here, we isolated a mutant strain, 8081-res, which has a restriction-deficient phenotype as shown by both in vivo and in vitro experiments. Strain 8081-res had lost the ability to produce YenI, and the mobilization frequency into 8081-res had increased about 10^3 times from that of the wild-type strain (Table 1). Transposition frequency from a suicide vector into 8081-res was about 6.7 × 10^-6, while that into the wild-type strain was practically zero (Table 1). Although we did not demonstrate where the mutation had taken place in 8081-res, it most probably was the YenI restriction endonuclease gene. It is, however, possible that a mutation in a putative regulatory gene of the R-M system would cause a similar phenotype, since such mutations in the R-M systems of PvuII and BamHI had caused a completely restrictionless phenotype and about a 1,000-fold decrease in endonuclease activity, respectively (20, 40).

The restriction-deficient strain greatly facilitated the genetic manipulation of Y. enterocolitica O:8. Close to 1% of the transposon insertion mutants had the rough phenotype and were resistant to bacteriophage ϕ80-18. All of the examined ϕ80-18-resistant Tn5 insertion mutants were similar to those shown in Fig. 2. The structure and composition of the O antigen and the core of Y. enterocolitica O:8 are not known. Although the core oligosaccharide is more conserved among gram-negative bacteria than is the O antigen (21, 24), the genetic organization of the rfa gene clusters of Salmonella typhimurium and E. coli K-12 revealed similarities as well as differences. The rfa gene clusters consist of distinct similar blocks of genes, as defined by the direction of transcription, which occupy about 18 kb (37). However, some of the genes (for example, rfaS whose function was not very clear, though it was suggested to play a role in core assembly) were found in E. coli but not in S. typhimurium and some other genes (for example, rfaL and rfaK) are poorly conserved between these two organisms (26, 33). It will be interesting to compare the genetic organization of the rfa genes of Y. enterocolitica O:8 to that of E. coli and S. typhimurium. Such a comparison will provide us with valuable information on the degree of conservation in the organization of the rfa genes between genera.

We greatly appreciate Victoria Shingler and Hans Wolf-Watz for their helpful suggestions in planning this work and for providing us with the plasmid pMMB70 and mini-Tn5 derivatives. We thank Anne Peippo for help in isolation and characterization of the Y. enterocolitica O:8 LPS-specific phage.

This work was supported by the Sigrid Jusélius Foundation, The Academy of Finland, and the Turku University Foundation.

REFERENCES


12. Colson, C., and A. Van Pel. 1974. DNA restriction and modification systems in Salmonella. I. SA and SB, two Salmonella typhi-


24. Kessler, C., and V. Manta. 1990. Specificity of restriction endonu-


37. Scholz, P., V. Haring, E. Scherzinger, R. Lurz, M. M. Bagdasaria-


