INFECTION AND IMMUNITY, Jan. 1984, p. 156–160
0019-9567/84/010156-05$02.00/0

Vol. 43, No. 1

Characterization of *Escherichia coli* Hemolysins Conferring Quantitative Differences in Virulence

RODNEY A. WELCH[1]* AND STANLEY FALKOW[2]

Department of Medical Microbiology, University of Wisconsin Medical School, Madison, Wisconsin 53706,[1] and Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305[2]

Received 8 July 1983/Accepted 3 October 1983

Recombinant plasmids encoding hemolysins (hly) isolated from four different *Escherichia coli* strains were found to be very similar by restriction endonuclease fragment analysis within the hemolysin region. Each of the four recombinant plasmids were used to transform a nonhemolytic fecal strain of *E. coli*. The comparative virulence of the transformants was tested in a rat model of peritonitis. Despite the physical similarity among the hemolysin recombinant plasmids, each conferred different levels of virulence to the normally avirulent fecal *E. coli* strain. Reciprocal exchange of similar restriction endonuclease fragments enabled the construction of hybrid hemolysin determinants with two hemolysins of disparate extracellular hemolysin production and relative virulence levels. The hybrid plasmids were introduced into the standard avirulent fecal strain and tested in the rat peritonitis model. The region conferring quantitative differences in extracellular hemolysin production and virulence was found to be within a region of less than 1 kilobase where the hly c cistron is encoded as well as the probable transcription initiation area for the entire hemolysin operon. A 750-base pair (bp) *Ava*I fragment from this region was isolated from a virulent hemolysin recombinant and inserted into a common *Ava*I site present in an avirulent hemolysin plasmid. This insertion resulted in an increase in the amount of extracellular hemolysin activity and the associated virulence when in the fecal *E. coli* background.

Escherichia coli represents one of the principal opportunistic pathogens of humans. Underlying disease is often the precedent to an extraintestinal *E. coli* infection. However, it is evident the individual strains causing these infections possess virulence factors aiding their proliferation. Recently, it has been advocated that the classic genetic approach to the identification of bacterial virulence factors is most effective (16). This approach relies on the construction of isogenic strains varying only in the presence of a putative virulence factor and the subsequent demonstration that the strains have quantitative as well as qualitative differences in relevant animal and in vitro models of pathogenicity. Recombinant DNA technology affords the construction of isogenic strains with a certainty of the genetic content, an approach that is unachievable with classic genetic methodologies (6). We demonstrated the successful use of this approach with the chromosomal hemolysin (hly) of *E. coli*, in which a recombinant plasmid coding for hly was clearly shown to confer an increase in the virulence of an *E. coli* strain when tested in a rat model of peritonitis (18). In addition, it was found that a second recombinant plasmid possessing a similar *E. coli* hly determinant did not confer the same level of virulence. Relying on the transfer of large naturally occurring hly plasmids from animal isolates, Smith and coworkers also observed variations in the degree of virulence these plasmids conferred on recipients (12–14). Data presented here will show that, despite close DNA sequence and genetic similarities, the hly genes of *E. coli* clearly differ in the amount of hly expressed, confirming an earlier observation of Rennie and Arbuthnott (10). In addition, we demonstrate how recombinant DNA methods can be utilized to determine what region of the hly determinant is responsible for differences in hly expression and virulence.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains serving as the source of the hly recombinant plasmids and the individuals who supplied these strains are identified in Table 1. The *E. coli* K-12 strain HB101 was used in the recombinant plasmid constructions. The human fecal *E. coli* strain, J198, was used in the rat peritonitis studies. This strain was acquired from the collection of Barbara Minshew, U.S. Public Health Service Hospital, Seattle, Wash.

Media and reagents. Blood agar base (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1 mM $CaCl_2$ and 5% defibrinated sheep blood was used as a plating medium. Luria broth was used for liquid cultures (8). Restriction endonucleases were acquired from New England Biolabs, Beverly, Mass., and Bethesda Research Laboratories, Gaithersburg, Md. T4 DNA ligase was purchased from New England Biolabs.

Plasmid isolation and recombinant methods. The general methods used for recombinant plasmid construction can be found in previous publications from this laboratory (3, 15). Specific restriction endonuclease fragments used in the construction of hybrid hly recombinants were isolated and purified by electroelution of the DNA fragment from an agarose gel slice of electrophoretically separated digest mixture. The conditions for agarose gel electrophoresis have been previously detailed (7). The electroelution was performed in 0.1× Tris acetate buffer (40 mM Tris, 20 mM sodium acetate, 2 mM disodium EDTA, pH 8.0). Confirmation of putative hybrid plasmid constructions was done by restriction endonuclease fragment analysis of plasmid DNA isolated by the method of Ish-Horiwicz and Burke (4).

Hemolysin assay. A modified protocol of Williams was used, in which the supernatants of cultures harvested at an

TABLE 1. Bacterial strains used in this study

Strain designation	Hemolysin recombinant plasmid	Origin of hemolysin determinant	Source
WAF100	pSF4000	Chromosomal determinant isolated from cosmid gene bank of the urinary tract isolate J96 (18)	B. Minshew
AN202-312	pAN202-312	Plasmid determinant from pHly152 isolated from mouse fecal strain (2)	W. Goebel
JS204	pJS204	Plasmid determinant from pHly185 isolated from a pig fecal strain	J. Stark and C. W. Shuster
WAF155	pWAF155[a]	*Sal*I fragment from cosmid recombinant p536 isolated from gene bank of a human O4 serotype isolate (2)	W. Goebel

[a] pWAF155 constructed in this laboratory.

optical density at 600 nm of 1.0 to 1.1 were subjected to serial twofold dilutions of lysis assay buffer (50 mM NaCl–10 mM $CaCl_2$) (20). Washed sheep erythrocytes (final concentration, 1.0%) were added to the supernatant dilutions held in round-bottomed wells of microtiter dishes. Titers are expressed as the reciprocals of the highest dilutions at which visible lysis of the erythrocytes was evident after 3 h of incubation at 37°C.

Rat intraabdominal sepsis model. Male Wistar rats, each weighing 160 to 180 g, were purchased from Simonsen Laboratories, Palo Alto, Calif., and infected with a modification of the inoculum and protocol described by Weinstein et al. (17). Human feces, rather than rat fecal material, was used. The inoculum consisted of a sterile 50% fecal and 10% barium sulfate (wt/vol) mixture suspended in 50% brain heart infusion broth. Bacterial suspensions adjusted by optical density to give a reproducible number of CFU were added to the adjuvant material. In the experiments detailed here, the inoculum contained 1×10^7 to 2×10^7 CFU. The bacteria-adjuvant suspension was injected intraperitoneally in a 0.25-ml volume with an 18-gauge needle and a 1-ml tuberculin syringe. The infected rats were housed individually and monitored twice daily for a 7-day period.

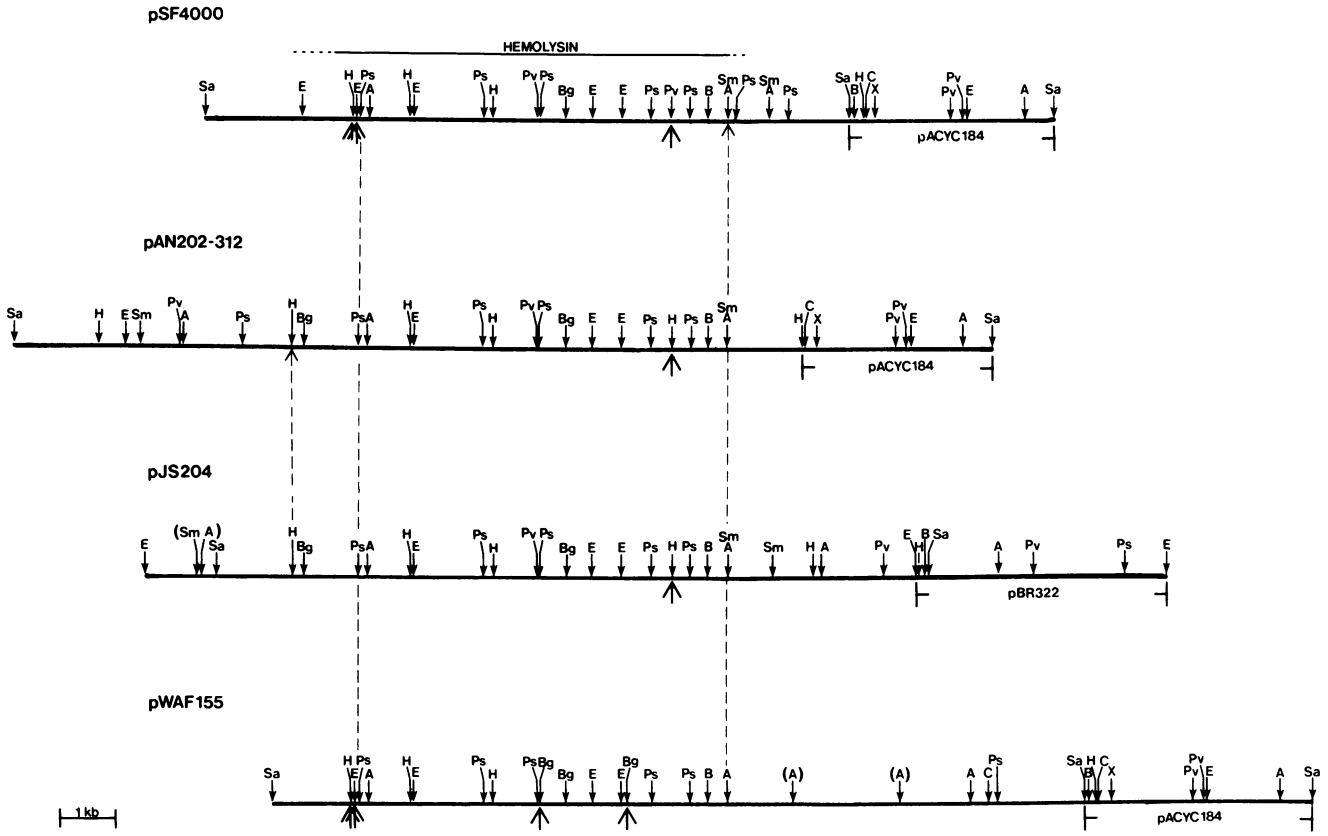

FIG. 1. Comparison of restriction endonuclease fragment maps of four hly recombinant plasmids. The dashed vertical lines extending through all four maps provide a vertical register for the *Ava*I and *Pst*I sites that represent the widest breadth of shared digestion sites for all four hly recombinant plasmids. The dashed line between the pAN202-312 and pJS204 maps represent the widest breadth of common restriction endonuclease sites seen between any two of the hly plasmids. The arrows beneath the horizontal bars represent those digest sites within the hemolysin-encoding region that are not commonly shared among the four plasmids. The arrows above the horizontal bar represent the location of digestion sites. Sa, *Sal*I; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; Bg, *Bgl*II; A, *Ava*I; Sm, *Sma*I; C, *Cla*I; B, *Bam*HI; X, *Xba*I. The *Sma*I site shown above the *Ava*I site to the right of the common *Bam*HI site for the plasmids indicates subclones of this region suggest that the *Ava*I and *Sma*I sites are identical. The parentheses surrounding the *Sma*I and *Ava*I sites for pJS204 indicate that the common identity or order of these two sites has not been determined. The parentheses surrounding the *Ava*I sites for pWAF155 indicate that a single *Ava*I site exists at either one of the two locations. The *Sma*I and *Pvu*II sites for pWAF155 have not been determined.

TABLE 2. Comparison of hemolysin production and rat mortality for strains containing different hemolysin recombinant plasmids

Plasmid designation[a]	*E. coli* origin of hemolysin determinant	Relative hemolysin zone size[b]	Hemolytic titer of culture supernatants[c]	No. of deaths/ total no. inoculated[d]	% Mortality
pSF4000	Human infection	+++	256	39/43	90
pAN202-312	Mouse feces	+	2	3/36	8
pJS204	Pig feces	++	64–128	9/20	45
pWAF155	Human infection	+	16	2/10	20

[a] All strains are in the background of J198, which has an O22 serotype and was isolated from the feces of a healthy human. Of 32 rats, 3 died when-inoculated with J198 (18). All vector plasmids are pACYC184 except pJS204, where pBR322 is used.

[b] Relative size ordering of the margins of hemolysis surrounding colonies grown overnight on blood agar medium containing 5% defibrinated sheep blood.

[c] Results of two separate experiments.

[d] Number of deaths occurring within 7 days after receiving intraperitoneal injection of 10^7 CFU of each strain.

RESULTS

The four recombinant plasmids encoding *E. coli* hly of different origins were compared by restriction endonuclease fragment analysis and found to share a number of DNA fragments of similar sizes based on their comigration in agarose electrophoresis gels. Figure 1 shows the restriction endonuclease fragment maps of the plasmids. The similarity resides principally in the area where the hly determinant for pSF4000 was localized by Tn*1*-mediated insertional mutagenesis (19). Although the majority of the restriction endonucleases tested revealed a close similarity within the area of the hly determinants, dissimilarities in each of the hly determinants were readily found utilizing only six different restriction endonucleases. Despite the apparent physical similarity in the hly-encoding regions, previous experiments revealed that the pSF4000 and pAN202-312 plasmids conferred differences in virulence for a fecal *E. coli* strain when used in a rat peritonitis model (18).

Consequently we examined which factors might be responsible for virulence differences. Differences in the amount of extracellular hly could account for the virulence differences because the hemolysis zone surrounding colonies of pSF4000-containing cells were markedly larger in size than that seen for pAN202-312 containing cells. To obtain a measurable parameter of these differences in hly, we adapted previously developed methods of *E. coli* hly quantification based on an endpoint dilution of the hemolytic activity (20). Besides the extracellular hly production encoded by pSF4000- and pAN202-312-containing cells, two additional *E. coli* hly recombinant plasmids were studied. The titers of extracellular hly (Table 2) were observed to directly reflect the relative diameters of hemolytic zones encoded by the four recombinant. Next, the relative virulence or the hly recombinants was examined in the rat peritonitis model. At approximately the same inoculum size of 10^7 CFU, the relative percentage of rat mortalities observed for the J198 strain harboring each of the plasmids paralleled their relative level of extracellular hly production.

The interpretation of the results involving the relative amounts of extracellular hly and virulence levels for the four recombinants is difficult because a common vector and an orientation of the cloned insert DNA was not utilized. However, the two plasmids for which a comparison is appropriate involved pSF4000 and pAN202-312. Both of these recombinants represent insertion of the hly-encoding restriction endonuclease fragment of approximately the same size in the identical orientation in the same vector. In addition, the rates of segregational loss of either plasmid in the absence of antibiotic selection is similar. The knowledge of the great similarity in the restriction endonuclease fragment pattern within the hly lent itself to an approach where reciprocal exchanges of restriction endonuclease fragments between these two plasmids would enable a localization and possibly resolution of the region(s) of the hly determinant responsible for the apparently related differences in expression of extracellular hly and virulence. Figure 2 shows the genealogy for the in vitro construction of the series of reciprocal hybrids. The particular hybrids sought involved reciprocal exchange in three specific areas of the hly determinant; the region to the left of the *Bgl*II site where a hly transport function is encoded, the area between the *Bgl*II and *Bam*HI sites where the majority of the 110,000-dalton hly structural gene polypeptide is encoded, and the area to the right of the *Bam*HI site where a 12,000-dalton polypeptide of unknown function is encoded that also includes the region where transcription initiation is believed to occur (2,

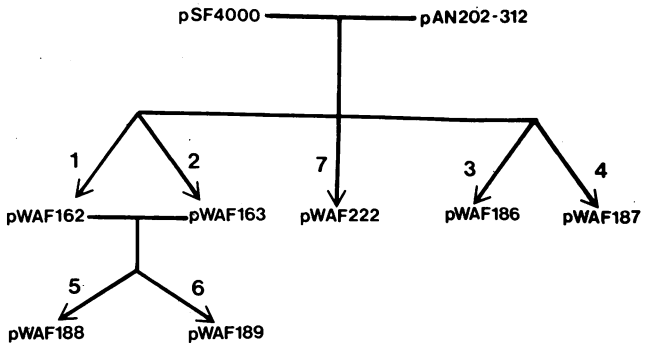

FIG. 2. Genealogy of the construction of hybrid hly recombinant plasmids. The construction of pWAF162 (construction 1) involved ligation of the pSF4000 *Bgl*II–*Bam*HI-C fragment together with pAN202-312 *Bgl*II–*Bam*HI-A and -B fragments; construction 2 involved the pSF4000 *Bgl*II–*Bam*HI-A and -B fragments with the pAN202-312 *Bgl*II–*Bam*HI-C fragment; construction 3 involved the pSF4000 *Bgl*II–*Cla*I-A fragment with the pAN202-312 *Bgl*II–*Cla*I-B fragment; construction 4 involved the pSF4000 *Bgl*II–*Cla*I-B fragment with the pAN202-312 *Bgl*II–*Cla*I-A fragment along with the *Bgl*II-B fragment; construction 5 involved the pWAF163 *Bgl*II–*Cla*I-A fragment with the pWAF162 *Bgl*II–*Cla*I-B fragment; construction 6 involved the pWAF163 *Bgl*II–*Cla*I-B fragment with the pWAF162 *Bgl*II–*Cla*I-A fragment along with the *Bgl*II-B fragment; construction 7 involved the pSF4000 *Ava*I-D fragment with the pAN202-312 *Bam*HI–*Cla*I-A fragment along with the *Bam*HI-*Cla*I-B fragment that was digested with *Ava*I after isolation. The designations of A, B, C, and D for DNA fragments refer to their relative decreasing size order as judged by agarose gel electrophoresis. The numbers given to the constructions, i.e., 1, 2, 3, signify the ordering of the hybrid plasmids as given from top to bottom in Fig. 3.

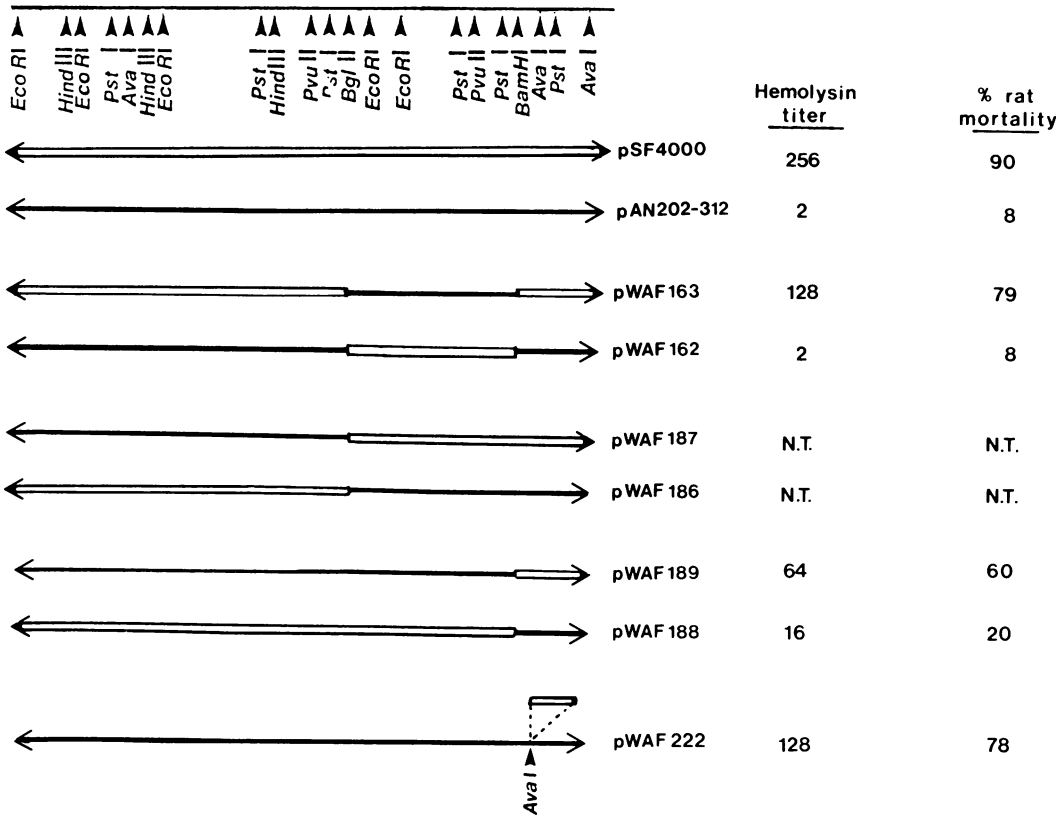

FIG. 3. Hybrid hly recombinants and their respective hly titers and virulence. The horizontal bar in the upper left of the figure is a restriction endonuclease fragment map of the hemolysin-encoding region of pSF4000. Shown below are drawings of the respective portions of hybrid hly recombinants constructed in vitro. The open bar represents the portion derived from pSF4000 and the solid bar the portion from pAN202-312. The arrows at the end of each bar refer to the continuation of that portion through to the vector plasmid pACYC184. All inserts are in the same orientation. The pWAF222 plasmid is pAN202-312 with the *Ava*I-D fragment of pSF4000 inserted at the single *Ava*I site to the left of the *Bam*HI site of pAN202-312. The corresponding hly titers encoded by each recombinant in the J198 genetic background are shown in the designated column. The hly titers were determined by the method described in the text. The percentage of rat mortality each recombinant plasmid confers in the J198 background is shown in the right-hand column. A group of at least 18 rats was used to test the virulence of each recombinant in the *E. coli* peritonitis model described in the text. N.T., Isolates not tested for their respective extracellular hly titers or virulence in the rat model of peritonitis. The zone of hemolysis surrounding pWAF187-containing strains is greater than that of pWAF186 strains.

9; R. A. Welch, unpublished data). The authenticity of hybrid constructions was tested by restriction endonuclease digest analysis by using combinations of enzymes permitting detection of sequences specific for either pSF4000 or pAN202-312, i.e., *Pvu*II, *Hin*dIII, *Ava*I, and *Bgl*II (see Fig. 1). Illustrations of the hybrid hly recombinants, their respective hly titers, and the percentage of rat mortality they confer when put in the J198 background are shown in Fig. 3.

To further delineate the region responsible for the expression effect, the pSF4000 *Ava*I-D fragment (smallest pSF4000 *Ava*I fragment; see Fig. 1) was inserted at the single *Ava*I site to the right of the *Bam*HI site of pAN202-312. This construction (WAF 222), although almost entirely pAN202-312, now confers an increased hly titer and a relatively high level of virulence (Fig. 3). The pSF4000 *Ava*I-D fragment cloned in a vector compatable to pAN202-312 does not enhance the pAN202-312 hly expression in *trans* (L. Segal, S. Pellet, and R. A. Welch, unpublished data).

DISCUSSION

It is clear from our studies, as well as a number of others, that the hly determinant present in the majority of hemolytic *E. coli* strains is of a common origin (1, 19). However, in this study we provide additional evidence that differences in the expression of hly is related to the virulence level of *E. coli* tested in a rat peritonitis model. There was little significant difference among human or animal *E. coli*-derived hly in terms of expression or virulence in the rat peritonitis model. Our data suggest that the source and apparent divergence of the hly structural gene sequence is less important in terms of virulence than in aspects of the regulation and secretion of the hly. This is best shown in the experiments involving the hybrid hly determinants we have constructed. The plasmids pWAF188 and pWAF189 represent the case in which the entire encoding sequence for the structural gene (hly a) and the hly transport function (hly b) were exchanged between two hly determinants exhibiting clear differences in structural gene sequence, extracellular hly production, and relative virulence. Unexpectedly, it was found that the region encoding the respective hly c cistrons and probably the transcription initiation regions were associated with the relative levels of hly secretion and degree of virulence. At least in the instance involving the two hly determinants studied here, the divergence of the hly a and hly b sequences as reflected by restriction endonuclease digest patterns cannot account for

the virulence differences. This has been proposed as a hypothesis to explain the phenomenon (1). The results involving the insertion of the pSF4000 *Ava*I-D fragment into pAN202-312 suggest that only some small portion of hly c or, perhaps more likely, a difference in the transcriptional or translational control region for hly c is responsible for the elevated expression of extracellular hly and subsequently the virulence. Considering what is known about the lytic activity and kinetics of the *E. coli* hly, this appears to be a logical finding. Several groups have provided evidence that the *E. coli* hly exhibits a one-hit phenomenon that is easily saturable (5, 11). This suggests a nonenzymatic, membrane insertional-type event similar to complement-mediated lysis. Therefore, it is likely that alterations in regulatory controls leading to the overall production or secretion of more hly would serve to enhance virulence just as well as any structural gene alterations which somehow influence the specificity or kinetics of lytic activity. This is not intended to discount the possibility that the apparent divergence of the hly a sequence has functional significance. It is clear that further physical and biochemical characterization of the cytolytic event is necessary.

ACKNOWLEDGMENTS

This work is supported by gift funds from the Ceteus Corp. and the Shaw Foundation. In addition, support was provided by grants from the University of Wisconsin-Madison Medical and Graduate Schools and Public Health Service grants AI 10885 and AI 20323 from the National Institutes of Health.

We thank Steve Moseley, Alison Weiss, and Renée Welch for their help in conducting the rat peritonitis experiments. We also thank Jim Stark for the gift of a strain containing pJS204 and providing us with a preliminary restriction endonuclease fragment map of that plasmid.

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. **Goebel, W., and J. Hedgpeth.** 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. J. Bacteriol. **151**:1290–1298.
3. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
4. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
5. **Jorgensen, S. E., R. Hammer, and G. K. Wu.** 1980. Effects of a single hit from the alpha hemolysin produced by *Escherichia coli* on the morphology of sheep erythrocytes. Infect. Immun. **27**:988–994.
6. **Koomey, J. M., R. A. Welch, and S. Falkow.** 1982. Potential contribution of recombinant DNA technology to the study of microbial pathogenicity. *In* A. G. Bearn (ed.), Antibiotics in the management of infections: outlook for the 1980s. Raven Press, New York.
7. **Meyers, J. A., D. Sanchez, L. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
8. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Noegel, A., U. Rdest, W. Springer, and W. Goebel.** 1979. Plasmid cistrons controlling synthesis and secretion of the exotoxin α-haemolysin of *Escherichia coli*. Mol. Gen. Genet. **175**:343–350.
10. **Rennie, R. P., and J. P. Arbuthnott.** 1974. Partial characterization of *Escherichia coli* haemolysin. J. Med. Microbiol. **7**:179–188.
11. **Rennie, R. P., J. H. Freer, and J. P. Arbuthnott.** 1974. The kinetics of erythrocyte lysis by *Escherichia coli* haemolysin. J. Med. Microbiol. **7**:189–195.
12. **Smith, H. W.** 1963. The hemolysins of *Escherichia coli*. J. Pathol. Bacteriol. **85**:197–211.
13. **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.
14. **Smith, H. W., and M. Lingood.** 1971. Observations on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with special reference to porcine diarrhea. J. Med. Microbiol. **4**:467–486.
15. **So, M., W. Dallas, and S. Falkow.** 1978. Characterization of an *Escherichia coli* plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. Infect. Immun. **21**:405–411.
16. **Sparling, P. F.** 1979. Use of microbial genetics in the study of pathogenicity: differentiation between correlation and causation, p. 249–253. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
17. **Weinstein, W. M., A. B. Onderdonk, J. G. Bartlett, and S. L. Gorbach.** 1974. Experimental intra-abdominal abcesses in rats: development of an experimental model. Infect. Immun. **10**:1250–1255.
18. **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.
19. **Welch, R. A., R. Hull, and S. Falkow.** 1983. Cloning and physical characterization of a chromosomal hemolysin from *Escherichia coli*. Infect. Immun. **42**:178–186.
20. **Williams, P. H.** 1979. Determination of the molecular weight of *Escherichia coli* α-haemolysin. FEMS Microbiol. Lett. **5**:21–24.