Physical Mapping of Hemolysin Plasmid pCW2, Which Codes for Virulence of a Nephropathogenic *Escherichia coli* Strain

CEES WAALWIJK, JOHANNES DE GRAAFF, AND DAVID M. MACLAREN

Department of Medical Microbiology, School of Medicine, and Department of Oral Microbiology, School of Dentistry.

Vrije Universiteit, 1081 BT Amsterdam, The Netherlands

Received 21 December 1983/Accepted 4 April 1984

The hemolysin plasmid pCW2, which enhances the virulence of *Escherichia coli*, has been mapped. Comparison of the region coding for hemolysin with other hemolysin determinants reveals differences that could explain differences in the ability to confer virulence.

Hemolysin is an extracellular protein that is produced by some strains of *Escherichia coli*. Its frequent occurrence among extraintestinal isolates indicates that hemolysin may be involved in the pathogenesis of such infections. Data from in vivo experiments support this view (7, 13). Furthermore, nonhemolytic mutants are less virulent than their hemolytic parents (12, 15).

The genes involved in the biosynthesis and secretion of hemolysin in *E. coli* thus far have been found to be clustered on a DNA segment of 3.2 × 10⁶ daltons (9). These hemolysin determinants may reside either on the bacterial chromosome or on large, transmissible plasmids (2, 7, 11, 16).

Molecular cloning has been reported for both the *E. coli* plasmid determinant and for chromosomal determinants (1, 3, 17), and hybridization experiments have demonstrated a high degree of homology, regardless of the origin of the hemolysin determinant. Nevertheless, their close structural and sequential relationship, defined differences are observed which seem to be located mainly within the structural gene for hemolysin (1). These differences may be reflected in the contribution of hemolysin determinants to virulence, since only the chromosomal hemolysin determinant engendered virulence in *E. coli* (17). More recently, even among various chromosomal determinants, both genetic and pathogenic differences have been observed.

Recently, we reported the existence of an hemolysin determinant that appears to exhibit some special features. First of all, this determinant is localized on plasmid pCW2, and unlike the majority of hemolysin plasmids, pCW2 is nonconjugative (14). Secondly, this determinant confers virulence upon *E. coli* (15) in contrast to the determinant of plasmid pHly152 (17). To identify whether these differences are caused by a different genetic organization, we constructed a physical map of pCW2.

pCW2 DNA was isolated by the method of Kado and Liu (6) with the following modifications. After lysis with alkaline sodium dodecyl sulfate and neutralization with acid phenol, the plasmid containing water phase was chromatographed on hydroxyapatite. Subsequently, DNA was eluted with 0.5 M EDTA (pH 7.5) and precipitated with ethanol. Redissolved plasmid DNA was digested with several restriction endonucleases. The sizes of the fragments generated by *EcoRI*, *BamHI*, *SalGI*, and *XhoI* are given in Table 1. To determine the sizes of the larger fragments in Table 1, digestion patterns were compared with those after digestion with a second enzyme. Alignment of the smaller fragments was done by partial digestions, and a rough map of pCW2 could be drawn from these results.

To confirm the correct location of restriction sites on this map, a similar strategy was used to construct maps of deletion derivatives of pCW2. These deletion derivatives, pCW13, pCW14, and pCW18, were obtained during "curing" experiments with novobiocin. Comparison of these digestion patterns with those obtained with pCW2 allowed us to draw a final map of pCW2, which is shown in Fig. 1.

Interestingly, two of the deletion mutations have one ending in common. Similar findings were also observed with chromosomal deletion mutations, and it has been suggested that these mutations are caused by insertion elements (4). Furthermore, hemolysin plasmids have been reported to contain up to six insertion-like sequences (18). These data suggest that the hemolysin determinant may be part of a transposon-like structure, which could explain its spread among chromosomal and unrelated extrachromosomal replicons (19) as well as its ability to move from one plasmid to another (8).

The location of the hemolysin determinant on the physical map of pCW2 was determined with the use of transposon Tn5 insertion mutations (Fig. 1 and 2). Since Tn5 does not contain *EcoRI* restriction sites (5), digestion with this enzyme results in loss of a fragment and concurrent appearance of a new fragment which is 5.4 kilobases larger. Results from these experiments (data not shown) demonstrate that

<table>
<thead>
<tr>
<th>Fragment</th>
<th><em>EcoRI</em></th>
<th><em>BamHI</em></th>
<th><em>SalGI</em></th>
<th><em>XhoI</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20.9</td>
<td>25.7</td>
<td>34.8</td>
<td>57.9</td>
</tr>
<tr>
<td>B</td>
<td>18.2</td>
<td>19.5</td>
<td>21.3</td>
<td>6.6</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>14</td>
<td>6.7</td>
<td>1.2</td>
</tr>
<tr>
<td>D</td>
<td>5.4</td>
<td>8.6</td>
<td>3.4</td>
<td>0.8</td>
</tr>
<tr>
<td>E</td>
<td>4.1</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>3.2/3.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Restriction enzyme digests were separated by horizontal agarose gel electrophoresis: 0.7% agarose in 89 mM Tris-89 mM boric acid-2.5 mM EDTA (pH 8.3). Electrophoresis was performed at 100 V and 100 mA for 3 h, and gels were stained with ethidium bromide (1 µg/ml).

* Restriction enzyme *EcoRI* was purchased from Boehringer Mannheim Corp., and *BamHI*, *SalGI*, and *XhoI* were purchased from Bethesda Research Laboratories. kbd. Kilobases.

* The sizes of the larger fragments were determined after digestion with a second restriction endonuclease. Total size of the plasmid in kilobases as determined from the sum of the fragment sizes for each enzyme was as follows: *EcoRI*, 67.5; *BamHI*, 67.8; *SalGI*, 67.5; and *XhoI*, 66.5.

* Corresponding author.
the Tn5 insertions have occurred within the two adjacent EcoRI fragments D and F2. More precise location of the insertions was performed with SalGI and BamHI, which both recognize one site within the transposon (5). These digestions localize the Tn5 insertions within a stretch of DNA of 3.8 kilobases between the BamHI site in EcoRI fragment D and the right-hand side of EcoRI fragment F2. As published previously, these three Tn5 insertion mutations result in the loss of both external and internal hemolytic activity (14). This suggests that either biosynthesis or processing of precursor molecules into active hemolysin is affected by the insertions. This was confirmed by comparing the map of the hemolysin determinant of pCW2 with those of other hemolysin determinants. All three insertions fall within the region that codes for the structural gene (i.e., hlyA) (Fig. 2). With respect to the sites recognized by restriction enzyme EcoRI, two differences were found between the hemolysin determinant of pCW2 and other plasmid-located hemolysin determinants. One of these was the absence of an EcoRI site within the structural gene, whereas the second difference was the location of the EcoRI site on the left of the hlyC gene. These differences were observed with only one restriction enzyme. Detailed restriction enzyme analysis of cloned DNA fragments is therefore needed to explain the observed differences in virulence exerted by these determinants. Experiments designed to clone the hemolysin determinant of pCW2 into vector pBR322 are presently in progress.

LITERATURE CITED


