INFECTION AND IMMUNITY, Dec. 1985, p. 682–686
0019-9567/85/120682-05$02.00/0

Vol. 50, No. 3

Relation between the Hemolytic Property and Iron Metabolism in *Escherichia coli*

GERHARD LEBEK* AND HANS-MARTIN GRUENIG

Institute for Hygiene and Medical Microbiology, University of Berne, CH-3010 Berne, Switzerland

Received 26 February 1985/Accepted 3 September 1985

The hemolytic activity of wild-type strains of *Escherichia coli* was measured by a standardized method in liquid broth. The system also allowed us to investigate the influence of various Fe^{3+} concentrations in the cultures on the amount of secreted hemolysin. We found that the hemolysin secretion of all strains was clearly reduced after addition of $FeCl_3$. However, the influence of additional iron chelators showed remarkable differences. The hemolytic activity of Hly plasmid-containing strains isolated in Berne significantly increased. Most of the strains with a chromosomal hemolysin determinant showed a similar effect, but to a lesser degree. Contrary to this, Hly plasmid-containing strains isolated in Essex and some strains with chromosomal hemolysin determinant were either not affected or even showed reduced hemolysin secretion after limitation of free iron ions in the broth. Our results suggest that (i) hemolysin secretion in *E. coli* is related to the bacterial iron metabolism, and (ii) hemolysin secretion is differentially regulated among *E. coli* strains.

In the past few years, results of investigations by several groups have demonstrated that hemolysin production contributes to *Escherichia coli* virulence (8–10, 12, 14, 21). Other virulence factors such as mannose-resistant hemagglutination and specific O and K antigens are often associated with hemolysin production in the same strain (9, 14). Preliminary studies showed that hemolytic *E. coli* strains occur more often among *E. coli* strains causing extraintestinal infections than among *E. coli* strains from the intestine (9–11, 15, 19).

The genes for the hemolytic property are either located on transmissible plasmids (3–5, 7, 19) or on the bacterial chromosome (1, 5, 11, 12, 14, 15, 20). Hly plasmids belong to different incompatibility groups (3) and have been isolated from various sources. The occurrence of these plasmids in human isolates is still relatively rare (6, 15, 20; H. M. Gruenig, Ph.D. thesis, University of Berne, Berne, Switzerland, 1984): strains isolated from people with urinary tract infections generally carry their Hly determinant on the chromosome (6). Both plasmid- and chromosome-mediated hemolysin determinants consist of cistrons *hlyA*, *hlyBa*, *hlyBb*, and *hlyC* (20). They show a high degree of sequence homology in cistrons *hlyBa*, *hlyBb*, and *hlyC* (1, 15). However, specific differences in cistron *hlyA*, which is the structural gene for hemolysin, could be found (1).

Despite numerous epidemiological, bacteriological, and genetic investigations, there are still unanswered questions concerning the role of the hemolytic property in the pathogenesis of *E. coli*. In addition, little about the regulation of hemolysin production and its secretion is known. Recent studies with Mu dl-directed *hly-lacZ* fusions, which allowed us to study the expression of *hly* genes by measuring β-galactosidase activity, showed that two promoters control the expression of the four *hly* genes (13). On the left side promoter p_L regulates transcription of *hlyC*, *hlyA*, and *hlyBa*, and on the right side promoter p_R regulates transcription of *hlyBb*.

In this paper the influence of various Fe^{3+} concentrations in broth on the amount of secreted hemolysin is shown. A modified method of Smith (18) was used to measure hemolytic activity of *E. coli* strains in liquid broth which was supplemented with either $FeCl_3$ or iron chelators.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* wild-type strains used in this work were isolates from feces and urinary tracts of urinary tract infection patients from the Berne area. Hly plasmids are listed in Table 1. *E. coli* K-12 WO 987 *araD lac aroB rpsL thi fepA* F^- ColV-EN41::Tn*10* *iut*$^+$ *iuc*$^-$, used for the analysis of hydroxamate production in a bioassay, plasmid pACYC 184, used as vector for gene cloning, and the restriction-free strain *E. coli* HB101, used for in vitro recombination experiments, were kindly provided by V. Braun, University of Tübingen, Tübingen, Federal Republic of Germany, and W. Goebel, University of Würzburg, Würzburg, Federal Republic of Germany, respectively.

Media and chemicals. Columbia broth (Becton Dickinson Labware) was used for all liquid cultures. $FeCl_3$ p.a. was purchased from E. Merck AG. 2,2′-Dipyridyl, ethylenediamine-di(*o*-hydroxyphenylacetic acid), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), and Conalbumin type 1 were obtained from Sigma Chemical Co. Restriction endonucleases and T4 DNA ligase were purchased from Böhringer GmbH.

Erythrocyte suspension. Sheep blood was stored in Alsever solution. The cells were used within 3 weeks of bleeding. Before use, the measurement of hemolytic activity the cells were washed three times with phosphate-buffered saline (pH 7.5) containing 8 g of sodium chloride per liter, 2 g of potassium chloride per liter, 2.89 g of $Na_2HPO_2 \cdot 12H_2O$ per liter, and 0.2 g of KH_2PO_2 per liter. The washed erythrocytes were then suspended in the same buffer at 20% hematocrit, and the suspensions were used immediately.

Measurement of hemolytic activity. The method of Smith (18), with the following modifications, was used. The first modification was that kinetic studies of hemolysin secretion by every strain were carried out to obtain the maximal amount of secreted hemolysin. This was especially important when studying hemolysin secretion under different culture conditions, which altered the growth rate of bacteria. The second modification was that by choosing appropriate

* Corresponding author.

TABLE 1. List of plasmids

Plasmid	Incompatibility group	Mol mass (kbp)[a]	Source (origin)	Reference
pGL680	F IV	70	Human (Berne)	Our data
pGL681	F VI	67	Human (Berne)	Our data
pGL682	F VI	105	Human (Berne)	Our data
pGL683	F VI	67	Human (Berne)	Our data
pGL684	F VI	67	Human (Berne)	Our data
pHly152	I_2	61	Mouse (Essex)	7
pSU316	F IV	73	Human (Essex)	3, 4
pSU212	F VI	109	Porcine (Essex)	4
pSU233		91	Porcine (Essex)	3, 4

[a] kbp, Kilobase pairs.

dilutions of culture fluids and a suitable concentration of erythrocyte suspension and by obtaining the exact measurement of the released hemoglobin in a spectrophotometer, we were able to find the precise amount of secreted hemolysin. As hemolysin secretion differs in the amount and the time point of maximal concentration, these modifications were essential. In addition, our method takes into consideration the relationship between hemolysin production and its inactivation (active hemolysin is quickly inactivated at room temperature).

(i) Preparation of inoculum and incubation. Approximately 5×10^6 viable organisms from overnight cultures of the strains to be tested were inoculated in prewarmed broth and incubated at 37°C. The measured hemolytic activity resulted from the amount of secreted hemolysin and the rate of inactivation of hemolysin, since hemolysin is not stable. The highest concentration of hemolysin was found after 2.5 h of incubation in most of the wild-type strains in the middle of the logarithmic phase of growth. For screening tests, therefore, all strains were incubated for this period.

(ii) Assay of hemolysis. The incubated cultures were centrifuged at 7,000 × *g* for 2 min at 4°C. To prevent multiplication of any strains, the supernatants were supplemented with 1 mg of streptomycin per ml. All strains tested were sensitive to this concentration. An equal volume of a 20% suspension of washed erythrocytes was added, and the mixtures were incubated for 2 h at 37°C. At the end of the incubation period, the remaining erythrocytes were sedimented by centrifugation at 3,500 × *g* for 2 min.

The amount of released hemoglobin in the supernatant solution was determined by measuring the absorbance of formed hemiglobin cyanide after addition of "Drabkin" potassium cyanide solution (16) at 546 mm in a model 2600 Beckman spectrophotometer. All strains were tested like this in the following three parallel assays in Columbia broth: (i) control without any addition; (ii) assay with addition of 100 μM $FeCl_3$ (suitable concentration which does not significantly alter the bacterial growth); (iii) assay with addition of 400 μM dipyridyl (this concentration produces remarkable inducing effects without completely inhibiting the bacterial growth). Hemolytic activity was expressed in nanomoles of hemoglobin released per minute per milliliter of culture (1 U) under the conditions described above. The results from the three different assays could not be directly compared with each other, because growth of bacteria is altered under varied metabolic conditions. Therefore, the results were normalized to equal numbers of bacteria and were then compared by expressing an inducing or reducing factor (see Table 3). The 100% level of lysis of erythrocytes was determined by addition of a portion of erythrocyte suspension to distilled water. Test solutions which caused more than 30% lysis of erythrocytes had to be diluted before addition of erythrocytes, since linearity is given only in this range.

Assay of siderophore production. Analysis of hydroxamate production by *E. coli* wild-type strains was carried out by the method of Csàky (2). In addition, the strains were tested in the following bioassay (17). A mutant of *E. coli* K-12 (E.C. K-12 WO987 *araD lac aroB rpsL thi fepA* F^- ColV-EN41::Tn*10* *iut*$^+$ *iuc*$^-$), deficient in enterochelin synthesis and uptake and aerobactin synthesis, was suspended in an agar medium containing human or sheep serum and poured out into petri dishes (10^4 to 10^5 CFU/ml). Since all the iron is bound to transferrin in the serum, the mutant strain could only grow if aerobactin was provided by the test strain, which was inoculated on small paper disks on top of the agar. The Csàky test and bioassay showed identical findings.

Isolation of plasmid DNA. The isolation of plasmid DNA was performed by the cleared lysate procedure followed by a dye buoyant density centrifugation of the cleared lysate as described by Goebel et al. (6).

In vitro recombination. Plasmid pGL681 and vector plasmid pACYC184 were treated with *Sal*I endonuclease. Ligation was performed with T4 DNA ligase, and the recombinant DNA was transformed in *E. coli* HB101.

Cleavage with restriction enzymes. Restriction enzyme cleavage was carried out as recommended by Böhringer GmbH.

RESULTS

Production of hemolysins and kinetics of hemolysin secretion. Maximal hemolysin secretion correlated with the middle of logarithmic growth in most strains (Fig. 1). Under the conditions used, this point of maximal hemolysin secretion was found to occur between 2 and 3 h, depending on the generation time of the strains and the composition of the broth. This was of particular importance in comparing the hemolytic activity of a strain in cultures with various Fe^{3+} concentrations. With the concentration of $FeCl_3$ used, the growth rate of the strains was not altered. Therefore, the corresponding results could be compared in a simultaneous test. However, the addition of iron chelators (400 μM dipyridyl) resulted in a significantly prolonged generation time (Fig. 2). It was therefore necessary to relate the number of bacteria to the amount of secreted hemolysin. We further analyzed the influence of iron and iron chelators on hemolysin secretion by *E. coli* wild-type strains. The results of this study are summarized in Table 3.

Strains with chromosomally located hemolysin determinants secreted very different amounts of hemolysin when grown in Columbia broth with no additive. However, strains

TABLE 2. Recombinant plasmids

Plasmid	Cistrons and resistance markers encoded by the plasmid[a]	Reference
pANN681	Hly determinant of pGL681 containing *hlyA, hlyC, hlyBa, hlyBb,* and flanking regions; mol mass, 13.2 kbp	Our data
pANN202-312	Hly determinant of pHly152 containing *hlyA, hlyC, hlyBa, hlyBb,* and Cm^r marker; mol mass, 9.6 kbp	6

[a] Cm^r, Chloramphenicol resistance; kbp, kilobase pairs.

containing Hly plasmids could be divided in two groups: Hly plasmid-containing strains from Berne, which secreted relatively low amounts, and Hly plasmid-containing strains from Essex, which secreted significantly higher amounts of active α-hemolysin. A significant reduction in hemolysin secretion was observed for all strains after addition of 100 μM $FeCl_3$, regardless of the initial hemolytic activity. Reduction of the Fe^{3+} concentration by addition of 400 μM dipyridyl increased the hemolysin secretion of most of the strains tested, especially of all Hly plasmid-containing strains isolated in Berne. In contrast, Hly plasmid-containing strains from Essex and some strains with chromosome-mediated hemolysin production were either not affected or even showed reduced levels of secreted hemolysin.

As could be demonstrated, other iron chelators showed the same effect. The differences between Hly plasmid-containing strains from Berne and Essex not only occurred in the wild-type strains, but also in *E. coli* K-12, which obtained the plasmids by conjugation. In addition, strains containing the cloned Hly determinant of pGL681 (pANN681) and pHly152 (pANN202-312) (6) differed in the same way.

Restriction maps of pANN681 and pANN202-312 are given in Fig. 3. They show almost identical restriction sites in the *hly* structural genes, but show differences in the flanking regions. It is therefore tempting to speculate that the genes responsible for the induction of hemolysin secretion are located in these flanking regions.

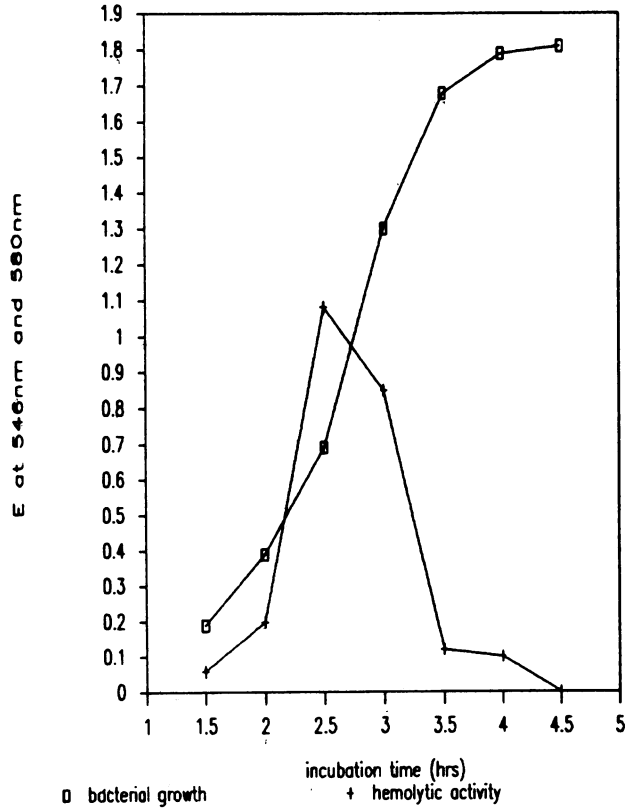

FIG. 1. Effect of incubation time on the amount of hemolysin secreted by *E. coli* 20'478. The hemolytic activity was measured at 546 nm, and the bacterial growth was measured at 580 nm. Maximal hemolytic activity was found after 2.5 h of incubation at 37°C in the middle of the logarithmic phase of growth. This time point still depends on the generation time of the strain and the amount of Fe^{3+} in the broth.

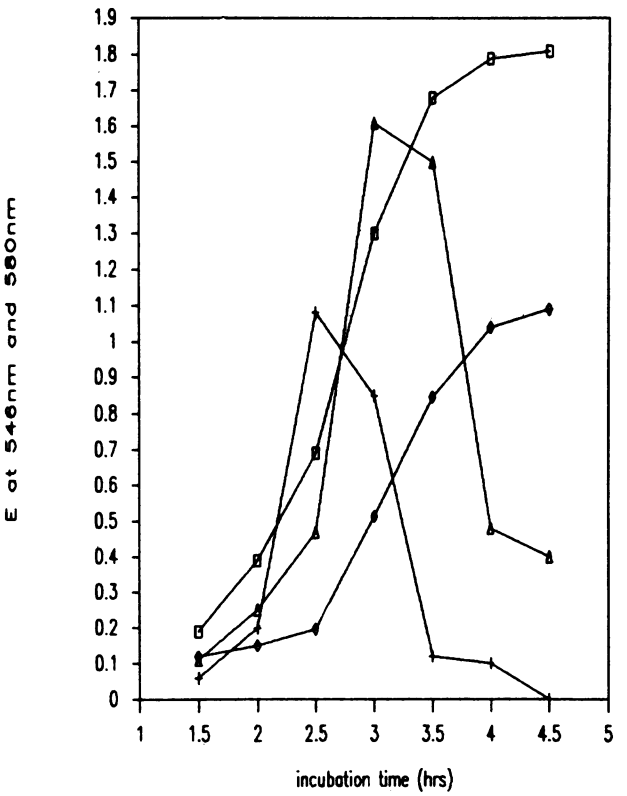

FIG. 2. Hemolytic activity (+) and bacterial growth (□) of *E. coli* 20'478 without addition of 2,2'-dipyridyl (△) and in the presence of the iron chelator (◇). The hemolytic activity was measured at 546 nm, and the bacterial growth was measured at 580 nm. The limitation of Fe^{3+} in the broth by the iron chelator dipyridyl leads to an increased hemolysin secretion despite reduced bacterial growth. Most of the strains tested showed a similar effect. Maximal hemolysin secretion was found 0.5 to 1 h later in samples supplemented with the iron chelator, as shown by the delayed growth.

Siderophore (hydroxamate) production among hemolytic *E. coli* wild-type strains. Among hemolytic *E. coli* strains siderophore (hydroxamate) production was a relatively rare property: none of the nine Hly plasmids in strains from Berne or Essex contained genes for hydroxamate production (17), and only 4 of 25 hemolytic strains with a chromosomal hemolysin determinant showed a positive result in the Csàky test. These four strains (*E. coli* 15141, 11464, 4553, and 4678) belonged to the group of wild-type strains which did not show a significantly induced hemolysin secretion in the presence of iron chelators. No differences in growth rates in cultures with limited Fe^{3+} could be detected between these four and the other hemolytic wild-type strains. This suggested that hemolysin secretion may be an alternative method for obtaining essential iron from the environment (e.g., from hemoglobin).

DISCUSSION

By using the method presented above it was possible to precisely quantify the amount of hemolysin secreted by *E. coli* strains. Consideration of the kinetics of hemolysin secretion, which altered with different Fe^{3+} concentrations for each strain, turned out to be especially important for the investigation of the influence of iron salts and iron chelators.

TABLE 3. Influence of iron salts and iron chelators on the amount of hemolysin secreted by *E. coli* wild-type strains

Strain	Source	Maximal hemolytic activity in Columbia broth	Increasing/reducing factor by addition of:	
			$FeCl_3$ (100 μM)	Dipyridyl (400 μM)
Plasmid-encoded secretion				
800 (pGL680)	Fecal	7	0.1×	12×
1175 (pGL681)	Fecal	2	—[a]	75×
9072 (pGL682)	Fecal	8	0.05×	12×
17910 (pGL683)	Fecal	5	0.1×	17×
18669 (pGL684)	Fecal	7	0.05×	11×
PM152 (pHly152)	Fecal	180	0.05×	0.1×
E.C. K-12 (pSU316)	Fecal	200	0.05×	1×
E.C. K-12 (pSU212)	Fecal	80	0.1×	0.2×
E.C. K-12 (pSU233)	Fecal	30	0.1×	0.3×
Chromasomally encoded secretion				
11'464	Fecal	48	0.1×	2×
13'246	Fecal	80	0.2×	2×
15'141	Fecal	9	0.05×	0.1×
15'623	Fecal	6	0.1×	2×
15'724	Fecal	1	—	4×
17'824	Fecal	0.5	—	3×
18'482	Fecal	9	0.1×	0.5×
18'674	Fecal	1	—	9×
20'477	Fecal	9	0.1×	1×
20'478	Fecal	60	0.1×	2×
1'630	UTI[b]	4	0.1×	2×
1'632	UTI	7	0.1×	1×
4'553	UTI	4	0.1×	1×
4'585	UTI	3	0.05×	3×
4'678	UTI	5	0.05×	1×
4'751	UTI	0.5	—	1×
4'821	UTI	1	—	2×
4'922	UTI	1	—	1×
5'232	UTI	9	0.1×	1×
5'841	UTI	4	0.1×	6×
6'045	UTI	2	—	1×
9'755	UTI	2	—	2×
10'071	UTI	1	—	3×
10'145	UTI	9	0.1×	1×
10'235	UTI	2	—	1×

[a] —, No hemolytic activity detectable.
[b] UTI, Urinary tract infection.

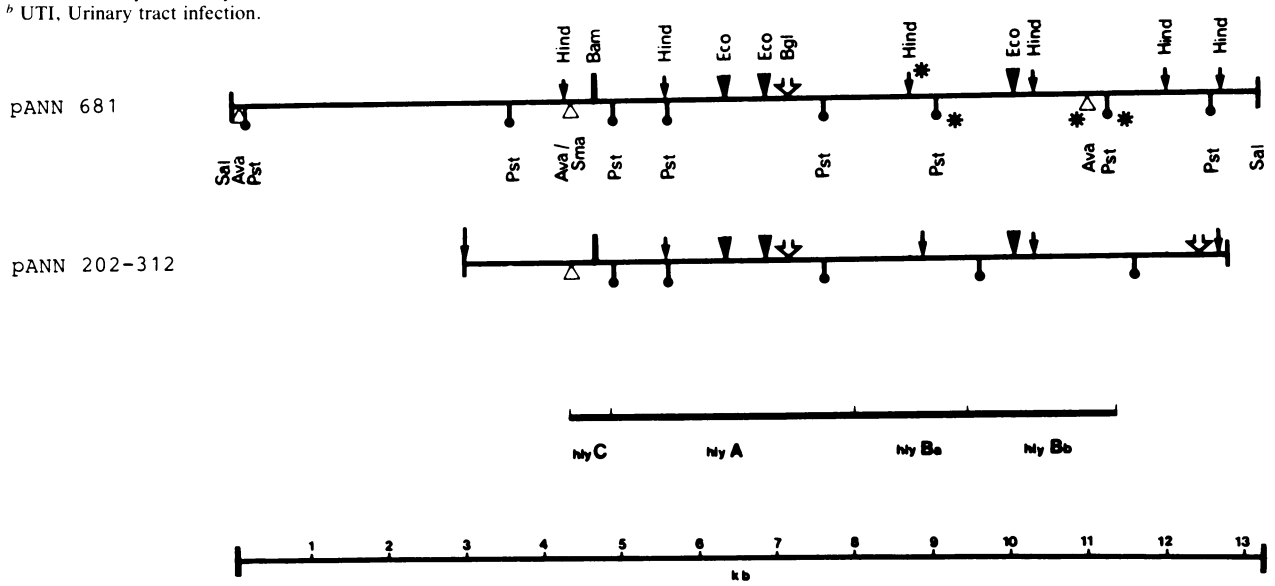

FIG. 3. Comparison of restriction maps of the hemolysin determinant of plasmids pGL681 (pANN681) and pHly152 (pANN202-312). The 13.2-kilobase segment of pANN681 and the 9.6-kilobase segment of pANN202-312, each containing the Hly determinant, are shown with the Hly cistrons placed as indicated by the functional analysis of deletion mutants (7; J. Hacker and C. Hughes, Curr. Top. Microbiol., in press). pANN681 and pANN202-312 showed almost identical restriction sites among the *hly* structural sites. Differences in the *hly* region are indicated by an asterisk. However, the flanking regions showed remarkable differences.

The reduced secretion by all strains in the presence of $FeCl_3$ points to a correlation of hemolysis with the bacterial iron metabolism. We propose that the offer of free iron ions causes a negative feedback effect on the hemolysin secretion. The increased hemolysin secretion by most strains in the presence of iron chelators supports this hypothesis. Particularly, Hly plasmid-containing strains isolated in our laboratory showed a strongly induced hemolysin secretion, whereas Hly plasmid-containing strains isolated in Essex, which already secreted a much higher amount in the assay without any addition, were either not affected or even showed reduced secretion. This points to different mechanisms in the regulation of *E. coli* hemolysin secretion. Hly plasmid-containing strains from Berne seem to be induced only at a very low iron concentration, whereas plasmid-containing strains from Essex do not show this regulatory effect. Our results suggest that the five Hly plasmids in strains isolated in Berne contain, in contrast to the plasmids in strains isolated in Essex, special genes in the flanking region of the *hly* genes, which control hemolysin production depending on the Fe^{3+} concentration in the culture. Most of the strains with chromosome-mediated hemolysin production show a similar effect. We assume that hemolysin secretion is one possibility for hemolytic strains to obtain essential iron ions from the environment and is therefore important for bacteria in infectious diseases.

Further investigations are currently in progress to enable us to better understand the regulatory mechanisms of hemolysin production and secretion.

ACKNOWLEDGMENTS

We thank C. Kempf for critically reading the manuscript and C. Lam for typing the manuscript.

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.
2. **Csàky, T. Z.** 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. **2**:450–454.
3. **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.
4. **De la Cruz, F., J. C. Zabala, and J. M. Ortiz.** 1979. Incompatibility among α-hemolytic plasmids studied after inactivation of the α-hemolysin gene by transposition of Tn 802. Plasmid **2**:507–519.
5. **De la Cruz, F., J. C. Zabala, and J. M. Ortiz.** 1983. Hemolysis determinant common to *Escherichia coli* strains of different O serotypes and origins. Infect. Immun. **41**:881–887.
6. **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., B. Royer-Pokora, W. Lindenmaier, and H. Bujard.** 1974. Plasmid controlling synthesis of hemolysin in *Escherichia coli*: molecular properties. J. Bacteriol. **118**:964–973.
8. **Hacker, J., C. Hughes, H. Hof, and W. Goebel.** 1983. Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. Infect. Immun. **42**:57–63.
9. **Hacker, J., G. Schröter, A. Schrettenbrunner, C. Hughes, and W. Goebel.** 1983. Hemolytic Escherichia coli strains in the human fecal flora as potential urinary pathogens. Zentralbl. Bakteriol. Mikrobiol. Hyg. I Abt. Orig. A **254**:370–378.
10. **Hughes, C., J. Hacker, A. Roberts, and W. Goebel.** 1983.Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *Escherichia coli*. Infect. Immun. **39**:546–551.
11. **Hughes, C., D. Müller, J. Hacker, and W. Goebel.** 1982. Genetics and pathogenic role of Escherichia coli hemolysin. Toxicon **20**:247–252.
12. **Hull, S. I., R. H. Hull, B. H. Minshew, and S. Falkow.** 1982. Genetics of hemolysin of *Escherichia coli*. J. Bacteriol. **151**:1006–1012.
13. **Juarez, A., M. Härtlein, and W. Goebel.** 1984. Study of regulation and transport of hemolysin by using fusion of the β-galactosidase gene (*lacZ*) to hemolysin genes. J. Bacteriol. **160**:161–168.
14. **Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow.** 1978. Association of hemolysin production, haemagglutination of human erythrocytes and virulence for chicken embryos of extraintestinal *Escherichia coli* isolates. Infect. Immun. **20**:50–54.
15. **Müller, D., C. Hughes, and W. Goebel.** 1983. Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli*. J. Bacteriol. **153**:846–851.
16. **Richterich, R., and J. P. Colombo.** 1978. Klinische Chemie, Theorie und Praxis, Intepretation, p. 333. S. Karger Verlag, Basel.
17. **Schoch, C., and G. Lebek.** 1984. Nachweis von plasmidisch kodierten Eisen-Transportmechanismen in Enterobacteriaceae. Hyg. Med. **9**:409–410.
18. **Smith, H. W.** 1963. The haemolysins of E. coli. J. Pathol. Bacteriol. **85**:197–211.
19. **Smith, H. W., and S. Halls.** 1967. The transmissible nature of the genetic factor in E. coli that controls hemolysin production. J. Gen. Microbiol. **47**:153–161.
20. **Wagner, W., M. Vogel, and W. Goebel.** 1983. Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. J. Bacteriol. **154**:200–210.
21. **Welch, R. A., A. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Hemolysin contributes to virulence of extraintestinal *E. coli* infections. Nature (London) **294**:665–667.