JOURNAL OF BACTERIOLOGY, Feb. 1983, p. 846–851
0021-9193/83/020846-06$02.00/0

Vol. 153, No. 2

Relationship Between Plasmid and Chromosomal Hemolysin Determinants of *Escherichia coli*

DOROTHEE MÜLLER, COLIN HUGHES, AND WERNER GOEBEL*

Institut für Genetik und Mikrobiologie, Universität Würzburg, D-8700 Würzburg, West Germany

Received 29 July 1982/Accepted 16 November 1982

Plasmid hemolysin (*hly*) determinants have been shown previously to comprise three cistrons (*hlyA*, *hlyB*, *hlyC*), coding for the synthesis and transport of hemolysin. Using recombinant plasmids as specific probes for these cistrons, we were able to analyze the chromosomal *hly* determinants of nine *Escherichia coli* strains which belonged to serotypes O4, O6, O18, and O75 and were isolated from urinary tract infections and fecal flora. The chromosomal *hly* genes shared extensive sequence homology with the cloned plasmid *hly* determinant. Nevertheless, small differences were observed, and these were found to lie mainly within cistron A (*hlyA*), which has been shown to determine the hemolysin protein itself. These fine variations were not specific for the O-serotype.

Hemolytic *Escherichia coli* strains can be isolated from normal animal intestinal flora and from intestinal and extraintestinal infections of humans. Whereas the percentage of hemolytic strains is usually low (ca. 10%) in the intestines of healthy individuals and patients with diarrheal diseases, it is very high (35 to 60%; 3, 6, 14) among those strains causing extraintestinal infections, such as those of the urinary tract (UTI) and blood. This has lead to the assumption that hemolysin (Hly), the extracellular protein synthesized by these strains (13, 19), may act as a virulence factor for extraintestinal infections. Recent data obtained from epidemiological studies (6, 10, 12) and in vivo virulence tests (21, 22) support this view.

Previous genetic and biochemical studies, which were performed mainly on fecal strains isolated from animals, indicated that hemolysin genes are carried on large transmissible plasmids (5, 8, 9, 18), and from one of these plasmids, pHly152, we were able to clone a DNA fragment of which about 7.5 kilobases (kb) were shown to be essential for hemolysin synthesis. This segment carries at least three cistrons involved in the synthesis and transport of hemolysin (15, 16, 19), and hybridization experiments performed with the cloned cistrons and several Hly plasmids demonstrated that all Hly plasmids carry virtually indistinguishable *hly* determinants regardless of their origin and overall relatedness (4). Recent studies on hemolytic *E. coli* isolated from humans have indicated that in practically all of these strains, the *hly* determinant seems to reside on the chromosome rather than on plasmids (7, 21; unpublished data). This paper describes the characterization of chromosomal *hly* determinants from various hemolytic *E. coli* strains and shows that these determinants are quite similar to those borne on plasmids.

MATERIALS AND METHODS

Bacteria. The *E. coli* strains studied are listed in Table 1 or have been described elsewhere (15).

Plasmids. Hemolytic plasmid pHly152 is large (62.1 kb) and self transmissible and belongs to the incompatibility group I_2 (15). The three cistrons necessary for hemolysin production, *hlyA*, *hlyB*, and *hlyC*, are spanned by several *Hin*dIII and *Eco*RI fragments (see Fig. 4), and the recombinant plasmids derived from these fragments and vector pACYC184 (2) have been described previously (15). We used pANN250 (containing *Eco*RI-G) to test for homology to *Eco*RI-G, *Hin*dIII-C, and *Hin*dIII-H; we used pANN215 (*Hin*dIII-C) to test for homology to *Hin*dIII-C and *Eco*RI fragments F, L, and G and pANN202 (*Hin*dIII-E) to test for homology to *Hin*dIII-E and *Eco*RI-F of pHly152.

Isolation of chromosomal DNA. Cells from a stationary culture in nutrient broth were centrifuged, washed once in 0.15 M NaCl–0.1 M EDTA and suspended in 25% sucrose–10 mM Tris-hydrochloride, pH 7.5. After the addition of a 1/20 volume of 0.25 M EDTA, the cells were treated with lysozyme (10 mg/ml) followed by 1% sodium dodecyl sulfate, both for 30 min at 37°C. The resulting lysate was shaken with pronase (100 μg/ml) for 3 h until clear and then shaken with the same volume of an isoamyl alcohol-chloroform (24:1) mixture for 40 min. The two phases were separated by low-speed centrifugation, and after the addition of absolute ethanol, the DNA was isolated out of the aqueous solution by subsequent spinning onto a glass rod. The DNA was then dried, dissolved in 10 mM Tris-hydrochloride (pH 7.5), and purified once in a CsCl gradient.

Isolation of plasmid DNA. Plasmid DNA was isolated as described previously (4, 11).

TABLE 1. Hemolytic *E. coli* strains

E. coli strain	Source[a]	Serotype
764	Feces (WG)	O18
768	Feces (WG)	O18
251	Urine (UK)	O18
536	Urine (WG)	O6
442	Urine (UK)	O6
367	Urine (UK)	O4
530	Urine (UK)	O4
341	Urine (UK)	O75
372	Urine (UK)	O75

[a] Strains were obtained from hospitals in West Germany (WG) and the United Kingdom (UK).

Preparation of radioactive probes for hybridization. Radioactive DNA probes were obtained by the nick translation of recombinant plasmids with a mixture of all four [α-^{32}P]-dNTPs as described previously (17).

Cleavage with restriction enzymes and electrophoresis of chromosomal DNA. The chromosomal DNA was treated with restriction enzymes *Eco*RI and *Hin*dIII, and the resulting fragments were separated by agarose gel electrophoresis on 1% agarose as described previously for plasmid DNA (4).

Hybridization and autoradiography. The transfer of DNA fragments from agarose gels to nitrocellulose filters and the washing and autoradiography of the filters after hybridization were performed as described previously (20). The filters were hybridized in 100% formamide (high stringency) at 43°C for 3 days.

RESULTS

Chromosomal carriage of the *hly* determinants in the *E. coli* strains analyzed. Nine hemolytic *E. coli* strains isolated from humans and belonging to O serotypes O4, O6, O18, and O75 (Table 1) were examined with regard to the genetic determinants responsible for hemolysin synthesis. Since previous studies on hemolytic *E. coli* strains, mainly isolated from animal sources (8, 9, 18), had shown the *hly* determinant to be located on transmissible plasmids, these strains were first screened for plasmids involved in hemolysin synthesis. This was performed by (i) the physical characterization of extrachromosomal DNA in cleared lysates (1a) and CsCl-ethidium bromide (EtBr) density centrifugation, (ii) the attempted transfer of the *hly* determinant into *E. coli* K-12 recipient strains by conjugation under various conditions (15), and (iii) the mobilization of possible non-self-transmissible hemolysin plasmids by a transfer factor. None of these strains carried plasmids as detected by the agarose gel electrophoresis of the cleared lysates of CsCl-EtBr gradients (Fig. 1). The diffuse DNA band seen in each cleared lysate on the agarose gel represents residual chromosomal DNA, which was not entirely removed during the clearing spin. In the cleared lysate of the *E. coli* K-12 strain containing pHly152 (62.1 kb), the additional plasmid band above the diffuse chromosomal band is clearly visible, although it is a low-copy-number plasmid. With this cleared lysate procedure, we could detect low-copy plasmids with sizes of up to 200 kb (even in wild-type strains). All Hly plasmids isolated until now have been of considerably smaller size, and their isolation from *E. coli* strains has been achieved without problems. Hemolytic K-12 transconjugants were not obtained when these strains were mated with *E. coli* K-12 either directly or after the introduction of F′ *lac* or R1 into the hemolytic wild-type strains. Conjugation was always carried out both in liquid broth and on solid agar. These conjugation experiments do not rule out transmissible Hly plasmids which transfer at frequencies of less than 10^{-6}, but such low transfer frequencies have never been obtained with known Hly plasmids (5, 8, 13). This evidence suggests that the *hly* determinants are on the chromosome.

Comparison of the chromosomal *hly* determinants from different *E. coli* strains with that of plasmid pHly152. Recent studies on the relationship between *hly* determinants carried on different plasmids have indicated high sequence homology regardless of the degree of similarity within other parts of the plasmids (4). It has been demonstrated (6, 10, 12) that among *E. coli* causing urinary tract infections hemolysin is predominantly synthesized by strains belonging to O serotypes O4, O6, O18, and O75, and data suggest that these hemolysins are generally determined not by plasmids but rather by chromosomal genes (7, 14). The data described above indicate that the *hly* determinants of the nine

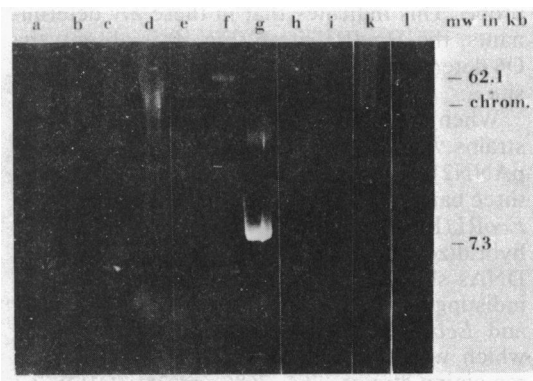

FIG. 1. Electrophoresis of cleared lysates of the nine Hly$^+$ *E. coli* isolates listed in Table 1 and of *E. coli* K-12 strains with Hly plasmid pHly152 (lane f) and recombinant plasmid pANN215 (lane g). Lysates of strains 764 (lane a), 768 (lane b), 251 (lane c), 536 (lane d), 442 (lane e), 367 (lane h), 530 (lane i), 341 (lane k), and 372 (lane l). mw, Molecular weight.

hemolytic *E. coli* strains studied, which belong to the same four O serotypes, are also of chromosomal origin. The homology of the chromosomal DNAs of these strains with the restriction fragments which cover most of the plasmid *hly* determinant, i.e., *Hin*dIII-E, *Hin*dIII-C, *Hin*dIII-H, *Eco*RI-F, *Eco*RI-L, and *Eco*RI-G (16; see Fig. 4), was tested by Southern hybridization. Hybridizations were performed with nick-translated ^{32}P-labeled vector (pACYC184) DNA and two recombinant DNAs carrying *Hin*dIII-C (pANN215) or *Eco*RI-G (pANN250) inserted into pACYC184. The inserts of these two recombinant plasmids cover most of *hlyA* and *hlyB* of the plasmid-encoded *hly* determinant (6a), 15; see Fig. 4). Chromosomal DNAs of the nine hemolytic *E. coli* strains were cleaved with *Eco*RI and *Hin*dIII. Nick-translated pACYC184 did not hybridize to any of these DNAs (Fig. 2), but hybridization occurred with specific fragments when the nick-translated *hly*-specific probes were used. The results of these hybridizations for five strains, each representing one hybridization pattern are shown in Fig. 3A and B. *Hin*dIII-C seems to be present in the *hly* determinants of five of the nine strains (the two O75 strains 341 and 372, the two O4 strains 367 and 530, and the O6 strain 442) since a fragment indistinguishable in size from *Hin*dIII-C was labeled when the *Hin*dIII-cleaved chromosomal DNAs of these strains were hybridized with nick-translated pANN215 [^{32}P] DNA. In the other four strains, three belonging to O18 and one to O6, a larger *Hin*dIII fragment was labeled. DNA of all three O18 strains hybridized in a fragment of the same size (4.1 kb; Fig. 3A), whereas DNA of the O6 isolate hybridized in an even larger one (11.5 kb), which was also labeled when *Hin*dIII-E (pANN202) was used as a probe. This indicates that in these *hly* determinants, the *Hin*dIII site b (Fig. 4) is absent, the O6 determinant lacking, in addition, the *Hin*dIII site a.

When the chromosomal DNAs of the nine strains were cut by *Eco*RI and hybridized with pANN215, homology was expected in at least three bands because *Hin*dIII-C was cut twice by *Eco*RI (Fig. 4). The two O6 strains, 536 and 443, hybridized in line with this assumption: both DNAs showed homology in bands which were indistinguishable in size from those of *Eco*RI-G and *Eco*RI-L (Fig. 3A) and in further bands which were larger than that of *Eco*RI-F. The remaining strains, 764, 768, and 251 (O18); 367 and 530 (O4); 341 and 372 (O75), hybridized in two bands (Fig. 3A). One of these, which is the same size (2.8 kb) in all strains, probably represents the equivalent of *Eco*RI-G because it was also labeled when this fragment was used as a probe. The second hybridizing band seems to be

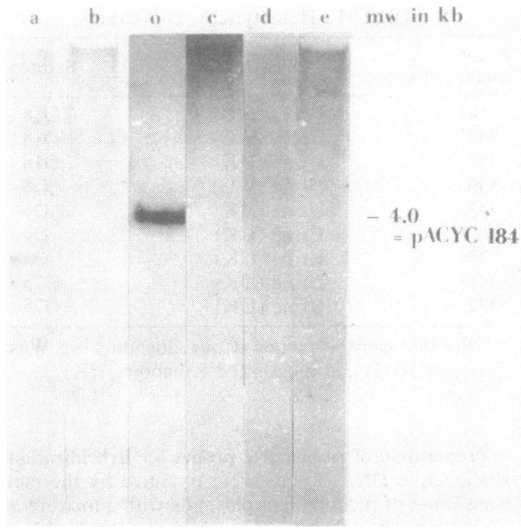

FIG. 2. Hybridization patterns obtained with *Hin*dIII-cleaved chromosomal DNA of five hemolytic *E. coli* strains of the four O types studied and ^{32}P-labeled nick-translated pACYC184 DNA. Lane a, strain 367 (O4); lane b, strain 372 (O75); lane c, strain 442 (O6); lane d, strain 536 (O6); and lane e, strain 764 (O18); lane O represents the control, i.e., hybridization of *Hin*dIII-cleaved pACYC184 with the labeled pACYC184 probe. mw, Molecular weight.

different in all strains, being always larger than *Eco*RI-F of pHly152 (Fig. 4), with the one exception of strain 251 (O18). None of the seven strains showed hybridization in a small band which could represent *Eco*RI-L. Therefore, we assume that in all these determinants the *Eco*RI site between *Eco*RI-F and *Eco*RI-L (Fig. 4) is missing and the homology to the *Eco*RI-L fragment resides in the bands hybridizing with *Eco*RI-F.

The *Hin*dIII fragment next to *Hin*dIII-C is *Hin*dIII-H (Fig. 4), and a fragment indistinguishable in size from this was labeled in the *Hin*dIII-cut chromosomal DNA of all nine strains when *Eco*RI-G (pANN250), which overlaps with *Hin*dIII-C and *Hin*dIII-H, was used as a hybridization probe. As expected, those DNAs which showed homology with *Hin*dIII-C in a *Hin*dIII fragment of the same size when pANN215 was used as a probe also hybridized to the same *Hin*dIII fragment when pANN250 (*Eco*RI-G) was used. The DNAs of those strains which hybridized to *Hin*dIII-C in a larger fragment also hybridized in the same large *Hin*dIII fragments when pANN250 was used as a probe (Fig. 3B). When the chromosomal DNAs of the nine hemolytic strains were cut with *Eco*RI and hybridized with pANN250, only two strains, 536 (O6) and 442 (O6), showed homology in a fragment indistinguishable in size from *Eco*RI-G (3.2 kb). The

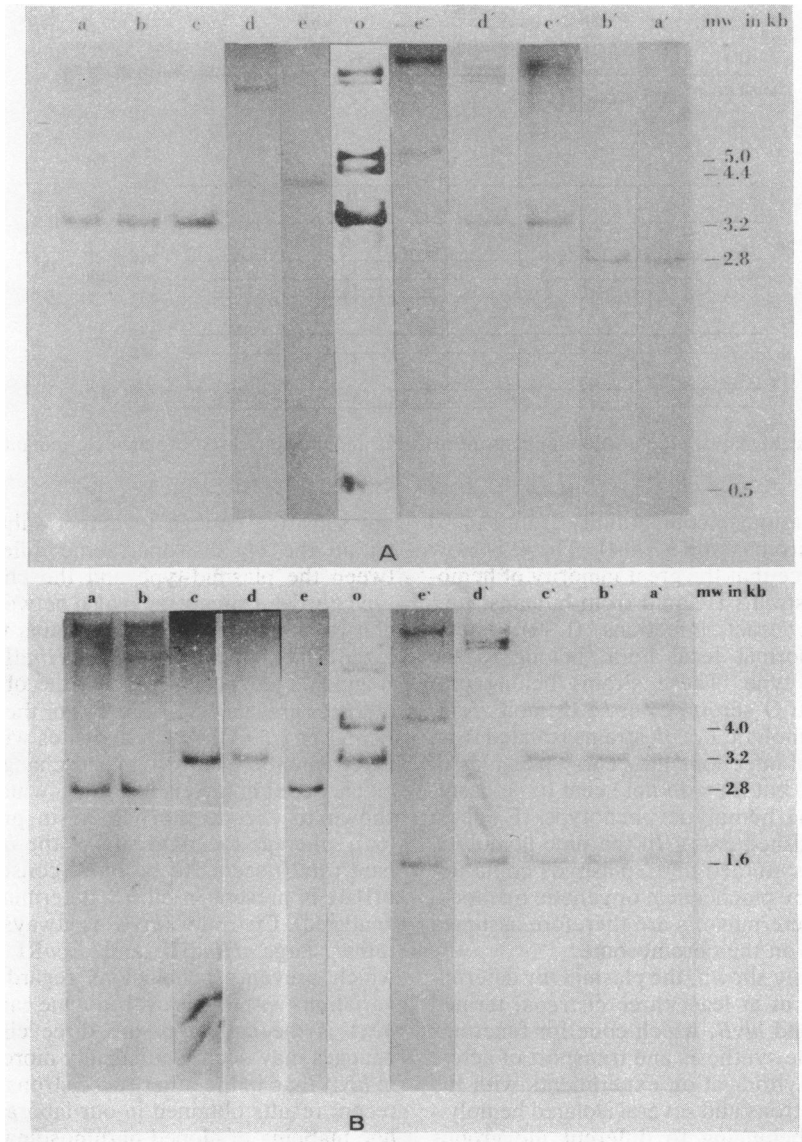

FIG. 3. Hybridization patterns obtained (A) with *Hin*dIII- (lanes a to e) and *Eco*RI (lanes e' to a')-cleaved chromosomal DNA isolated from five hemolytic *E. coli* strains representing the four O serotypes studied and ^{32}P-labeled nick-translated pANN215 DNA and (B) with *Eco*RI- (lanes a to e) and *Hin*dIII- (lanes e' to a')-cleaved chromosomal DNA of the same strains and ^{32}P-labeled pANN250 DNA. Lanes a and a', strain 367; lanes b and b', strain 372; lanes c and c', strain 442; lanes d and d', strain 536; lanes e and e', strain 764; lane o, pHly152 DNA cleaved with *Eco*RI and pANN250 cleaved with *Hin*dIII (B). The hybridization patterns of strains 530 (same as 367), 341 (same as 372), 768 (same as 764), and 251 are not shown in this figure, but are included in Fig. 4. mw, Molecular weight.

three O18 strains 764, 768, and 251; the two O75 strains, 341 and 372; and the O4 strains, 367 and 530, hybridized in a fragment (2.8 kb) which was smaller than *Eco*RI-G (Fig. 3B). These findings concur with the homology observed when *Hin*dIII-C was hybridized to *Eco*RI-cut DNA as described above. Figure 4 summarizes the similarities and differences observed between the chromosomal *hly* determinants analyzed and the known plasmid *hly* determinant (4, 15).

DISCUSSION

The data presented here and elsewhere show that the genes determining hemolysin synthesis in *E. coli* may be carried either on transmissible

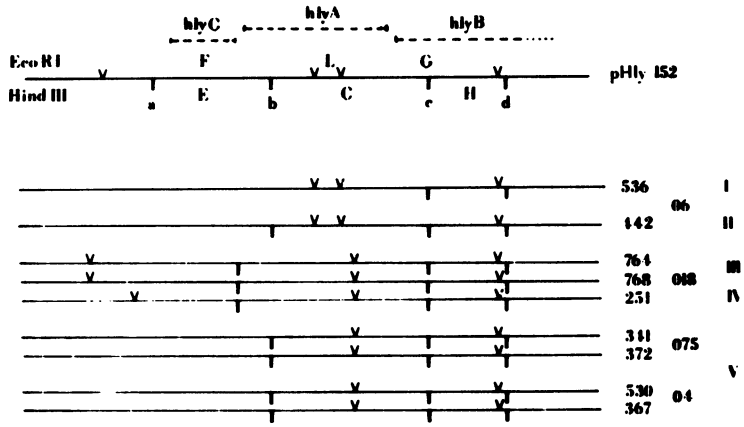

FIG. 4. Physical maps of the *hly* determinants of pHly152 and the nine chromosomal determinants analyzed.

plasmids of various incompatibility groups (4, 5) or on the chromosome (7, 14). There is now ample evidence that the great majority of hemolytic *E. coli* strains isolated from humans, i.e., from urinary tract infections (UTI), bacteremias, or normal fecal flora, belong to the chromosome type. These strains belong predominantly to O serotypes 4, 6, 18, and 75 (6, 12). Some hemolytic *E. coli* strains isolated from extraintestinal infections may carry plasmids of various sizes, but they do not seem to be correlated with the hemolytic phenotype (F. de la Cruz, unpublished data). In the nine hemolytic *E. coli* strains studied here, plasmids could not be identified by biochemical or genetic methods, and the *hly* determinants are therefore assumed to be located on the chromosome.

As previously shown, the plasmid *hly* determinant consists of at least three cistrons, termed *hlyC*, *hlyA*, and *hlyB*, which code for functions involved in the synthesis and transport of active hemolysin. Hybridization experiments with the cloned *hly* cistrons and several isolated hemolytic plasmids belonging to different *inc* groups have indicated that the *hly* determinants are very similar in all plasmids, regardless of the extent of homology within other parts of these extrachromosomal genomes (4).

In the present study, these investigations have been extended to chromosomal *hly* determinants of *E. coli*. Again using recombinant plasmids carrying various parts of the plasmid-type *hly* determinant as hybridization probes, we could show by Southern hybridization that the chromosome-type *hly* determinants in all strains tested show extensive sequence homology with the plasmid-type *hly* determinant.

Hybridization always occurs with specific DNA fragments when distinct segments bearing *hlyA* or *hlyB* of the plasmid-type *hly* determinant are used as probes. Although this method was not sensitive enough to detect subtle changes within the *hly* cistrons, some differences between the plasmid-type and the chromosome-type *hly* determinants and also between different chromosome-type *hly* determinants were recognized. Due to the use of cloned *Hin*dIII or *Eco*RI fragments as specific probes, the observed differences are caused by the loss or the addition of *Eco*RI or *Hin*dIII restriction sites within the *hly* determinants. The recognized changes seem to be clustered in the cistron *hlyA*, which has been shown to encode the hemolysin protein itself (6a), whereas the right site of the *hly* determinant (*hlyB*) seems to be more conserved (*Hin*dIII-H is present in all *hly* determinants so far analyzed). Cistron C (*hlyC*) is always located on rather large *Hin*dIII and *Eco*RI fragments, which prevents conclusions regarding genetic variations within this cistron. One can argue that *hlyA* is the largest of the three cistrons, and changes may occur statistically more frequently in *hlyA* than in the other two cistrons. However, recent results obtained in our laboratory by the fine mapping of cloned chromosomal *hly* determinants from four different O serotypes confirm that *hlyA* is the most variable cistron (1). From the data presented here and from unpublished data, it appears that these changes within *hlyA* are not random but fixed for a given type of *hly* determinant. We have tried to point this out in Fig. 4 by arranging the analyzed *hly* determinants into five groups. Although we are aware that the number of *hly* determinants analyzed here is rather small, preliminary data from this and another laboratory (F. de la Cruz, personal communication) indicate that the *hly* determinants of other *E. coli* strains also fall into one of these groups. This observation is of particular interest in the light of recent findings which show that *hly* determinants from different sources exhibit differences in their virulence in

animal models (21, 22; unpublished data). In UTI, *hly* synthesis is predominantly found among strains belonging to the four *E. coli* serotypes O4, O6, O18, and O75. The analysis of the *hly* determinants from such strains, however, shows that specific types of *hly* determinants do not seem to be correlated with particular O serotypes.

ACKNOWLEDGMENTS

We acknowledge the financial support obtained through SFB 105 Teilprojekt A-12 and thank Anthony Roberts of Charing Cross Hospital, London, and G. Schröter of the University Würzburg for providing and serotyping strains. We are also grateful to Fernando de la Cruz for communicating as-yet-unpublished data.

LITERATURE CITED

1. **Berger, H., J. Hacker, A. Juarez, C. Hughes, and W. Goebel.** 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. J. Bacteriol. **152**:1241–1247.

1a. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids. Res. **7**:1513–1523.

2. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multy copy DNA cloning vehicles derived from the P15 A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.

3. **Cooke, E. M., and S. P. Ewins.** 1975. Properties of strains of *Escherichia coli* isolated from a variety of sources. J. Med. Microbiol. **8**:107–111.

4. **De la Cruz, F., D. Müller, J. M. Ortiz, and W. Goebel.** 1980. Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. J. Bacteriol. **143**:825–833.

5. **De la Cruz, F., J. C. Zabala, and J. M. Ortiz.** 1979. Incompatibility among α-haemolytic plasmids studied after inactivation of the α-haemolytic gene by transposition of Tn802. Plasmid **2**:507–519.

6. **Evans, D. T., D. G. Evans, C. Höhne, M. H. Noble, E. V. Haldane, H. Lior and L. S. Young.** 1981. Haemolysin and K-antigens in relation to serotype and haemagglutination type of *Escherichia coli* isolated from extraintestinal infections. J. Clin. Microbiol. **13**:171–178.

6a. **Goebel, W., and J. Hedgpeth.** 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. J. Bacteriol. **151**:1290–1298.

7. **Goebel, W., A. Noegel, U. Rdest, D. Müller, and C. Hughes.** 1981. Structure and epidemiological spread of the haemolysin determinant of *Escherichia coli*, p. 43–50. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Publishing Corp., New York, N.Y.

8. **Goebel, W., B. Royer-Pokora, W. Lindenmaier, and H. Bujard.** 1973. Plasmids controlling synthesis of hemolysin in *Escherichia coli*. I. Molecular properties. J. Bacteriol. **118**:964–973.

9. **Goebel, W., and H. Schrempf.** 1971. Isolation and characterization of supercoiled circular deoxyribonucleic acid from β-hemolytic strains of *Escherichia coli*. J. Bacteriol. **106**:311–317.

10. **Green, C. P., and V. L. Thomas.** 1981. Haemagglutination of human type O erythrocytes, haemolysin production and serogrouping of *Escherichia coli* isolates from patients with acute pyelonephritis, cystitis and asymptomatic bacteriuria. Infect. Immun. **31**:309–315.

11. **Grinstedt, J., P. M. Bennett, S. Higginson, and M. H. Richmond.** 1978. Regional preference of insertion of Tn501 and Tn802 into RP1 and its derivatives. Mol. Gen. Genet. **166**:313–320.

12. **Hughes, C., R. Phillips, and A. P. Roberts.** 1982. Serum resistance among *Escherichia coli* strains causing urinary tract infection in relation to the carriage of hemolysin, colicin and antibiotic resistance determinants. Infect. Immun. **35**:270–275.

13. **LeMinor, S., and E. LeCoueffic.** 1975. Studies on haemolysins of Enterobacteriaceae. Ann. Microbiol. (Paris) **126**:313–332.

14. **Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow.** 1978. Association of hemolysin production hemagglutination of human erythrocytes and virulence for chicken embryos at extraintestinal *Escherichia coli* isolates. Infect. Immun. **20**:50–51.

15. **Noegel, A., U. Rdest, and W. Goebel.** 1981. Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. **145**:233–247.

16. **Noegel, A., U. Rdest, W. Springer, and W. Goebel.** 1979. Plasmid cistrons controlling synthesis and excretion of the exotoxin α-hemolysin of *E. coli*. Mol. Gen. Genet. **175**:343–350.

17. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.

18. **Smith, H. W., and S. Halls.** 1967. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. J. Gen. Microbiol. **47**:153–167.

19. **Springer, W., and W. Goebel.** 1980. Synthesis and secretion of hemolysin by *Escherichia coli*. J. Bacteriol. **144**:53–59.

20. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

21. **Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Haemolysin contributes to virulence of extraintestinal *E. coli* infections. Nature (London) **294**:665–667.

22. **van den Bosch, J. F., P. Postma, J. De Graaff, and D. M. Maclaren.** 1981. Haemolysis by urinary *Escherichia coli* and virulence in mice. J. Med. Microbiol. **14**:321–331.