

## Actin enhances the haemolytic activity of *Escherichia coli*

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**Actin is a major cytoskeletal protein of mammalian muscle and non-muscle cells. Exposure of cells to soluble factors that damage cell membranes results in the release of actin into the extracellular spaces. The  $\alpha$ -haemolysin (HlyA) of *Escherichia coli* is the prototype RTX (repeat in toxin) toxin and is thought to be important in virulence because of its ability to lyse cells by formation of pores in the cell membrane. These studies were conducted to determine if actin influences growth and haemolytic activity of *E. coli*. Growth of *E. coli* in the presence of actin resulted in culture supernatant haemolytic activity that was 2.4-, 2.7- and 3.3-fold greater than that of *E. coli* grown in medium containing BSA, non-supplemented medium, or medium containing heat-denatured actin, respectively. The enhanced haemolytic activity occurred only when actin was present during the growth phase and there was no effect when actin was added to culture supernatants containing haemolysin. The increased haemolytic activity by actin was concentration-dependent, detectable in early-exponential-phase growth, and associated with increased concentrations of secreted HlyA by Western blotting. Actin induced a 2.9-fold increase in alkaline phosphatase activity in *E. coli* CC118 with a *TnphoA* insertion in the *hlyB* determinant of the recombinant haemolysin plasmid pWAM04. These results indicate that extracellular actin enhances haemolysin production by *E. coli* and may have implications in the pathogenesis of *E. coli* infections.**

**Keywords:** actin, *Escherichia coli*, haemolysin

### INTRODUCTION

Actin is the predominant protein of the microfilament cytoskeleton and the most abundant intracellular protein in mammalian cells. Actin accounts for 10–20% of protein in most cells and as much as 35% of total protein in platelets (Harris & Weeds, 1978; Pollard & Cooper, 1986; Stossel, 1978). Actin exists in two intracellular pools, soluble monomers (globular or G-actin) or polymerized filaments (filamentous or F-actin) (Cooper, 1991; Theriot, 1994). When soluble actin is released from damaged cells, polymerization is favoured in the presence of increased extracellular salt concentrations (Haddad *et al.*, 1990). Excessive cell damage from tissues undergoing necrosis or severe inflammation can result in the release of relatively large quantities of actin into the extracellular space and peripheral cir-

culatation (Goldschmidt-Clermont *et al.*, 1988; Lee *et al.*, 1985; Smith *et al.*, 1988). An actin-scavenger system composed of actin-binding and actin-severing proteins is present in serum of humans and animals and serves to remove extracellular actin (Dueland *et al.*, 1991; Harris *et al.*, 1980; Lee & Galbraith, 1992). Depletion of the actin-scavenger system can result in accumulation of extracellular actin, thus contributing to increased blood viscosity, endothelial damage, microvascular thrombosis and delayed clearance of inflammatory exudate (Haddad *et al.*, 1990; Vasconcellos *et al.*, 1994).

*Escherichia coli*  $\alpha$ -haemolysin (HlyA) is the prototype of the RTX (repeat in toxin) family of bacterial toxins (Felmlee *et al.*, 1985; Welch *et al.*, 1992). Gram-negative bacterial pathogens of humans and animals that produce structurally similar RTX toxins include *Actinobacillus actinomycetemcomitans* (Kolodrubetz *et al.*, 1989; Kraig *et al.*, 1990), *Pasteurella haemolytica*, *Actinobacillus pleuropneumoniae*, *Proteus vulgaris* and

**Abbreviations:** FBS, foetal bovine serum; SRBC, sheep red blood cell.

*Morganella morganii* (Devenish *et al.*, 1989; Eberspacher *et al.*, 1990; Koronakis *et al.*, 1987). Certain RTX toxins are host-specific and cytolytic against a narrow range of target cells, while *E. coli* haemolysin is toxic to a wide range of mammalian cell types (Beutin, 1991; Bhakdi *et al.*, 1990; Cavalieri & Snyder, 1982; Forestier & Welch, 1991; Trifillis *et al.*, 1994). The sublethal effects of HlyA on leucocytes include depletion of ATP and the release of inflammatory mediators and cytokines from leucocytes and platelets (Bhakdi *et al.*, 1990; Cavalieri & Snyder, 1982; Konig *et al.*, 1994). The cytotoxic activity of HlyA, as with other RTX toxins, results from the formation of 1–2 nm, cation-specific, transmembrane pores that result in leakage of the cytoplasmic membrane, osmotic swelling and cell lysis (Konig *et al.*, 1994; Menestrina *et al.*, 1994; Moayeri & Welch, 1994).

One consequence of cytolysis is the release of intracellular contents, including cytoplasmic proteins, into the extracellular space (Clinkenbeard *et al.*, 1989). Exposure of a susceptible cell line (BL3) to culture supernatants from *E. coli*, *P. haemolytica* and *A. pleuropneumoniae* results in the release of actin into culture supernatants (R. J. Basaraba & A. N. Byerly, unpublished results). During infections, host proteins like actin may interact with bacteria within inflammatory exudate and further influence bacterial pathogenicity. However, the effect that actin and other host proteins have on bacterial virulence factor expression, specifically RTX toxins, has not been adequately investigated (Clinkenbeard *et al.*, 1995; Waurzyniak *et al.*, 1994). In these studies, we show that actin enhances the expression of HlyA by *E. coli*.

## METHODS

**Bacteria and plasmids.** *Escherichia coli*, strain K-12, CC118 [*araD139*  $\Delta$ (*ara leu*)7697  $\Delta$ *lacX74* *phoA* $\Delta$ 20 *galE galK thi rpsE rpoB* *argE<sub>am</sub>* *recA1* (Manoil & Beckwith, 1985) was used as the host strain for *TnphoA* mutagenesis. The haemolysin recombinant plasmid pWAM04 (provided by Dr Rodney Welch, University of Wisconsin, USA) carries the *hlyCABD* genes that convey a haemolytic phenotype to host bacteria (Welch & Pellett, 1988). *E. coli* 5-20701 (O138: F:107 H14W, *Stx*<sup>+</sup> and *Stb*<sup>+</sup>) was isolated from the faeces of a pig with diarrhoea. Bacteria were grown on sheep blood agar and colonies were resuspended to an OD<sub>570</sub> of 0.250 in 0.01 M PBS (0.145 M NaCl; 0.15 M sodium phosphate). Bacterial suspensions (100  $\mu$ l,  $1.3 \times 10^6$  c.f.u. ml<sup>-1</sup>) were inoculated into 30 ml RPMI-1640 medium with or without 0.1% foetal bovine serum (FBS) (Sigma) and incubated for 6 h at 37 °C on a rotary shaker. Bacteria were removed by centrifugation at 16000 g for 1 min and the supernatant was filtered through 0.22  $\mu$ m pore-size acetate syringe filters (MSI, Micron Separations) and stored at -70 °C.

**Actin.** Actin was purified from bovine skeletal muscle using the method of Spudich & Watt (1971). Purified bovine skeletal muscle actin in buffer A (2 mM Tris/HCl, 0.2 mM Na<sub>2</sub>ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl<sub>2</sub>, pH 8.0) was filter-sterilized, polymerized by dialysis in 0.01 M PBS, and stored as F-actin at 4 °C at a concentration greater than 10 mg ml<sup>-1</sup>. G-actin was prepared by dialysis of F-actin in buffer A and by centrifugation for 30 min at 100000 g to remove residual F-

actin. G-actin was lyophilized in the presence of 2 mg sucrose (mg actin)<sup>-1</sup> and stored at -70 °C for long-term storage (Frieden, 1982). Protein concentrations were determined using the Pierce Micro BCA assay.

**Culture conditions.** Thirty millilitre aliquots of RPMI-1640 medium containing either F-actin, heat denatured F-actin, G-actin or BSA fraction V (ICN Biomedicals) (30  $\mu$ g ml<sup>-1</sup>) were inoculated with *E. coli* 5-20701 ( $1.3 \times 10^6$  c.f.u. ml<sup>-1</sup>) and incubated at 37 °C on a rotary shaker. Aliquots of bacterial suspension were removed from 0 to 12 h at 2 h intervals and at 24 h. Bacteria were removed by centrifugation and the supernatants were filtered and stored at -70 °C until assayed for haemolytic activity.

To determine the influence of FBS supplementation on haemolytic activity, *E. coli* was grown for 6 h in RPMI-1640 or RPMI-1640 supplemented with 0.1% FBS and/or 30  $\mu$ g F-actin ml<sup>-1</sup>. Bacteria were removed by centrifugation and the supernatant was filtered and stored at -70 °C until assayed for haemolytic activity.

The effect of various actin concentrations was evaluated with parallel cultures of *E. coli* in RPMI-1640 containing 0.1% FBS supplemented with 0–50  $\mu$ g F-actin ml<sup>-1</sup>. Haemolytic activity of 6 h sterile filtered culture supernatants was determined for each actin concentration.

In separate experiments, F-actin (30  $\mu$ g ml<sup>-1</sup>) was added to culture supernatants of RPMI-1640 and RPMI-1640 supplemented with 0.1% FBS prior to assaying for haemolytic activity.

**Haemolysin assay.** Haemolytic activity of culture supernatants was determined by lysis of 1% sheep red blood cell (SRBC) suspensions (Feder *et al.*, 1994). Whole sheep blood was collected in heparin, washed in PBS by centrifugation, and stored at 4 °C at a packed cell volume of 10–15% in citrate-phosphate-dextrose (280–300 mOsm kg<sup>-1</sup>). A standard curve of haemolytic activity was constructed by adding 0, 2, 5, 10, 20, 30, 50, 70, 90 and 100  $\mu$ l culture supernatants and the final volume of each well was adjusted to 100  $\mu$ l with RPMI-1640 medium. For each assay, 100  $\mu$ l of a 1% suspension of SRBCs in 10 mM Tris/HCl/saline, pH 7.4, was placed in triplicate wells of V-bottom 96-well microtitre plates (Dynatech Laboratories). Plates were incubated for 2 h at 37 °C, centrifuged for 5 min at 80 g, and 100  $\mu$ l supernatant was transferred to flat-bottom 96-well microtitre plates (Corning). The haemoglobin content was determined by measuring the A<sub>410</sub> using a microplate reader (V Max microplate reader; Molecular Devices). Relative haemoglobin concentrations were compared to the maximal haemoglobin release following osmotic lysis of SRBCs with distilled water.

Haemolysis was expressed as the A<sub>410</sub> or the reciprocal of the haemolytic units, where one haemolytic unit equals the microlitres of culture supernatant required to lyse 50% of a 100  $\mu$ l volume of a 1% suspension of SRBCs (Feder *et al.*, 1994). The number of haemolytic units in the culture supernatant was calculated from the standard curve using the ELISA plate reader software (Softmax; Molecular Devices).

**Bacterial growth.** Growth kinetics of *E. coli* were evaluated in parallel cultures in RPMI-1640 containing 0.1% FBS supplemented with F-actin, heat-denatured F-actin or BSA (30  $\mu$ g ml<sup>-1</sup>). Cultures were grown at 37 °C on a rotary shaker and aliquots for plate counts and OD<sub>570</sub> measurement were obtained at 2 h intervals from 0 to 24 h.

**Electrophoresis and Western blot analysis.** Culture supernatants were concentrated 20 $\times$  (Centricon 30; Amicon), boiled in electrophoresis buffer, subjected to SDS-PAGE using

4% stacking and 7.5% resolving gels, and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated with pooled anti-HlyA mAbs B7 and C10 (1:10000 and 1:5000, respectively) provided by Dr Rodney Welch (Pellett *et al.*, 1990). Actin-antibody complexes were detected by sequential reaction of membranes with anti-mouse antibody conjugated with horseradish peroxidase (Sigma), followed by 4-chloro-4-naphthol for colour development.

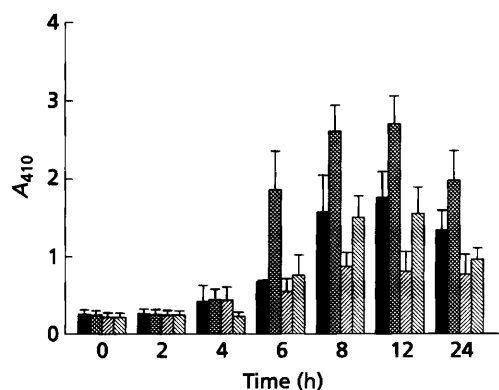
**TnphoA mutagenesis.** Plasmid pWAM04 was introduced into *E. coli* CC118 by transformation. TnphoA mutagenesis was carried out on this strain by the methods of Manoil & Beckwith (1985). Kanamycin-resistant, alkaline-phosphatase-positive colonies were plated on blood agar to assess the haemolytic phenotype and on Luria agar that contained ampicillin to monitor retention of the plasmid. Strains which were resistant to kanamycin and ampicillin and were non-haemolytic were selected. Plasmid DNA was isolated by the method of Birnboim & Doly (1979). The site of insertion of TnphoA was determined by DNA sequence analysis using the f-mol kit (Promega) and a primer specific to the phoA-end of TnphoA. One isolate, NH1, which contained a TnphoA insertion within hlyB was selected. The alkaline phosphatase spectrophotometric assays were carried out as described by Manoil & Beckwith (1985).

**Data analysis.** Data were analysed by repeated measures analysis and analysis of variance using SAS version 6.11 (SAS Institute, Cary, NC, USA). Tests for significance were by protected least significant differences.

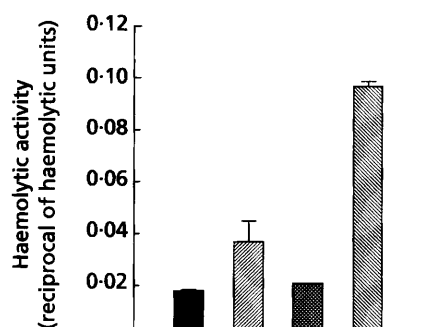
## RESULTS

### Influence of actin on haemolytic activity

The haemolytic activity of *E. coli* culture supernatants increased in a time-dependent fashion with peak activity between 8 and 12 h (Fig. 1). The relative trends for rate



**Fig. 1.** Comparison of haemolytic activity of culture supernatants of *E. coli* grown in the presence of protein supplements. The haemolytic activity of culture supernatants of *E. coli* grown in the presence of F-actin (▨), heat-denatured actin (▩) and albumin (□) (30 µg ml<sup>-1</sup>) was compared with that of non-supplemented RPMI (■). Haemolysis by *E. coli* culture supernatant was expressed as haemoglobin released from lysed SRBCs incubated with undiluted culture supernatant. Treatments from each experiment were done in triplicate and averaged. Data represent the mean of four separate experiments  $\pm$  SEM.



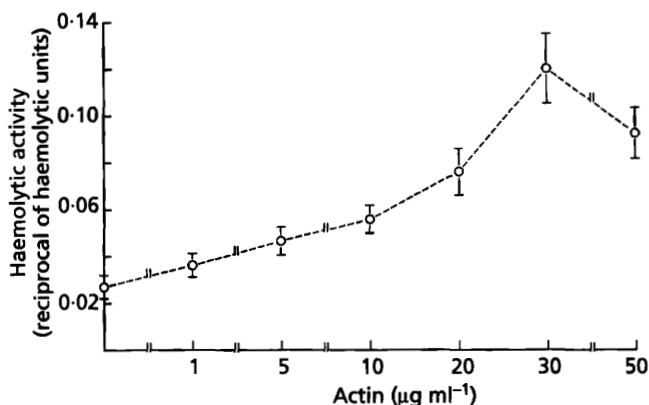
**Fig. 2.** Synergistic effect of serum and actin on the haemolytic activity of *E. coli* culture supernatants. The figure shows the haemolytic activity of culture supernatant of *E. coli* grown in RPMI-1640 (■), RPMI-1640 with 30 µg F-actin ml<sup>-1</sup> (▨), RPMI-1640 + 0.1% FBS (□) or RPMI-1640 + 0.1% FBS and 30 µg F-actin ml<sup>-1</sup> (▩). Treatments from each experiment were done in triplicate and averaged. The data represent the mean of four separate experiments  $\pm$  SEM.

of increase and the decline in haemolytic activity were similar for all treatments. However, at 6 h growth, culture supernatants containing actin had 2.7, 2.4 and 3.3 times more haemolytic activity than RPMI-1640, RPMI-1640 supplemented with BSA, and RPMI-1640 supplemented with heat-denatured actin, respectively. These differences were significant ( $P \leq 0.01$ ) at 6 as well as 8 h. At 12 h, actin-supplemented cultures differed only from those supplemented with heat-denatured actin ( $P \leq 0.01$ ). No significant differences were present between RPMI-1640 and RPMI-1640 supplemented with either heat-denatured actin or BSA at any time point.

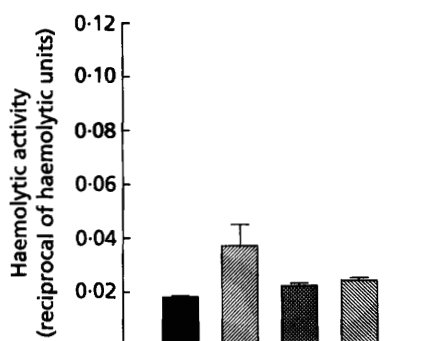
Supplementation of RPMI-1640 containing 0.1% FBS with actin increased the haemolytic activity of *E. coli* supernatant 4.6-fold compared to supernatant from RPMI-1640 containing only 0.1% FBS (Fig. 2). Supplementation of cultures with 0.1% FBS did not enhance the haemolytic activity compared to RPMI-1640 alone. Addition of 30 µg actin ml<sup>-1</sup> to cultures grown in RPMI-1640 medium increased the haemolytic activity 2.6-fold compared to non-supplemented RPMI-1640 cultures.

Actin-mediated enhancement of *E. coli* haemolysis activity in RPMI-1640 containing 0.1% FBS was concentration-dependent (Fig. 3). There was no significant difference ( $P \geq 0.05$ ) in haemolytic activity of culture medium with 0, 1, 5 and 10 µg actin ml<sup>-1</sup>. Differences were significant ( $P \leq 0.05$ ) when actin was added at 20 µg ml<sup>-1</sup> or greater, with maximal haemolytic activity at 30 µg actin ml<sup>-1</sup>. There was a slight decrease in haemolytic activity at 50 µg actin ml<sup>-1</sup>, although differences were not significant ( $P \geq 0.05$ ). *E. coli* culture supernatant supplemented with a 10-fold higher actin concentration (300 µg ml<sup>-1</sup>) had haemolytic activity similar to 30 µg ml<sup>-1</sup> and 50 µg ml<sup>-1</sup> samples (data not shown).

Addition of 30 µg actin ml<sup>-1</sup> to supernatants of *E. coli* grown in RPMI-1640 alone or RPMI-1640 with 0.1%



**Fig. 3.** Actin-concentration-dependent expression of *E. coli* haemolytic activity. *E. coli* was grown in the presence of RPMI-1640+0.1% FBS to which increasing concentrations of F-actin were added. The haemolytic activity of culture supernatant was determined by the haemoglobin released from the lysis of SRBCs. Data represent the mean of samples done in triplicate, averaged from five separate experiments  $\pm$  SEM.

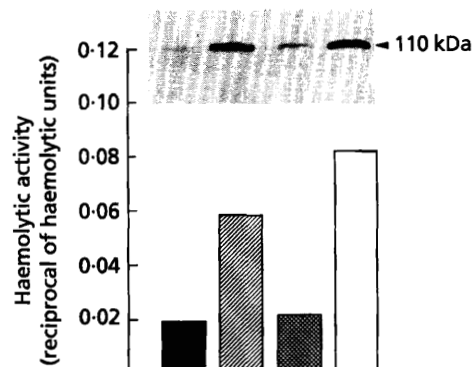


**Fig. 4.** Failure of actin to influence haemolytic activity of pre-formed haemolysin in *E. coli* culture supernatant. The haemolytic activity of culture supernatant of *E. coli* grown in the presence of RPMI-1640 supplemented with 30 µg F-actin ml<sup>-1</sup> (▩) was enhanced compared to that of *E. coli* grown in RPMI-1640 alone (■), RPMI-1640+0.1% FBS (▨) or RPMI-1640 containing 0.1% FBS to which 30 µg F-actin ml<sup>-1</sup> was added (▩), prior to incubation with SRBCs. Data represent the mean of triplicate samples averaged from four separate experiments  $\pm$  SEM.

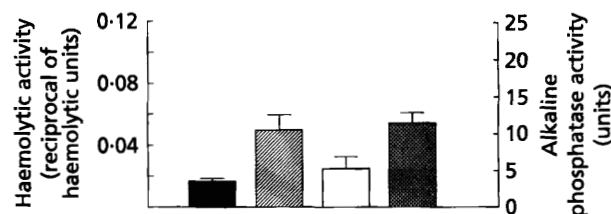
FBS immediately prior to the assay had no additional influence on the haemolytic activity (Fig. 4).

#### Effect of actin on *E. coli* growth

There were no significant differences in the growth of *E. coli* in supplemented and non-supplemented RPMI-1640 medium throughout the growth period. For 6 h cultures, the OD<sub>570</sub> was 0.253, 0.254, 0.256 and 0.248 in RPMI-1640, RPMI-1640 supplemented with F-actin, RPMI-1640 with heat-denatured actin and RPMI-1640 supplemented with albumin, respectively. These data were



**Fig. 5.** Actin-mediated enhancement of haemolytic activity corresponds to increased HlyA secretion. The lower panel shows the enhanced haemolytic activity by actin from a representative experiment (lower panel), and the corresponding Western blot analysis of culture supernatant shows increased immunoreactivity to HlyA (upper panel). *E. coli* was grown in RPMI-1640 (■), RPMI-1640+30 µg F-actin ml<sup>-1</sup> (▨), RPMI-1640+0.1% FBS (▩) or RPMI-1640+0.1% FBS+30 µg F-actin ml<sup>-1</sup> (□). Each lane in the upper panel contains the equivalent of 2 ml concentrated supernatant.



**Fig. 6.** Enhanced expression of HlyB-alkaline phosphatase fusion protein in NH1 grown in the presence of actin. The *E. coli* strain NH1 carries a copy of TnphoA in hlyB of the recombinant plasmid pWAM04, generating an HlyB-alkaline phosphatase fusion protein. Growth of NH1 in the presence of F-actin (▩) enhanced the alkaline phosphatase activity 2.9-fold compared to NH1 grown in RPMI-1640 alone (□). *E. coli* strain CC118 containing the recombinant pWAM04 plasmid without the TnphoA insertion served as a control and haemolytic activity of culture supernatant was determined following growth in RPMI-1640 alone (■) or in RPMI-1640 supplemented with 30 µg F-actin ml<sup>-1</sup> (▨). Data represent mean  $\pm$  SEM of four separate experiments.

confirmed with plate counts from a representative experiment (data not shown).

#### Electrophoresis and Western blot analysis

Representative culture supernatants with differing haemolytic activities were subjected to SDS-PAGE and immunoblot analysis (Fig. 5). A single band of 110 kDa was detected in *E. coli* culture supernatants by pooled mAbs B7 and C10. Culture supernatants supplemented with F-actin or F-actin and FBS had the greatest haemolytic activity (Fig. 5, lower panel) and the greatest relative concentration of HlyA as represented by band density (Fig. 5, upper panel).

### TnphoA mutagenesis

*E. coli* strain NH1 carries a copy of TnphoA in *hlyB* generating a HlyB-alkaline phosphatase fusion protein. In strain NH1, expression of alkaline phosphatase is under the control of the *hlyCABD* operon regulatory signals. When NH1 was tested for response to actin, it had a 2.9-fold increase in alkaline phosphatase activity when grown in the presence of F-actin, compared to when grown in RPMI-1640 alone (Fig. 6). *E. coli* strain CC118 containing the recombinant *hly* plasmid pWAM04 without the TnphoA insert, grown in the presence or absence of F-actin, was included as a control and had no measurable alkaline phosphatase activity but had comparable haemolytic activity that was 2.2-fold greater in the presence of actin than that of bacteria grown in RPMI-1640 alone.

### DISCUSSION

This study demonstrates that the haemolytic activity of *E. coli* culture supernatant was enhanced when bacteria were grown in the presence of actin. This enhancement was not due to an effect on bacterial growth, but was associated with increased secretion of HlyA in culture supernatants. The dose responsiveness and the saturation of the effect by high concentrations of actin are consistent with an influence of actin on haemolysin production and secretion rather than changes in HlyA stability or the effects on *E. coli* growth from the added protein. The effect of actin on *E. coli* haemolysin secretion is particularly relevant since actin is ubiquitous and is released from intracellular pools following cell damage, including that from pore-forming cytotoxins such as HlyA (R. J. Basaraba & A. N. Byerly, unpublished results).

When haemolytic activity was examined over time, actin-supplemented cultures had increased activity at all time points compared to non-supplemented and BSA- and heat-denatured-actin-supplemented cultures. Haemolytic activity declined at a rate that was similar for all treatments, suggesting that the differences in haemolytic activity between treatments were not due to differences in rate of degradation or changes in haemolysin stability. Proteins, chaotropic agents or cations may stabilize HlyA and similar toxins or prevent the self-aggregation of these proteins, thus increasing the active concentration in solution (Clinkenbeard *et al.*, 1995; Confer & Durham, 1992; Gentry *et al.*, 1986; Rennie *et al.*, 1974; Waurzyniak *et al.*, 1994). Calcium pretreatment of HlyA changes irregular toxin aggregates to regular spherical structures (Rennie *et al.*, 1974). Calcium also activates HlyA and is necessary for binding of haemolysin to erythrocytes and thus influences activity as a post-translational modification (Boehm *et al.*, 1990). Interestingly, calcium is an integral part of the actin protein structure and may therefore interact with HlyA through its calcium-binding sites (Frieden, 1982). A variation in free calcium concentration in these studies appeared to have no significant influence on haemolytic activity when treatments were compared to media

controls. Actin preparations were dialysed and polymerized in a calcium-free PBS buffer prior to use to avoid the influence of changes in extracellular cation concentrations.

To determine if actin influenced haemolytic activity by interacting directly with HlyA or target cells, actin was added to culture supernatant containing haemolysin immediately prior to assaying for haemolytic activity. The lack of effect of actin on haemolysin post-secretion suggests that F-actin has its influence at the time HlyA is produced in the early growth phase or immediately following secretion.

A specific effect of F-actin on *E. coli* haemolytic activity is suggested by the loss of its influence when denatured by heat and by the significant increase in activity when monomeric G-actin is polymerized to F-actin. This observation has *in vivo* relevance since extracellular bacteria are probably exposed to higher concentrations of F-actin at sites of inflammation or tissue necrosis (Vasconcellos *et al.*, 1994). G-actin within the cytoplasm is soluble and is released from damaged cells, while F-actin is insoluble and closely associated with cell membranes (Cano *et al.*, 1992; Fechheimer & Zigmond, 1993). Polymerization of G-actin to F-actin is favoured, however, in the presence of high salt concentrations that exist in extracellular spaces (Haddad *et al.*, 1990; Vasconcellos *et al.*, 1994). Therefore, purified G-actin in these studies was polymerized by dialysis against PBS containing physiological salt concentrations.

The synergistic effect between actin- and serum-supplemented medium was unexpected. A concentration of serum was chosen that, when added to culture medium, had no effect on bacterial growth or haemolytic activity. However, when actin was added to the serum-containing medium, haemolytic activity increased significantly. Serum contains the actin-binding and scavenger proteins gelsolin and vitamin-D-binding protein (GC-globulin) (Lee & Galbraith, 1992; Lind *et al.*, 1986). Besides binding actin monomers and severing actin filaments, gelsolin is a potent actin nucleating protein thus favouring polymerization. Actin-binding proteins under these conditions may interact with actin and further influence actin polymerization thus amplifying the effect of F-actin on haemolysin activity (Lind *et al.*, 1986). The effect of actin on haemolytic activity is dependent on the form of actin since incubation of *E. coli* in the presence of actin in the G-form has no effect on haemolytic activity.

To establish that the enhanced haemolytic activity by F-actin was not due to nutrient enrichment, *E. coli* growth rate was compared in the presence of equal concentrations of BSA, heat-denatured actin and non-supplemented media. The similarity in the rate of bacterial growth between protein-supplemented and non-supplemented culture media indicated that F-actin had little influence on growth, yet significantly influenced expression of haemolytic activity of the culture supernatant.

Western blot analysis of culture supernatants was used

to determine if F-actin-enhanced haemolytic activity was associated with increased secretion of HlyA in the culture supernatant. The relative increase in immunoreactivity of HlyA in supernatants containing actin was consistent with increased HlyA concentration rather than a change in activity of secreted HlyA caused by F-actin. Although haemolytic activity is generally related to HlyA concentration, extracellular haemolysin can be inactivated without significant degradation of the HlyA protein (Oropeza-Wekerle *et al.*, 1989; Rennie *et al.*, 1974). This loss of haemolytic activity is partially due to a lack of binding of HlyA to target cells (Boehm *et al.*, 1990; Oropeza-Wekerle *et al.*, 1989). Culture supernatants with equal HlyA concentrations could therefore differ in haemolytic activity. However, an increase in haemolytic activity in actin-supplemented culture supernatants in these studies was associated with an increase in HlyA production that was reflected in the culture supernatant as increased HlyA immunoreactivity.

The secretion of active, extracellular HlyA requires the expression of four genes (*hlyCABD*) constituting the *hly* operon (Welch *et al.*, 1992). An alteration in the expression of one or more of these gene products required for active transport or activation of HlyA would be reflected in the expression of haemolytic activity in the culture supernatant. Additionally, post-translational modifications of secreted protein can alter haemolytic activity (Boehm *et al.*, 1990; Oropeza-Wekerle *et al.*, 1989). The insertional mutagenesis of the *hly* operon by *TnphoA* enabled the use of this reporter to confirm that growth of *E. coli* in the presence of actin induced the expression of the genes and thus production of HlyA. These data support the Western blot data, indicating that the increased secretion of HlyA was due to increased production of HlyA in response to actin supplementation. Furthermore, the increased alkaline phosphatase fusion protein concentration in response to F-actin indicates that the actin enhancement effect is not an artifact of the haemolysis assay. The laboratory strain of *E. coli*, CC118, carrying the pWAM04 recombinant haemolysin plasmid showed a similar increased response to F-actin both in the haemolysin assay and with the *TnphoA* insertion as did pathogenic *E. coli* isolates. These data suggest that the ability of *E. coli* to respond to F-actin is common to at least those pathogenic and laboratory strains that carry the *hly* genes.

The concentration responsiveness of actin-associated haemolytic activity, the saturation by high actin concentrations, the loss of activity by heat denaturation and the induction of HlyA production in response to actin suggest specific binding of actin to surface-exposed structures on bacterial cells. Such an interaction has been described for *Listeria monocytogenes* and *Shigella flexneri*, which express specific actin-binding outer-membrane proteins that are important in virulence. The outer-membrane proteins ActA of *L. monocytogenes* and IcsA of *S. flexneri* induce polymerization of cytoplasmic actin and the formation of actin tails used for intracellular motility and cell invasion (Bernardini *et al.*, 1989; Goldberg & Sansonetti, 1993; Kocks *et al.*, 1992).

The interaction between actin and surface structures of *E. coli* is supported by preliminary studies in our laboratory showing that certain pathogenic strains induce polymerization of G-actin in solution. However, attempts to demonstrate surface-bound polymerized actin on *E. coli* by immunofluorescence and immunohistochemistry using an anti-actin mAb have been inconclusive (R. J. Basaraba & A. N. Byerly, unpublished results).

Enteropathogenic *E. coli* induces polymerization of intracellular actin at sites of attachment to small and large intestinal epithelium, producing the characteristic attaching effacing (AE) lesion. A dense plaque of polymerized actin develops at the site of attachment of AE bacteria to cell membranes (Knutton *et al.*, 1987; Rothbaum *et al.*, 1982; Ulshen & Rollo, 1980). This pattern of actin accumulation is specific to enteropathogenic *E. coli* species that express the *eaeA* gene encoding the 94 kDa outer-membrane protein intimin (Donnenberg *et al.*, 1993a, b). The polymerization of intracellular actin by intimin is mediated through multiple transmembrane signalling events as the result of intimate attachment between the bacteria and host cells (Kenny & Finlay, 1997).

Additionally, the alteration of intracellular actin cytoskeleton by some strains of *E. coli* can be mediated by secreted toxins. Cytotoxic necrotizing factor is a monomeric protein secreted by certain *E. coli* strains isolated from humans and animals with diarrhoea and extra-intestinal infections (Blanco *et al.*, 1997; Caprioli *et al.*, 1987). Cytotoxic necrotizing factor induces assembly of intracellular F-actin stress fibres by modification of the GTP-binding protein Rho (Fiorentini *et al.*, 1995; Oswald *et al.*, 1994). The current study suggests that besides alteration of intracellular actin pools by *E. coli* through cell contact or secreted toxins, some pathogenic strains of *E. coli* can interact with extracellular actin. The exposure of bacteria to extracellular actin may occur as the result of host cell lysis by exotoxins like HlyA or other mediators of necrosis and inflammation. These interactions with actin may influence the potential pathogenesis of *E. coli* by masking surface antigens, increasing adhesion or modulating virulence factor expression as shown in the current studies (L'Hote *et al.*, 1996; Olsen *et al.*, 1989; Shen *et al.*, 1995).

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