INFECTION AND IMMUNITY, Jan. 1982, p. 32-37
0019-9567/82/010032-06$02.00/0

Vol. 35, No. 1

# Hemolysin Plasmid Coding for the Virulence of a Nephropathogenic *Escherichia coli* Strain

CORNELIS WAALWIJK,[1]* JOHANNES F. VAN DEN BOSCH,[1] DAVID M. MACLAREN,[1] AND JOHANNES DE GRAAFF[2]

*Department of Medical Microbiology, School of Medicine,[1] and Department of Oral Microbiology, School of Dentistry,[2] Free University, 1007 MC Amsterdam, The Netherlands*

Received 5 June 1981/Accepted 31 August 1981

The nephropathogenic *Escherichia coli* strain P673 was shown to harbor two plasmids with molecular sizes of 70 and 41 megadaltons, respectively. The 70-megadalton plasmid, pCW1, coded for tetracycline resistance, whereas hemolysin production was coded by the 41-megadalton plasmid, pCW2. Plasmid pCW1 proved to be self-transmissible, in contrast to pCW2. Transfer of the hemolysin character was associated with the appearance of a 110-megadalton plasmid, pCW3. The incompatibility of pCW3 with both native plasmids and restriction enzyme analysis led to the conclusion that pCW3 is a cointegrate of pCW1 and pCW2. pCW2, carrying the hemolytic determinant, is involved in the nephropathogenic character of strain P673, because (i) elimination of pCW2 from P673 was associated with a loss of virulence and (ii) the nephropathogenicity of the avirulent mutant could be restored by reintroduction of pCW2 DNA as part of a cointegrate structure.

Hemolysin production is a rather general phenomenon of *Escherichia coli* strains. In some cases, the ability to produce hemolysin has been shown to be transmissible to nonhemolytic *E. coli* strains as well as to *Salmonella* and *Shigella* strains (5, 7, 8, 20, 21). It has been found possible to "cure" *E. coli* strains for hemolysin production (15, 21). From these results, it has been concluded that hemolysin production in *E. coli* can be mediated by plasmids. Furthermore, chromosomal inheritance of the hemolytic phenotype has been observed as well (13, 16; Waalwijk and de Graaff, unpublished data).

Several studies have shown that hemolysin production is more frequently found in *E. coli* isolates from urinary tract infections and other extraintestinal sources than in fecal isolates from healthy persons (2, 14, 23). These results indicate that hemolysin may be a virulence factor of *E. coli*, and various animal models have been used to test this hypothesis.

Hemolytic strains induce experimental pyelonephritis in rats without obstruction of the renal collecting tubules, whereas nonhemolytic strains do not (6). After intranasal instillation of hemolytic strains, mice rapidly succumb due to a hemorrhagic lung edema (5, 12). Furthermore, hemolysin is produced by 85% of the extraintestinal isolates that are virulent in the chicken embryo test, whereas none of the avirulent strains is hemolytic (13). On the other hand, nonhemolytic mutants obtained after mutagenesis are no longer virulent in these animal models (5, 6, 11).

Recently, we described a hematogenous mouse model that differentiates *E. coli* strains according to their virulence level: group I strains are avirulent; nephropathogenic group II strains are specifically virulent for mouse kidneys; and group III strains exhibit a more generalized virulence for various organs (24, 25). In both groups of virulent strains, the frequency of hemolytic strains is significantly higher than in the avirulent group of strains. However, only in nephropathogenic strains, the elimination of the hemolytic property resulted in almost all cases in a loss of virulence (25).

In this study, the role of hemolysin in the virulence of a nephropathogenic strain was studied by elimination and subsequent reintroduction of the hemolytic property.

## MATERIALS AND METHODS

**Bacterial strains.** Table 1 lists the bacterial strains used.

**Plasmid isolation.** Plasmid DNA was isolated by a modification of the methods described by Birnboim and Doly (1) and Portnoy et al. (17). Cells were grown into log phase in 50 ml of nutrient broth (Oxoid, London) to $10^9$ cells/ml, harvested, washed with 50 ml of 50 mM Tris-hydrochloride buffer, pH 8.0, containing 10 mM EDTA (TE), and resuspended in 1 ml of TE. Two milliliters of lysis buffer (TE plus 6% sodium dodecyl sulfate, pH 12.45) was added, and the tubes were mixed by rapid but gentle inversion. After 20 min

TABLE 1. *E. coli* strains used

| Strain | Relevant properties[a] | Plasmid size (Md) | Virulence[b] | Derivation | Source |
|---|---|---|---|---|---|
| P673 | Tc; Hly | 70; 41 | II | Natural isolate | L. Emödy (5) |
| P673/II | Sm; Lac$^-$; Tc | 70 | I | P673 (Act D)[c] | L. Emödy (5) |
| J53-1 | Nx | None | I | | N. Datta |
| W1485Nx | Nx | None | NT | | Phabagen Collection[d] |
| J53-1 (p673) | Nx; Tc; Hly | 110 | I | P673 × J53-1 | L. Emödy (5) |
| CW15 | Nx; Tc; Hly | 110 | NT | P673 × W1485Nx | This study |
| CW16 | Nx; Tc | 70 | NT | P673 × W1485Nx | This study |
| CW22 | Hly | 41 | II | P673 (Act D) | This study |
| CW35 | Sm; Lac$^-$; Tc; Hly | 110 | II | CW22 × P673/II | This study |

[a] Tc, Sm, and Nx, resistance to tetracycline, streptomycin, and nalidixic acid, respectively; Hly, production of hemolysin.

[b] Determined in mice after intravenous injection: I, avirulent; II, nephropathogenic; NT, not tested.

[c] Act D, cured with 40 μg of actinomycin D per ml.

[d] Phabagen Collection, Laboratory for Microbiology, Utrecht, The Netherlands.

at 37°C, the samples were neutralized, and single-stranded DNA was precipitated by the addition of 1.5 ml of 3 M sodium acetic acid, pH 4.8. After incubation for 16 h in an ice bath and centrifugation for 10 min at 9,000 × *g*, the supernatant was transferred to another tube. The plasmid DNA was precipitated by the addition of 2.5 volumes of cold ethanol (96%) and placed at −20°C for at least 1 h. The precipitate was collected by centrifugation and washed once with cold ethanol (70%). After drying under vacuum, the precipitate was suspended in 200 μl of 10 mM Tris-hydrochloride, pH 7.5. Plasmid DNA was analyzed by electrophoresis in Tris-borate-buffered agarose gels (17). Molecular weights were estimated by comparing the mobilities of the plasmids with those of plasmids of known molecular size ranging from 36 to 120 megadaltons (Md).

**Plasmid elimination.** Curing was performed according to the method of Mitchell and Kenworthy (15). Strain P673 was grown in nutrient broth containing 40 μg of actinomycin D per ml (Sigma Chemical Co.), and after 24 or 48 h, subcultures were plated on blood agar. Tetracycline-susceptible mutants were identified by replica plating on nutrient agar containing 20 μg of tetracycline per ml.

**Conjugation.** Mating experiments were carried out by mixing equal volumes of log-phase-grown cultures of donor and recipient strains and overnight incubation of the mixture at 37°C. Filter matings were performed by filtering 2 ml of overnight cultures of both donor and recipient cells through a membrane filter (0.45-μm pore size) followed by incubation of the filter on nutrient agar plates at 37°C for 18 h. Filters were washed with fresh medium, and transconjugants were selected on blood agar supplemented with either 50 μg of nalidixic acid or 50 μg of streptomycin per ml. After overnight incubation at 37°C, these plates were replicated on nutrient agar containing 20 μg of tetracycline per ml. Tetracycline-resistant and hemolytic transconjugants were isolated.

**Digestion of plasmid DNA with restriction enzyme.** The restriction enzyme *Eco*RI was purchased from Boehringer Mannheim Corp. Plasmid DNA was digested for 2 h at 37°C in 100 mM Tris-hydrochloride, pH 7.5, containing 50 mM NaCl and 10 mM $MgCl_2$. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM. Digested DNA was analyzed on 0.8% agarose slab gels.

**Virulence test.** The virulence level of the strains was tested by determination of the number of viable bacteria in mouse kidneys for 8 h after intravenous injection of approximately $2 \times 10^8$ log-phase cells, as described previously (23). When *E. coli* strains are tested in this way, three different patterns can be observed. Avirulent group I strains show a rapid initial decrease in viable count, followed by a slower reduction. With group II strains (nephropathogenic strains), however, the initial decrease is followed by an increase at 3 to 4 h after injection. Finally, group III strains have a general virulence with high viable counts in all organs.

## RESULTS

**Influence of hemolysin on the virulence level.** Strain P673 was shown to be virulent in the mouse lung test and the chicken embryo test (5) as well as nephropathogenic in our mouse model (25; Fig. 1). Because hemolysin production has often been reported to be associated with virulence, we tested the virulence level of nonhemolytic mutants obtained after treatment with actinomycin D.

Treatment of P673 with actinomycin D frequently resulted in derivatives that had lost resistance to tetracycline or the ability to produce hemolysin. With the nonhemolytic mutant P673/II, a virulence pattern was obtained that is characteristic for avirulent group I strains (24; Fig. 1). On the other hand, CW22, a mutant that has lost tetracycline resistance, behaved similarly to the parent strain P673 (Fig. 1).

These data suggest that hemolysin is essential for the virulence of P673. We therefore deter-

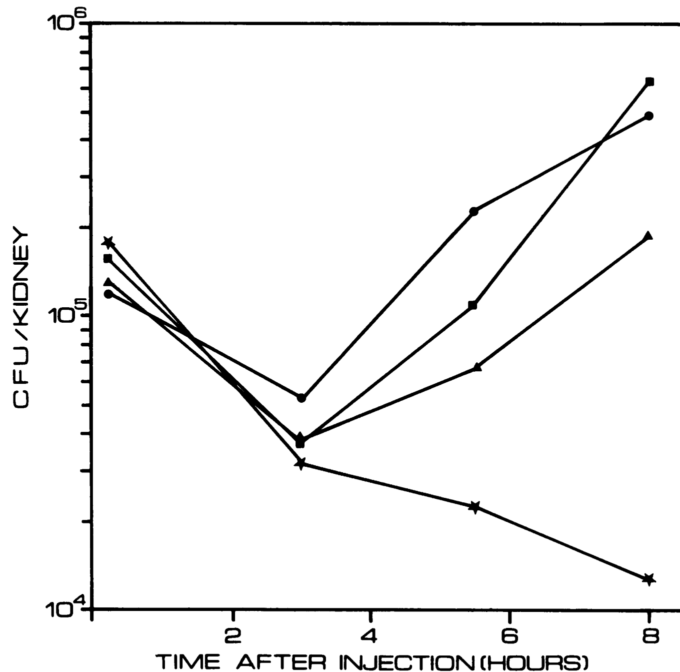


FIG. 1. Kinetics of the viable count in mouse kidney after intravenous injection of $2 \times 10^8$ bacteria. Symbols: (●) strain P673; (★) nonhemolytic mutant P673/II; (■) tetracycline-susceptible mutant CW22; (▲) hemolytic transconjugant CW35. Using standard deviations calculated for nephropathogenic strains and avirulent strains (24), the Student *t* test shows the differences between either of the hemolytic strains and the nonhemolytic strain to be significant ($P < 00.1$). CFU, Colony-forming units.

mined the virulence level of a hemolytic *E. coli* K-12 transconjugant obtained after mating between donor P673 and recipient J53-1. Introduction of the hemolytic property into this otherwise avirulent strain did not result in an increase in virulence (Table 1). Because *E. coli* K-12 has been reported by others (5, 13) to be less suitable for the in vivo expression of virulence factors, we reintroduced the hemolytic property into the nonhemolytic mutant P673/II. This avirulent mutant was mated with the hemolytic donor CW22 (Table 1). Hemolytic transconjugants were selected on blood agar plates supplemented with 50 μg of streptomycin and 20 μg of tetracycline per ml. The resulting transconjugant, designated CW35, was tested in the mouse model. The virulence level obtained after intravenous injection resembled the nephropathogenic character of the parent strain P673 (Fig. 1).

**Plasmid analysis.** Emödy et al. (5) have reported that the hemolysin produced by strain P673 can be transferred to *E. coli* K-12. We could confirm the plasmid nature of this hemolytic factor in mating experiments between donor P673 and either J53-1 or W1485Nx as recipient. From these experiments we obtained two types of *E. coli* K-12 transconjugants. The first type exhibited resistance to tetracycline. The second type was tetracycline resistant as well as hemolytic. Hemolytic transconjugants that were susceptible to tetracycline were never observed. At least 20,000 colonies from each of 10 different matings were examined. All hemolytic transconjugants also expressed resistance to tetracycline. The tetracycline resistance transferred at a frequency of approximately $10^{-3}$, whereas tetracycline-resistant as well as hemolytic transconjugants were obtained at a frequency of about $10^{-5}$. These results suggest that hemolysin and tetracycline resistance are coded by different plasmids.

Therefore, we examined the extrachromosomal DNA content of these strains. On agarose gels, two different plasmids, designated pCW1 and pCW2, were detected in P673 (Fig. 2) and had molecular sizes of about 70 and 41 Md, respectively. By curing with actinomycin D, either tetracycline-susceptible or nonhemolytic variants were obtained at frequencies of about 5 $\times 10^4$ and about $2 \times 10^{-3}$, respectively. These mutants were screened for plasmid content. Most mutants still carried two plasmids with apparently unaltered mobilities (data not shown). However, one of the tetracycline-sus-

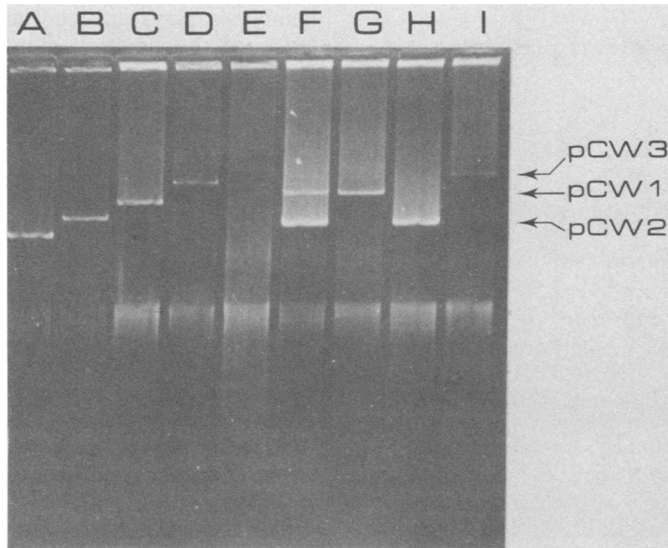


FIG. 2. Electrophoresis pattern of plasmid DNA on a 0.7% agarose slab gel. (A–E) *E. coli* K-12 strains harboring the marker plasmids RP4 (36 Md), R702 (46 Md), R1 (62 Md), RA1-1 (85 Md), and Rts1 (120 Md), respectively; (F) strain P673; (G) P673/II; (H) CW22; (I) CW35.

ceptible mutants had lost pCW1, whereas in one of the nonhemolytic mutants the 41-Md plasmid, pCW2, was lost (Fig. 2).

Transconjugants of J53-1 and W1485Nx that exhibited only resistance to tetracycline were found to carry a plasmid that comigrated with pCW1. However, in transconjugants that both were resistant to tetracycline and produced hemolysin, plasmids comigrating with pCW1 and pCW2 were never observed. We tested 10 transconjugants from 4 independent mating experiments. They all carried a single plasmid species of about 110 Md. In the nephropathogenic transconjugant CW35, obtained after conjugation between donor CW22 and recipient P673/II (Table 1), a plasmid of similar size was observed (Fig. 2). The large plasmid in this strain was designated pCW3. The molecular weight of pCW3 suggested that it might be a cointegrate structure of pCW1 and pCW2. If so, one would expect pCW3 to be incompatible with both pCW1 and pCW2. To test this, we transferred pCW3 into P673/II, which carries only pCW1, as well as into a streptomycin-resistant mutant of CW22 that carries only pCW2. Hemolytic transconjugants, selected on blood agar plates containing 50 μg of streptomycin per ml, were obtained at a frequency of about $5 \times 10^{-4}$. All transconjugants ($n$ = 8) from the mating between J53-1 (p673) (5) and P673/II and all transconjugants ($n$ = 3) from the mating between CW15 (Table 1) and CW22 carried a 110-Md plasmid as the only plasmid species. This indicates that pCW3 is incompatible with both pCW1 and pCW2. Further evidence for the presumed cointegrate structure of pCW3 came from restriction enzyme analysis of pCW1, pCW2, and pCW3. The DNA fragment profile generated after digestion of pCW3 with restriction enzyme *Eco*RI was almost identical to the fragment profile obtained after digestion with *Eco*RI of a mixture of pCW1 and pCW2. Only a 4.7-Md fragment of pCW1 and a 2.0-Md fragment of pCW2 were not observed, and a novel fragment of 6.7 Md was detected (Fig. 3).

## DISCUSSION

Because hemolysin is more often associated with virulent than avirulent *E. coli* strains, it is thought to be a virulence factor of pathogenic strains. In this study, we examined the influence of hemolysin on the virulence of the natural isolate P673. In the hematogenous mouse model, this strain behaved similar to nephropathogenic strains.

Mutants that had lost the ability to produce hemolysin were no longer virulent. These results indicate that the hemolysin of P673 plays a decisive role in the expression of its virulence. We therefore studied the effect of acquisition of this hemolysin by an otherwise avirulent *E. coli* K-12 strain. Transfer of the hemolytic property to this benign organism did not result in an increase of virulence. This is consistent with other studies concerning the introduction of virulence factors into *E. coli* K-12 (e.g., 5, 13). The acquisition of virulence factors seems to be insufficient to convert *E. coli* K-12 into a form

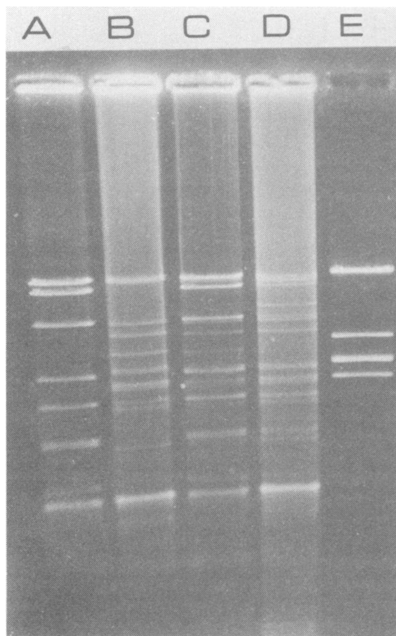


FIG. 3. Agarose (0.8%) gel electrophoresis of plasmid DNA digested with restriction enzyme *Eco*RI. (A) Strain CW22; (B) P673/II; (C) P673; (D) CW35; (E) DNA fragments generated after digestion of phage lambda DNA with *Eco*RI (fragment sizes were taken from Thomas and Davis [22]).

that is virulent in the various virulence assays.

We therefore examined the effect of hemolysin production on virulence in isogenic strains. The nonhemolytic mutant P673/II was no longer virulent for mouse kidneys, in contrast to the hemolytic parent, P673. Such a loss of virulence accompanying the loss of hemolysin has been reported earlier (5, 6, 11, 24). In these studies, like in the present one, nonhemolytic mutants were obtained by treating the hemolytic parents with mutagens or curing agents such as actinomycin D. It must be emphasized, however, that treatment of P673 with actinomycin D frequently resulted in nonhemolytic mutants that carried plasmids with apparently unaltered mobilities. This suggests that actinomycin D can induce mutations. The observed loss of virulence could be caused by a mutation introduced simultaneously with, but independently of, the loss of hemolysin production. To exclude the possibility that the loss of virulence in P673/II was caused by such a mutation, rather than by the loss of the hemolysin plasmid, we determined the virulence level of P673/II after hemolysin had been reintroduced. The resulting hemolytic transconjugant, CW35, exhibited the nephropathogenic character of the parent strain, P673. From these results, it can be concluded that hemolysin plays an important role in the virulence of the nephropathogenic *E. coli* strain P673. The question of whether hemolysin itself or another product encoded by the plasmid pCW2 is the virulence factor remains to be solved.

Emödy et al. (5) reported the transfer of hemolysin from P673 to *E. coli* K-12. In our experiments, this transfer was always accompanied by transfer of tetracycline resistance. From these results, we concluded that both properties were plasmid mediated. On agarose gels, two different plasmids, pCW1 (70 Md) and pCW2 (41 Md), were detected in P673. From plasmid analysis of tetracycline-susceptible mutants and nonhemolytic mutants, it could be concluded that pCW1 coded for tetracycline resistance, whereas hemolysin production resided on pCW2.

The mating between donor CW22, carrying the hemolysin plasmid pCW2, and the recipient P673/II, carrying the plasmid pCW1, resulted in the transconjugant CW35. In this strain, no plasmids could be observed that comigrated with pCW1 or pCW2. However, a novel plasmid of 110 Md, designated pCW3, was detected.

The following observations suggest that pCW3 is a cointegrate composed of the plasmids pCW1 and pCW2: (i) pCW3 carried the tetracycline resistance gene of pCW1 and the hemolysin gene of pCW2; (ii) pCW3 was found to be incompatible with both pCW1 and pCW2; (iii) the DNA fragments of pCW3 generated after digestion with restriction endonuclease *Eco*RI were nearly all identical to the DNA restriction fragments generated after digestion of a mixture of pCW1 and pCW2.

pCW3 could have arisen by transfer of pCW2 from CW22 into P673/II, followed by cointegration. However, in mating experiments between donor P673 and *E. coli* K-12, we never observed transfer of the hemolytic property without simultaneous transfer of tetracycline resistance. From these matings, only two types of transconjugants could be obtained. In tetracycline-resistant transconjugants, a plasmid that comigrated with pCW1 was observed. We therefore conclude that pCW1 is a conjugative plasmid. Hemolytic transconjugants, however, always expressed resistance to tetracycline as well. These transconjugants invariably carried a plasmid similar in size to the cointegrate pCW3. Since the simultaneous transfer of tetracycline resistance and hemolysin occurred at a frequency of about $10^{-5}$, transfer of pCW2, if it occurs at all, must occur at a lower frequency. However, lower frequencies cannot be detected for nonselectable markers like hemolysin. Furthermore, cointegration is not obligatory for the generation of a hemolytic transconjugant.

On the other hand, the cointegrate pCW3 could have arisen through the following sequence of events: (i) pCW1 is transferred from P673/II into CW22; (ii) in CW22, a cointegrate between pCW1 and pCW2 is formed; and (iii) this cointegrate is retransferred to P673/II.

Cointegration between plasmids has been observed in different species (3, 9, 10). Hooykaas et al. (10) found that nonconjugative plasmids could be mobilized by conjugative ones when the latter carried transposable DNA elements that were able to jump onto the nonconjugative plasmid. These cointegrates could have arisen by recombination between small homologous regions that were present in both plasmids. On the other hand, cointegration could be the result of transposition. In a recently presented molecular model of transposition of translocatable elements (19), cointegrates are obligatory intermediates in the transposition process. The involvement of transposable elements in the cointegration process of pCW1 and pCW2 is supported by the fact that cointegration can take place in different ways. Occasionally, hemolytic transconjugants were isolated that, despite the presence of a cointegrate, did not express tetracycline resistance. These results will be published elsewhere.

The presence of a transposable DNA sequence in hemolysin plasmids is further suggested by the finding that hemolysin plasmids from different incompatibility groups share a common DNA sequence that codes for hemolysin production (4). Furthermore, hemolysin determinants are able to jump from one plasmid onto the other (18) or even into the chromosome (16).

ACKNOWLEDGMENTS

We are grateful to J. D. A. van Embden (National Institute of Public Health, Bilthoven, The Netherlands) for his help and advice and to L. Emödy (Pécs, University Medical School, Hungary) for providing us with his strains. We also thank H. T. Kortbeek for his excellent technical assistance.

LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513–1523.
2. **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.
3. **Cornelis, G.** 1980. Transposition of Tn951 (Tn*lac*) and cointegrate formation are thermosensitive processes. J. Gen. Microbiol. 117:243–247.
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. **Emödy, L., T. Pál, N. V. Safonova, B. Kuch, and N. K. Golutva.** 1980. Alpha-haemolysin: an additive virulence factor in *Escherichia coli*. Acta Microbiol. Acad. Sci. Hung. 27:333–342.
6. **Fried, F. A., C. W. Vermeulen, M. J. Ginsburg, and C. M. Cone.** 1971. Etiology of pyelonephritis: further evidence associating the production of experimental pyelonephritis with hemolysis in *Escherichia coli*. J. Urol. 106:351–354.
7. **Goebel, W., B. Royer-Pokora, W. Lindenmaier, and H. Bujard.** 1974. Plasmids controlling synthesis of hemolysin in *Escherichia coli*: molecular properties. J. Bacteriol. 118:964–973.
8. **Goebel, W., and H. Schrempf.** 1971. Isolation and characterization of supercoiled deoxyribonucleic acid from beta-hemolytic strains of *Escherichia coli*. J. Bacteriol. 106:311–317.
9. **Helmuth, R., R. Stephan, E. Bulling, W. J. van Leeuwen, J. D. A. van Embden, P. A. M. Guinée, D. Portnoy, and S. Falkow.** 1981. R-factor cointegrate formation in *Salmonella typhimurium* phage type 201. J. Bacteriol. 146:444–452.
10. **Hooykaas, P. J. J., H. den Dulk-Ras, and R. A. Schilperoort.** 1980. Molecular mechanism of Ti plasmid mobilization by R plasmids: isolation of Ti plasmids with transposon-insertions in *Agrobacterium tumefaciens*. Plasmid 4:64–75.
11. **Hull, R. A., S. I. Hull, B. H. Minshew, and S. Falkow.** 1980. Genetics of hemolysin of *Escherichia coli*, p. 1283–1285. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. II. American Society for Microbiology, Washington, D.C.
12. **Kétyi, I., L. Emödy, T. Kontrohr, A. Vertényi, S. Pácsa, T. A. Avdeeva, N. V. Safonova, and N. K. Golutova.** 1978. Mouse lung oedema caused by a toxic substance of *Escherichia coli* strains. Acta Microbiol. Acad. Sci. Hung. 25:307–317.
13. **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 of extraintestinal *Escherichia coli* isolates. Infect. Immun. 20:50–54.
14. **Minshew, B. H., J. Jorgensen, M. Swanstrum, G. A. Grootes-Reuvekamp, and S. Falkow.** 1978. Some characteristics of *Escherichia coli* strains isolated from extraintestinal infections of humans. J. Infect. Dis. 137:648–654.
15. **Mitchell, I. G., and R. Kenworthy.** 1977. Attempted elimination of plasmid-determined haemolysin, K88 antigen and enterotoxin from *Escherichia coli* pathogenic for pigs. J. Appl. Bacteriol. 42:207–212.
16. **Noegel, A., U. Rdest, and W. Goebel.** 1981. Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. 145:233–247.
17. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. 31:775–782.
18. **Royer-Pokora, B., and W. Goebel.** 1976. Plasmids controlling synthesis of hemolysin in *Escherichia coli*. Mol. Gen. Genet. 144:177–183.
19. **Shapiro, J. A.** 1979. Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc. Natl. Acad. Sci. U.S.A. 76:1933–1937.
20. **Smith, H. W., and S. Halls.** 1967. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. J. Gen. Microbiol. 47:153–161.
21. **Smith, H. W., and M. A. Linggood.** 1971. Observations on the pathogenic properties of K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. J. Med. Microbiol. 4:467–485.
22. **Thomas, M., and R. Davis.** 1975. Studies on the cleavage of bacteriophage λ DNA with *Eco*RI restriction endonuclease. J. Mol. Biol. 91:315–328.
23. **Vahlne, G.** 1945. Serological typing of the colon bacteria. Acta Pathol. Microbiol. Scand. Suppl. 62, p. 81–103.
24. **van den Bosch, J. F., J. de Graaff, and D. M. MacLaren.** 1979. Virulence of *Escherichia coli* in experimental hematogenous pyelonephritis in mice. Infect. Immun. 25:68–74.
25. **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.