Analysis of Hemolytic Determinants of Plasmid pHly185 by Tn5 Mutagenesis

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A nonconjugative hemolysin plasmid, pHly185, was isolated from *Escherichia coli* 185. Tn5 mutagenesis produced nonhemolytic derivatives that either expressed no hemolytic activity or did not secrete activity. These Tn5 insertions were located in three contiguous *EcoRI* fragments. Insertions inactivating hemolysin structural gene(s) were identified on all three *EcoRI* fragments. Mutations affecting the secretory function were clustered in one portion of a single *EcoRI* fragment.

Alpha-hemolysin is an extracellular protein toxin produced by some strains of *Escherichia coli* (21). The genetic information for hemolysin may reside either on plasmids or on the chromosome (8, 10, 14, 16, 17, 19, 22). This communication presents observations on the hemolysin plasmid pHly185 obtained from an *E. coli* strain of porcine origin (16). The hemolytic phenotype coded by this plasmid consists of at least two functions, synthesis and transport. The areas of the plasmid coding for these processes were mapped by restriction analysis of Tn5 insertion mutations.

The original hemolytic strain, 185Hly (21), contained two plasmids: a conjugative plasmid carrying resistance to tetracycline and chloramphenicol and a nonconjugative plasmid coding for the hemolytic phenotype (Hly). The hemolytic determinants from strain 185Hly were transferred by mating into a background without restriction and modification functions, strain JS600 *hsdR hsdM*, a nalidixic acid-resistant derivative of strain C600. Transconjugants were selected for hemolysis and streptomycin resistance. A single hemolytic colony was picked from the lawn of recipients, restreaked on selective medium, and designated JS1. All hemolytic transconjugants were resistant to tetracycline and chloramphenicol. Subsequent attempts to transfer Hly by conjugation in the absence of other plasmids were unsuccessful. pHly185 was clearly not conjugative, but the plasmid could be mobilized by an R plasmid present in the original strain. Strain JS1 was cured of antibiotic resistance with ethidium bromide (5). The cured hemolytic strain, JS2, was used as a source of plasmid DNA (12) to transform strain JS600 *hsdR hsdM* to eliminate the possibility of mutagenic effects of ethidium bromide on the back-

ground. The preparation of competent cells and the transformation conditions used have been described by Dagert and Ehrlich (6). A single hemolytic transformant was purified, and plasmid DNA from this strain was used for subsequent analysis. The hemolytic strain was designated JS3.

pHly185 DNA was digested with the restriction endonucleases *EcoRI*, *HindIII*, *Sall*, *XhoI*, *BamHI*, and *SmaI*. The data depicting the distribution and sizes of the resulting fragments are summarized in Table 1. Summation of the fragment sizes indicated that the plasmid contained an average composition of 73,900 base pairs (b.p.; 70,000 to 82,000), equivalent to approximately 48.7 megadaltons.

The lambda phage vector described by Berg (1, 2) and Berg et al. (3), λ b221 cI857::Tn5, provided an efficient method for introduction of Tn5 insertion mutations into the recipient strain, JS3. The kanamycin-resistant colonies were replicated on selective media, grown at 42°C and then screened by replica plating on sheep blood agar containing kanamycin (30 μ g/ml) at 30°C. Nonhemolytic colonies were purified and tested for lysogeny by spotting a lawn with a suspension of λ b221 cI857. Nonlysogenic, kanamycin-resistant, nonhemolytic strains were selected for further investigation. The frequency of transposition of kanamycin resistance was 4.2×10^{-3} per phage particle, in agreement with previous values (1, 2). A total of 56 of 1,500 kanamycin-resistant clones were not hemolytic (4%), suggesting a Tn5 insertion into the region of hemolysin production.

Linkage of the Tn5 insertion mutations to the Hly plasmid was tested by transformation with purified plasmid DNA, P1 transduction, and identification of the inserted sequence in specific

TABLE 1. Restriction endonuclease fragments of plasmid pHly185^a

Frag- ment	Restriction endonuclease fragment size (b.p.) ^b					
	<i>EcoRI</i>	<i>HindIII</i>	<i>SalI</i>	<i>XhoI</i>	<i>BamHI</i>	<i>SmaI</i>
A	23,000	23,000	24,500	26,500	30,000	18,200
B	16,500	8,140	13,500	16,100	27,250	17,400
C	6,900	5,800	11,800	16,100	22,600	11,000
D	5,600	5,530	11,000	11,900	2,550	10,400
E	5,300	4,300	6,700	1,120		4,750
F	4,790	4,300	1,360			4,100
G	4,300	4,220	1,200			2,550
H	3,180	3,780				2,050
I	3,000	3,700				1,640
J	2,500	3,100				1,380
K	650	2,900				
L		2,450				
M		1,960				
N		1,510				

^a Restriction endonuclease digests of 10- μ g samples of plasmid DNA were prepared. Plasmid DNA was prepared by a modification of the alkaline lysis procedure (4, 12, 15, 20) from 2.5-ml overnight cultures grown in brain heart infusion (Difco Laboratories). Larger amounts of DNA were obtained with a scaled-up version of that procedure followed by cesium chloride dye buoyant density ultracentrifugation. Restriction endonuclease digests were separated by horizontal agarose gel electrophoresis; 0.8% agarose (Marine Colloids) was prepared in a buffer containing 40 mM Tris-20 mM sodium acetate-2 mM EDTA and adjusted to pH 8.3 with acetic acid. Electrophoresis was performed at 50 V and 20 mA overnight, or at 75 V and 50 mA for 4 h. The agarose slab was stained with ethidium bromide (1 μ g/ml) and then destained with water.

^b *BamHI*, *HindIII*, *SalI*, *SmaI*, and *XhoI*, were purchased from Bethesda Research Laboratories. Endonuclease digestion was performed by using the buffers and conditions described by the manufacturer. *EcoRI* was prepared and digestions performed as described by Green et al. (11). The total size (in b.p.) of the plasmid as determined from the sum of the fragment sizes for each endonuclease was: *EcoRI*, 75,720; *HindIII*, 74,710; *SalI*, 70,060; *XhoI*, 71,720; *BamHI*, 82,400; *SmaI*, 73,470.

restriction endonuclease fragments from pHly185. The methods and media used for P1-mediated transduction with P1 *vir* have been described previously (15). P1 lysates of Tn5 mutagenized strains were used to infect strain JS3. Transductants were selected for kanamycin resistance (Km^r) and screened for Hly⁻ on blood agar plates containing kanamycin. A co-transduction frequency between Km^r and Hly⁻ of greater than 95% was observed in all experiments. Therefore, the Tn5 insertions were likely to be localized in the genes coding for hemolysin production and release.

The sites of Tn5 insertions were identified by analysis of restriction endonuclease fragments

of plasmid DNAs from the mutant strains. Plasmid DNA from insertion mutants was purified by cesium chloride buoyant density centrifugation and used to transform the *E. coli* strains JS600 or RR1 *hsdS20* (7). Purified pHly::Tn5 DNA was transformed into strain RR1 to eliminate the remote possibility that restriction fragments and physiological observations might have resulted from some form of residual lambda vector DNA present in the JS600 background. In each case, the fragment patterns of plasmids from strain RR1 were identical to those of plasmids isolated from the strain JS600 background. Plasmid DNA from 42 transposition mutants and from the parental strain were digested with *EcoRI*. The Tn5 insertion sequence contains no *EcoRI* restriction sites (13), and those fragments containing Tn5 insertions were identified by the loss of a fragment present in the digest of intact pHly185 and the concurrent appearance of a new fragment with a molecular size equal to that of the missing fragment and

FIG. 1. Identification of *EcoRI* fragments of pHly185 containing Tn5 insertions that inactivate hemolytic functions. The *EcoRI* fragment containing a Tn5 insertion was identified by loss of a fragment present in the *EcoRI* digest of pHly185 (lane 1) and the concurrent appearance of a new band containing Tn5 plus the missing fragment. Plasmid DNA from mutants Tn5-21 or Tn5-15 (lane 2) indicate the loss of a 650-b.p. fragment (not visualized) and the appearance of a new fragment, c, 6,300 b.p. in size. Plasmid DNA from mutant 2 (lane 3) demonstrates the loss of a 3,180-b.p. fragment, e, and the appearance of a new 9,000-b.p. fragment, b. The *EcoRI* digest of mutant Tn5-4 (lane 4) illustrates the loss of a 5,300-b.p. fragment, d, and a new fragment, a, 11,000 b.p. in size.

FIG. 2. (a) Location of Tn5 insertions on the physical map of pHly185. The locations of Tn5 insertion mutations in the Hly region of pHly185 were determined by analysis of *EcoRI* and *HindIII* digestions of plasmid DNA. Specific sites of insertion were obtained for all mutants illustrated except mutant Tn5-4 (indicated by a question mark and noting 2 possible insertion sites). *EcoRI* and *HindIII* sites are illustrated by the arrows above and below the line, respectively. A phenotypic map corresponding to the mutants is shown below the physical location of the insertion. Dotted lines indicate that the extent of the DNA required for a given phenotype is uncertain. Hly^{ex} represents extracellular hemolysin, and Hlyⁱⁿ refers to intracellular hemolytic activity. *EcoRI* fragments E, H, and K, are 5,300, 3,180, and 650 b.p. respectively. *HindIII* fragments K and M are 3,100 and 2,450 b.p., respectively. (b) Comparison of the physical and genetic maps of plasmids pHly152 and pHly185. The *EcoRI* fragments of each plasmid are illustrated above the line representing the segment of plasmid DNA, and the *HindIII* fragments below the line. To accentuate the physical and functional similarities of pHly152 and pHly185, the physical map of pHly152 presented by Noegel et al. (19) was reoriented so that the cistron coding for transport is depicted on the left (*cisB*), and the cistrons for the production of functional hemolysin (*cisA* and *cisC*) are on the right. The sizes of restriction endonuclease fragments (in parentheses) are in megadaltons.

Tn5 (approximately 5,400 b.p.). We identified three *EcoRI* fragments in which Tn5 had inserted which contained information necessary for hemolysin production and release. Representative gel electrophoresis profiles of the *EcoRI* fragments are shown in Fig. 1.

The relative positions of Tn5 insertions were assessed by analysis of *HindIII* digests of the mutant plasmids. *HindIII* cleaves pHly185 into 14 fragments (Table 1). The Tn5 sequence contains two *HindIII* sites (3). Hence, an insertion into a *HindIII* fragment results in three products after digestion by this enzyme: a central fragment from Tn5, and two flanking fragments in which the sizes are determined by the position of the insertion. Each of the flanking fragments contained an additional 1,115 b.p. derived from Tn5 DNA. Tn5 insertions were found in only two *HindIII* fragments, 3,100 and 2,450 b.p. The

insertions (11 analyzed) residing in the 3,100-b.p. *HindIII* fragment represented insertions in all of the three *EcoRI* fragments. Therefore, the 3,100-b.p. *HindIII* piece was considered to span a sequence encompassing two *EcoRI* sites. The central fragment of Tn5 was observed in digests of all insertion mutants. Only one strain, Tn5-4 (Fig. 1, lane 3), demonstrated the loss of the *HindIII* 2,450-b.p. fragment.

The data indicated that the three *EcoRI* fragments containing Tn5 insertions were overlapped by the *HindIII* 3,100-b.p. fragment. The linear sequence of the *EcoRI* fragments was thought to be *EcoRI* 3,180, *EcoRI* 650, and *EcoRI* 5,300. Evidence supporting this sequence was derived from analysis of the new fragments observed in the *HindIII* digests that contained the 1,115-b.p. ends of Tn5. Restriction digests of plasmid DNA from mutant Tn5-12, with an

insert in *EcoRI* 3,180, contained two new *HindIII* fragments of approximately equal sizes and, therefore, must represent an insertion close to the center of the *HindIII* 3,090. Tn5-21 contained two unequal fragments of 3,400 and 2,270 b.p., and this insertion can be considered to be to the right of that in Tn5-12, because this insert was located in *EcoRI* 650. By comparison with Tn5-12, the new larger *HindIII* fragment from Tn5-21 represented the left side of *HindIII* 3,100, and the smaller new fragment represented the right side. Mutants Tn5-15, Tn5-17, and Tn5-28, also with inserts located in *EcoRI* 650, yielded *HindIII* digest patterns that were indistinguishable from each other, containing new fragments of sizes 2,140 and 3,390 b.p. The smaller fragment constituted the right portion of the *HindIII* 3,100-b.p. fragment. This series of insertions was positioned to the right of the Tn5-21 insertion. Estimates of the *HindIII* products and their sizes were used to order the remainder of the Tn5 mutants both to the right and left of the Tn5-12 insertion. These results were oriented in a physical map (Fig. 2a) of the Tn5 insertions, relative to each other and to the *EcoRI* and *HindIII* restriction sites.

The production of intracellular hemolysin was tested in the Tn5 mutants to determine whether any of the insertions had occurred in genes coding for functions related to the externalization process. There were 42 Tn5 Hly⁻ mutants tested and 19 were found to produce intracellular hemolysin. The mutants producing internal hemolysin were found to have Tn5 insertions only in the *EcoRI* 3,180 fragment. Those mutants that produced no internal or external hemolysin were found to have Tn5 insertions in all three *EcoRI* fragments, *EcoRI* 5,300, *EcoRI* 3,180 and *EcoRI* 650. The data imply that at least two physiological functions are necessary for the hemolytic phenotype and that the secretory functions are coded on a region contiguous to the structural gene for hemolysin.

Another hemolysin plasmid, pHly152, has been studied with Tn3 insertion mutants and recombinant plasmids (18). Genetic analysis demonstrated three cistrons necessary for hemolysis (19). Restriction mapping of the region showed that the genetic material was located on three contiguous *EcoRI* fragments. In the present study, parallel restriction digests were performed on pHly152 DNA (data not shown) isolated from an *E. coli* K-12 strain, and the fragment distribution showed no similarities to that derived from pHly152 nor do these fragment patterns resemble those published for this plasmid (19). However, the previous studies with pHly152 suggest that the arrangement of genetic units on this Hly plasmid bears similarities to pHly185. With pHly152, three *EcoRI* fragments

with information for Hly production and export were also identified (18, 19). According to these studies, three cistrons transcribed from a single promoter code for the hemolytic phenotype (7). A comparison of the restriction site structure of pHly185 with data reported for pHly152 is summarized in Fig. 2b. The size of these fragments was different but the arrangement was similar. The structural genes for hemolysin coded by both plasmids overlapped all three *EcoRI* fragments, and the export genes were in *EcoRI* fragments of similar sizes. The homology of the Hly genes coded by pHly152 and pHly185 is currently unknown. However, known hybridization data suggested homology between Hly genes on several plasmids (7-9).

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