

## Enteropathogenic *Escherichia coli* Protein Secretion Is Induced in Response to Conditions Similar to Those in the Gastrointestinal Tract

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**The pathogenicity of enteropathogenic *Escherichia coli* (EPEC) is associated with the expression and secretion of specific bacterial factors. EspB is one such secreted protein which is required to trigger host signaling pathways resulting in effacement of microvilli and cytoskeletal rearrangements. These events presumably contribute to the ensuing diarrhea associated with EPEC infections. EPEC encounters several environmental changes and stimuli during its passage from the external environment into the host gastrointestinal tract. In this paper we show that the secretion of EspB is subject to environmental regulation, and maximal secretion occurs under conditions reminiscent of those in the gastrointestinal tract. Thus, secretion is maximal at 37°C, pH 7, and physiological osmolarity. In addition, maximal secretion requires the presence of sodium bicarbonate and calcium and is stimulated by millimolar concentrations of Fe(NO<sub>3</sub>)<sub>3</sub>. The secretion of the four other EPEC-secreted proteins appears to be modulated in a manner similar to that of EspB. Our results also show that secretion is not dependent on CO<sub>2</sub>, as originally reported by Haigh et al. (FEMS Microbiol. Lett. 129: 63–67, 1995), but that CO<sub>2</sub> more likely acts as a component of the medium buffering system, since CO<sub>2</sub> dependence was abolished by the use of alternative buffers.**

Enteropathogenic *Escherichia coli* (EPEC) is a gram-negative bacterium which causes diarrhea in humans and is responsible for the deaths of up to a million infants per annum. During infection, EPEC forms microcolonies which adhere to jejunal epithelial cells (36, 37). Microcolony formation and efficient attachment to host cells involves the bundle-forming pilus (5, 12). Attachment leads to the localized effacement of the underlying microvilli, while more intimate association is correlated with host cytoskeletal reorganization and the generation of pedestal-like structures upon which the adherent bacteria reside (8, 21, 22, 32). How these bacterial events contribute to the resulting diarrhea is still unknown. Both in vitro and in vivo studies have identified several bacterial and host components participating in these EPEC-induced alterations of the host cell. EPEC-host cell interaction induces host protein phosphorylation events and inositol phosphate and calcium fluxes, as well as cytoskeletal rearrangements (1, 7, 8, 10, 20, 26, 32). Several of these events are dependent on the secretion of at least two EPEC-secreted proteins (EspA and EspB, formerly EaeB) and a dedicated type III secretion apparatus (15, 18, 19). In addition to EspA and EspB, EPEC secretes at least three other proteins, one of which, EspC, is a member of the immunoglobulin A protease family (42). The bacterial outer membrane protein intimin (EaeA) is essential in organizing host cytoskeletal rearrangements and generating the pedestal-like structure on which the bacteria reside (16, 17, 32, 33). Intimin is required for full bacterial virulence, and its expression is regulated by the *per* regulon (6, 13). This regulatory locus is located on a 60-MDa pMAR plasmid, as are the genes encoding the bundle-forming pilus (13, 41, 43). The *per* regulon comprises four reading frames (*perA*, *B*, *C*, and *D*),

and maximal expression of intimin requires all four gene products. However, expression of PerC alone can induce intimin expression (13). PerA appears to be a member of the AraC family of bacterial regulators (13), which includes VirF, a transcriptional activator of the *Yersinia* virulence regulon (3). The *per* locus also plays a role in EPEC protein secretion (18) and bundle-forming pilus regulation (31).

The pathogenicity of many bacteria is associated with the expression of specific proteins, some of which are secreted, including the *Shigella* Ipa and *Yersinia* Yop proteins (reviewed in references 29 and 45). Expression of such genes is tightly regulated and responds to environmental stimuli, such as temperature, pH, and nutrient availability (reviewed in references 9, 27, and 39). Such regulation permits the coordinated and timely expression of proteins required for growth and survival under changing environmental conditions. Although *Yersinia* secretes proteins, it is now becoming apparent that under in vivo conditions some of these proteins are transferred directly into host cells (35). *Yersinia* Yop protein secretion is dependent on calcium concentrations (44), although it has been proposed that this mimics mammalian cell contact (35).

In this study we examined the secretion of the EPEC-secreted proteins in response to various external conditions to determine if secretion is regulated in response to changing environmental conditions. Our results demonstrate that secretion is regulated by temperature, pH, and osmolarity. We also identified several other conditions which are required for maximal protein secretion or which inhibit the secretory process. The results of these studies are discussed in relation to in vivo environmental conditions encountered by EPEC during its passage through the gastrointestinal tract.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and chemicals.** The wild-type EPEC strain E2348/69 and the plasmidless JPN15 strain were used in this study (24, 25). The multicopy plasmid CVD450 encodes the *per* regulon (13). Tetracycline (25 µg/ml final concentration) was used to select and maintain bacteria carrying CVD450. Me-

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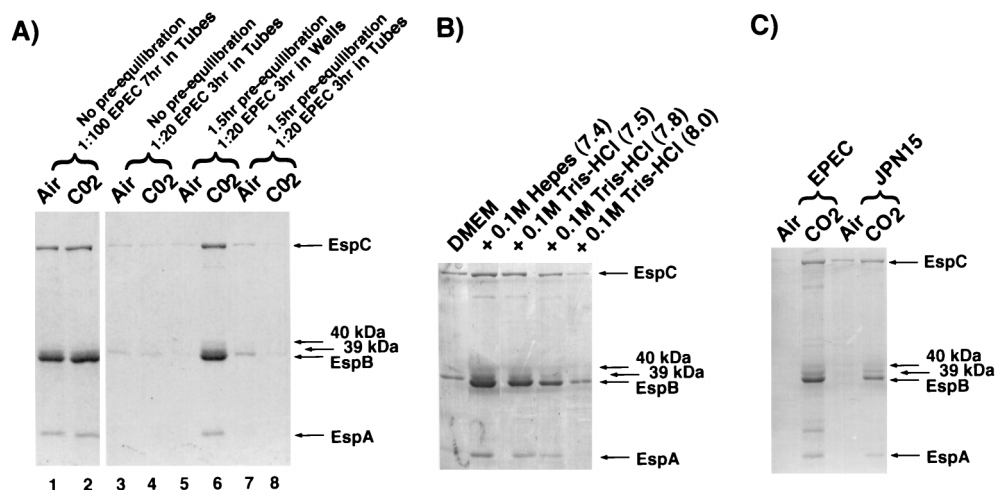


FIG. 1. EPEC secretion is not dependent on  $\text{CO}_2$ . (A) Overnight EPEC cultures grown in LB were diluted 1:100 (lanes 1 and 2) or 1:20 (lanes 3 to 8) in 2 ml of DMEM and grown for 7 h (lanes 1 and 2) or 3 h (lanes 3 to 8) at  $37^\circ\text{C}$  in air or 5%  $\text{CO}_2$  atmosphere in glass tubes (lanes 1 to 4, 7, and 8) or six-well plates (lanes 5 and 6). The DMEM (lanes 5 to 8) was pre-equilibrated in a  $\text{CO}_2$  incubator for 90 min prior to infection. (B) Two milliliters of DMEM (untreated or buffered with 0.1 M HEPES [pH 7.4] or 0.1 M Tris [pH 7.4, 7.8, or 8]) was placed in six-well plates and pre-equilibrated for 90 min in a  $37^\circ\text{C}$  air incubator prior to infection with a 1:20 dilution of overnight EPEC culture. Cultures were grown for 3 h. (C) Two milliliters of DMEM was placed in six-well plates and pre-equilibrated for 90 min in a  $37^\circ\text{C}$  air incubator prior to infection with a 1:20 dilution of overnight wild-type EPEC culture or the plasmid-cured strain JPN15. Wild-type EPEC was grown for 3 h, while growth of JPN15 was continued until it reached the same optical density as the wild type. One-milliliter supernatant samples were isolated, and the proteins were precipitated by the addition of TCA (see Materials and Methods). Samples were resolved by SDS-PAGE (12%) and visualized by Coomassie blue. The positions of the secreted EspA (25 kDa), EspB (37 kDa), 39-kDa, 40-kDa, and EspC (110 kDa) proteins are indicated by arrows.

media used in this study were Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (Gibco), Luria broth (LB), and M9 minimal medium. LB and M9 medium were made up as described by Sambrook et al. (38), with the addition of 0.8 mM  $\text{MgSO}_4$  to the M9 medium. Buffering agents used were HEPES (Sigma), morpholinopropanesulfonic acid (MOPS) (Sigma), and Tris (Boehringer Mannheim).

**SDS-PAGE analysis of secreted proteins.** EPEC was inoculated into 2 ml of LB and grown overnight at  $37^\circ\text{C}$  without shaking. EPEC was routinely diluted 1:100 in the appropriate medium. During assessment of the contribution of salts in secretion, LB-grown EPEC was washed in salt-free medium prior to inoculation. The bacterial cultures were grown in a 5%  $\text{CO}_2$  atmosphere unless otherwise indicated. The optical density of the culture was monitored at  $A_{600}$ , and supernatant samples were taken at the same growth phase ( $A_{600}$  from 0.5 to 0.7). Bacteria were removed by centrifugation ( $16,000 \times g$ , 5 min), and supernatant proteins were precipitated by the addition of trichloroacetic acid (TCA) (10% [vol/vol]; BDH) for 60 min on ice. Precipitated proteins were pelleted by centrifugation at  $4^\circ\text{C}$  ( $16,000 \times g$ , 10 min) and resuspended in Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 10% (vol/vol) saturated Tris base (Boehringer Mannheim). Samples were resolved on 12% polyacrylamide gels as described by Laemmli (23) and were visualized by Coomassie R-250 blue staining (0.25% in 40% methanol–10% acetic acid solution; ICN Biochemicals).

**ELISA of secreted EspB.** Supernatant samples were collected as described above. Microtiter plates (Immunolon 2; DynaTech) were seeded with the supernatant volume derived from bacteria at  $A_{600}$  of 0.01, and the volume was increased to 200  $\mu\text{l}$  with sterile DMEM. Twofold serial dilutions were made, and the plates were incubated overnight at  $4^\circ\text{C}$  for protein binding. Plates were washed twice with distilled water and blocked with 200  $\mu\text{l}$  of 0.1% Tween-20 (BDH) in phosphate-buffered saline (PBS) for 1 h at  $37^\circ\text{C}$ . Each plate was washed three times with distilled water. Rabbit anti-EspB antibodies were diluted 1:5,000 in 0.05% Tween-20–PBS, and 100  $\mu\text{l}$  was added per well. After being incubated for 1 h at  $37^\circ\text{C}$ , the wells were washed as before. Peroxidase-conjugated affinity-purified goat anti-rabbit immunoglobulin G antibodies (ImmunoResearch Laboratories Inc.) were diluted 1:3,000 in 0.05% Tween-20–PBS, and 100  $\mu\text{l}$  was aliquoted per well. After 1 h of incubation at  $37^\circ\text{C}$ , the wells were washed three times with distilled water and the enzyme-linked immunosorbent assay (ELISA) was developed by adding 200  $\mu\text{l}$  of developing solution (30 mg of *o*-phenylenediamine dihydrochloride [Sigma] in 30 ml of 0.1 M citric acid [pH 4.4]). The reaction was stopped by adding 100  $\mu\text{l}$  of 3 N sulfuric acid. The ELISA plates were then read at 490 nm. The dilution factor required to obtain an absorbance of approximately 0.1 was determined and was used to gauge the difference in protein secretion levels relative to the positive control.

**Data imaging.** Photographic negatives or raw data of the figures were scanned into Adobe Photoshop with an AGFA studio scanner or a Kodak professional RFS 2035 Plus film scanner in which they were labeled before being printed with a Mitsubishi S3600-30U color printer.

**Compositions of media.** Composition of DMEM was as follows:  $\text{Fe}(\text{NO}_3)_3$ , 0.25  $\mu\text{M}$ ;  $\text{CaCl}_2$ , 1.4 mM;  $\text{KCl}$ , 5.4 mM;  $\text{MgSO}_4$ , 0.8 mM;  $\text{NaCl}$ , 110 mM;  $\text{Na}_2\text{HPO}_4$ , 1 mM; and  $\text{NaHCO}_3$ , 44 mM. Composition of M9 medium was as follows:  $\text{KH}_2\text{PO}_4$ , 22 mM;  $\text{Na}_2\text{HPO}_4$ , 48 mM;  $\text{NH}_4\text{Cl}$ , 18.4 mM;  $\text{MgSO}_4$ , 0.8 mM; and  $\text{NaCl}$ , 8.55 mM. M9 is supplemented with 0.45% glucose and Casamino Acids (0.2% final concentration), while DMEM contains 0.45% glucose, essential amino acids, vitamins, and phenol red (Gibco). Reconstituted DMEM (RD-MEM) contained the same components as DMEM except for the omission of vitamins.

## RESULTS

**EPEC protein secretion is dependent on  $\text{CO}_2$  buffering.** Recently, Haigh et al. (14) reported that the secretion of EspB was dramatically reduced when cultures were grown in air compared to when they were grown in a 5%  $\text{CO}_2$  atmosphere. However, under conditions used in our laboratory we found no difference in secretion levels in these two environments (Fig. 1A, lanes 1 and 2). Our growth conditions differed from those of Haigh et al. in several aspects, including the following: (i) a fivefold-smaller inoculum, (ii) incubation for 7 h in glass tubes rather than 3 h in six-well plates, and (iii) no pre-equilibration of the media in air or in  $\text{CO}_2$  incubators. We therefore compared secretion under both sets of conditions. We found that if we used a fivefold-higher inoculum, secretion was practically abolished under our growth conditions (Fig. 1A, lanes 3 and 4). Although we confirmed the finding of Haigh et al. (Fig. 1A, lanes 5 and 6), we found the results not conclusive, as growing EPEC in glass tubes instead of six-well plates abolished secretion (Fig. 1A, lanes 7 and 8). These results suggested that induction of EPEC secretion is a more complicated matter and not simply dependent on  $\text{CO}_2$ , but that more likely  $\text{CO}_2$  is required as a buffering agent. To investigate this possibility, we buffered DMEM with either 0.1 M HEPES or Tris-HCl and grew EPEC in an air atmosphere under the conditions described by Haigh et al. Figure 1B shows that, as reported, growth in the air atmosphere in the absence of a buffering agent did not induce high-level secretion. However, buffering the medium to pH 7.4 to 7.5 with these alternative buffers

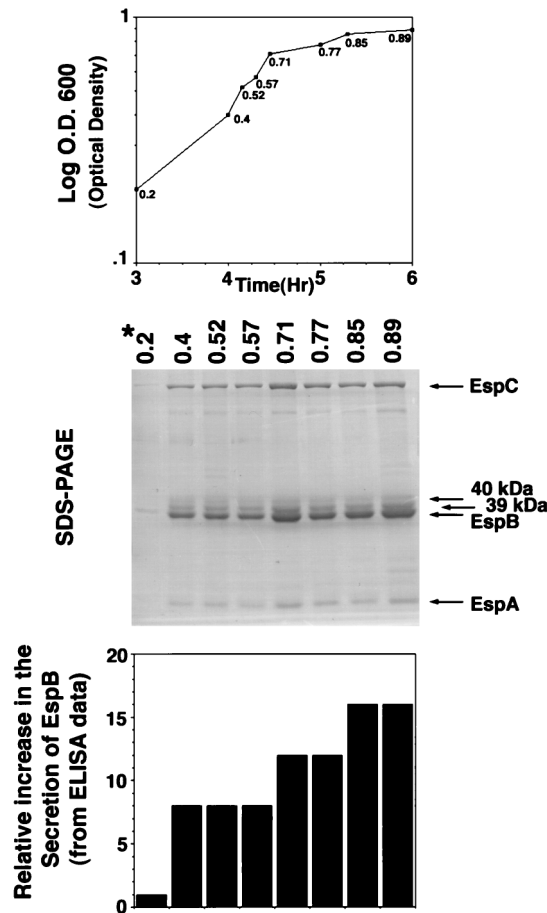


FIG. 2. Secretion is induced by early logarithmic growth phase. EPEC was diluted 1:100 into 3 ml of DMEM in 10-ml glass tubes and grown at 37°C. At various time intervals a sample was removed for optical density determination (upper panel). A volume of supernatant derived from bacteria at an  $A_{600}$  of 0.4 was TCA precipitated, except for the lane indicated by an asterisk, where only the volume derived from bacteria at an  $A_{600}$  of 0.2 was precipitated due to limiting volume, and analyzed by SDS-PAGE (middle panel). The lower panel depicts the results obtained from ELISA studies quantifying the relative secretion levels of secreted EspB at the time intervals indicated in the upper panel. ELISA studies were carried out on supernatant samples (in triplicate) derived from bacteria at  $A_{600}$  of 0.01 with anti-EspB antibodies (see Materials and Methods).

induced high levels of secretion independent of  $\text{CO}_2$ , and secretion was reduced as the pH was increased (Fig. 1B).

In addition, Haigh et al. (14) reported that pMAR001, a plasmid-cured EPEC strain lacking the *per* regulon, had wild-type secretion levels in DMEM when grown in a  $\text{CO}_2$  atmosphere. However, we previously reported that high-level EPEC protein secretion required the *per* regulon (18), and thus, we reexamined these observations. We used a similar mutant strain, JPN15, also cured of the large pMAR plasmid encoding the *per* regulon, and found that in contrast to the report of Haigh et al., secretion by JPN15 was reduced compared to secretion by EPEC (Fig. 1C) in the presence of  $\text{CO}_2$ .

#### EPEC protein secretion levels vary during growth phase.

We next investigated the relationship between secretion and EPEC growth phase. Thus, DMEM was inoculated with EPEC and samples were taken at various times for optical density determination. In addition, supernatant samples were taken for ELISA and SDS-PAGE analysis. Figure 2 shows that whereas protein secretion is only readily detectable by mid-

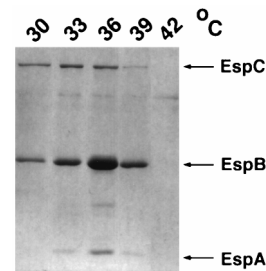


FIG. 3. Protein secretion is temperature regulated. EPEC was diluted 1:100 in DMEM and grown to the same optical density at either 30, 33, 36, 39, or 42°C in an air incubator. Supernatant samples derived from equal numbers of bacteria were isolated, and the proteins were precipitated by the addition of TCA (see Materials and Methods). Samples were resolved by SDS-PAGE (12%) and visualized by Coomassie blue.

logarithmic phase ( $A_{600} = 0.4$ ) with SDS-PAGE, the more sensitive ELISA detected secreted EspB by early log phase ( $A_{600} = 0.2$ ). From mid-log phase ( $A_{600} = 0.4$ ) onwards the rate of secretion appears to be constant, as doubling the number of bacteria only doubled the level of secreted EspB.

**Stringent temperature regulation of EPEC protein secretion.** Previously, we reported that EPEC secretion is inhibited at 30°C (18). As EPEC is expected to encounter a temperature shift following entry into the host, we examined the effect of a range of temperatures on protein secretion. To assess this, EPEC-inoculated DMEM was incubated in an air atmosphere at either 30, 33, 36, 39, or 42°C prior to the isolation and concentration of the supernatant proteins. Analysis of the resulting supernatant protein profiles by SDS-PAGE demonstrated that EPEC protein secretion is maximal at around 36°C and is significantly reduced at 33 or 39°C and undetectable at 42°C (Fig. 3).

**EPEC protein secretion varies in different media.** The above results indicate that EPEC is capable of responding to certain environmental factors, such as temperature, to modulate protein secretion. An indication that EPEC secretion is responsive to additional environmental signals came from the observation that M9 or LB did not support protein secretion but that this inhibition could be alleviated if the bacteria were transformed with the multicopy plasmid CVD450 (Fig. 4). This plasmid carries the *per* regulatory locus (13), which has been shown to influence EPEC protein secretion (18). Indeed, the presence of CVD450 even induced higher secretion levels from EPEC grown in DMEM (Fig. 4).

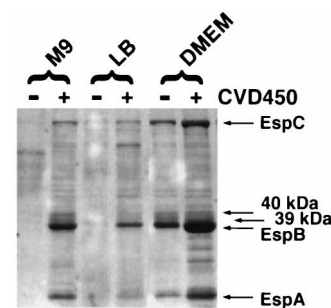


FIG. 4. EPEC cannot secrete in LB or M9 medium except when transformed with CVD450. EPEC or EPEC/CVD450 (*per*) was diluted 1:100 in DMEM, LB, or M9 medium and incubated for 8 h at 37°C in an air incubator. One-milliliter supernatant samples were isolated, and the proteins were precipitated by the addition of TCA (see Materials and Methods). Samples were resolved by SDS-PAGE (12%) and visualized by Coomassie blue.

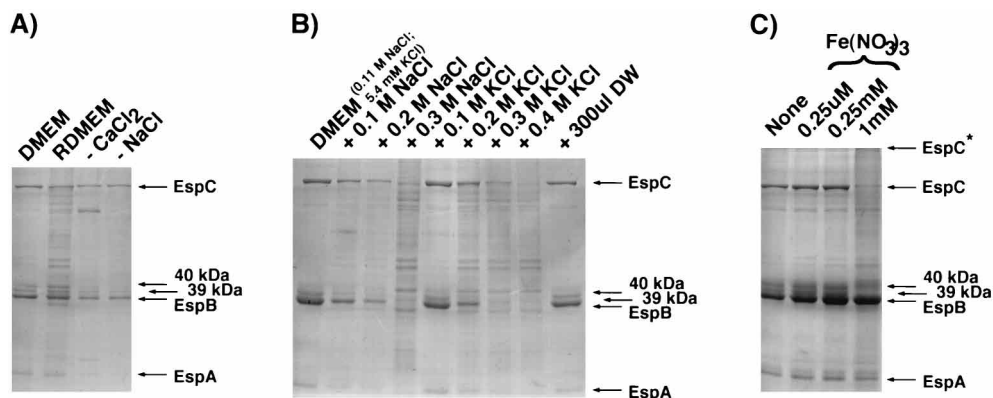


FIG. 5. Factors in DMEM influencing EPEC protein secretion. DMEM was reconstituted (RDMEM) as described in Materials and Methods, omitting or adding various components as indicated. EPEC was washed in the same volume of salt-free medium and diluted 1:100 in DMEM or RDMEM lacking  $\text{CaCl}_2$  or NaCl (A), in the presence of increasing concentrations of NaCl or KCl (B), or in the absence or presence of various concentrations of  $\text{Fe}(\text{NO}_3)_3$  (C). Cultures were grown to an  $A_{600}$  of 0.5 to 0.6 in a 5%  $\text{CO}_2$  incubator at 37°C; 1-ml supernatant samples were isolated, and the proteins were precipitated by the addition of TCA (see Materials and Methods). Samples were resolved by SDS-PAGE (12%) and visualized by Coomassie blue. The addition of 1 mM  $\text{Fe}(\text{NO}_3)_3$  led to the appearance of a high-molecular-mass band, possibly an EspC aggregate, which is indicated in panel C.

**Protein secretion is regulated by calcium and osmolarity.** To investigate the components of DMEM that induce EPEC protein secretion, we examined the contribution of individual components of this medium on EPEC secretion. DMEM was reconstituted omitting individual components. The various RDMEMs were inoculated with EPEC and incubated to the same growth phase in a 5%  $\text{CO}_2$  incubator. Supernatant samples were isolated and analyzed by SDS-PAGE. In order to quantitate differences in the levels of protein secretion, we also carried out ELISA studies, probing with anti-EspB antibodies (see Materials and Methods).

The level of secretion in RDMEM was the same as that in the commercial DMEM (Fig. 5A; ELISA data not shown). As EPEC secretion did not require the vitamins present in DMEM (data not shown), they were omitted in further experiments. We found that secretion was reduced if NaCl or  $\text{CaCl}_2$  (normally 110 and 1.4 mM, respectively) was omitted from RDMEM, but without any significant effect on bacterial growth (Fig. 5A), indicating a role for these inorganic salts in maximal secretion. ELISA indicated that secretion was reduced by four- and twofold, respectively, in the absence of these salts (data not shown). The observation that omission of NaCl inhibited secretion suggested that this process might be sensitive to osmoregulation. To investigate this possibility, the osmolarity of the media was increased by adding higher concentrations of NaCl or KCl. Figure 5B shows the progressive inhibition of secretion with increasing salt concentrations.

Omitting  $\text{Fe}(\text{NO}_3)_3$ , normally at a concentration of 0.25  $\mu\text{M}$ , from the media reduced secretion levels (Fig. 5B) by an estimated twofold by ELISA. In contrast, addition of increasing concentrations of  $\text{Fe}(\text{NO}_3)_3$  led to higher secretion levels (twofold by ELISA) (Fig. 5C). Surprisingly, 1 mM  $\text{Fe}(\text{NO}_3)_3$  appeared to specifically inhibit EspC secretion, though an aggregate which might represent an EspC complex is apparent at the top of the gel lane (Fig. 5C).

We found that the presence of the KCl,  $\text{MgSO}_4$ , and  $\text{NaH}_2\text{PO}_4$  was essential for bacterial growth, and thus, the role of these salts in modulating secretion levels could not be addressed.

**Dependence of secretion on pH and sodium bicarbonate.** Initial observations (Fig. 1B) indicated that pH affects EPEC secretion. As EPEC experiences large pH changes during its passage through the gastrointestinal tract, we decided to ex-

amine more closely the effect of pH on secretion. MOPS at a concentration of 0.1 M was added to DMEM, and the pH was adjusted by the addition of NaOH or HCl. MOPS was chosen in this experiment, as it has a wider buffering capacity than HEPES or Tris-HCl. The buffered media were inoculated as before, and their ability to support EPEC secretion was examined. Figure 6A shows that buffering the medium pH from 6.35 to 7.6 supported high levels of secretion but that secretion was greatly reduced at pH 6 or 8. Bacterial growth could not be supported at higher or lower pHs.

To investigate a role for  $\text{NaHCO}_3$  (present at 44 mM) in DMEM, we first buffered the medium with 0.1 M MOPS (pH 7.5) prior to varying the  $\text{NaHCO}_3$  concentration, as  $\text{NaHCO}_3$ - $\text{CO}_2$  buffers the medium. Figure 6B shows that reducing the concentration of  $\text{NaHCO}_3$  from 44 to 10 mM had no significant effect on secretion, whereas further reduction inhibited secretion (Fig. 6B).

**Ammonium chloride inhibits EPEC secretion.** As shown earlier (Fig. 4), M9 medium is not conducive to high levels of secretion unless EPEC carries CVD450. Thus, to determine if there was a single component in the M9 medium that inhibited EPEC secretion, we reconstituted the medium, omitting individual components. Omitting individual M9 salts did not induce EPEC protein secretion (data not shown). We assessed whether the addition of DMEM components to M9 medium could induce EPEC protein secretion. The addition of such components did not induce secretion (data not shown). We also investigated whether any M9 component would inhibit EPEC secretion when added to DMEM and found that  $\text{NH}_4\text{Cl}$  reduced secretion (Fig. 7).

## DISCUSSION

In this study we investigated the apparent contradiction of the results we obtained to those of Haigh et al. (14), who reported a dependence of EspB secretion on  $\text{CO}_2$ . We determined that secretion was not dependent on  $\text{CO}_2$ , but that  $\text{CO}_2$  was probably required to act as a buffering agent with the  $\text{NaHCO}_3$  in the media in order to maintain the pH at approximately 7.4. Thus, in the absence of  $\text{CO}_2$  the medium pH remains high (pH 7.8 to 8): conditions unfavorable for secretion. High levels of secretion were detected if the medium was buffered with HEPES or Tris-HCl (pH 7.4 to 7.5) in the ab-

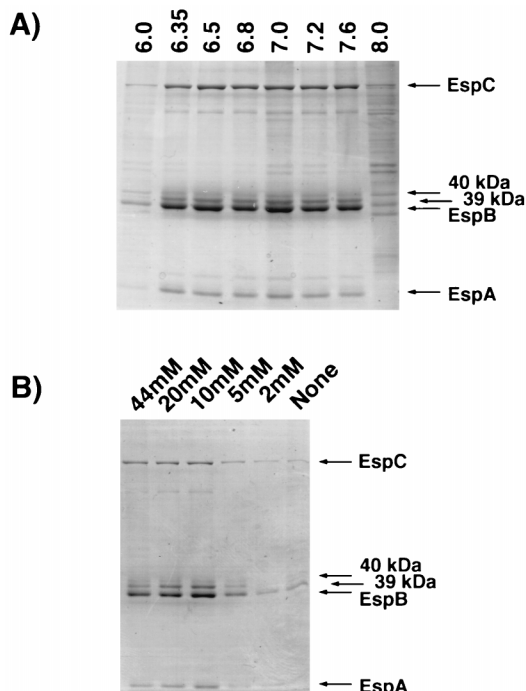


FIG. 6. EPEC protein secretion is influenced by pH and sodium bicarbonate ( $\text{NaHCO}_3$ ) concentration. (A) MOPS (0.1 M) was added to DMEM, and the pH was adjusted by the addition of HCl or NaOH. (B) DMEM was reconstituted, omitting  $\text{NaHCO}_3$ , 0.1 M MOPs was added, and the pH was adjusted to 7.4 before adding various concentrations of  $\text{NaHCO}_3$ . In both experiments overnight EPEC cultures were diluted 1:100 and grown to an optical density of 0.5 to 0.6 in an air incubator at  $37^\circ\text{C}$ . One-milliliter supernatant samples were isolated, and the proteins were precipitated by the addition of TCA (see Materials and Methods). Samples were resolved by SDS-PAGE (12%) and visualized by Coomassie blue.

sence of  $\text{CO}_2$ , although increasing the pH to 7.8 and 8 reduced secretion levels. Thus, EPEC does not appear to use  $\text{CO}_2$  as a regulatory factor in protein secretion, but rather protein secretion appears to be sensitive to external pH. Since EPEC encounters extremes of pH during its migration through the gastrointestinal tract, we assessed the effect of various pHs on secretion by using MOPS as a buffering agent. We found that EPEC protein secretion was modulated by external pH. Whereas high-level secretion occurred between pHs 6.35 and 7.6, secretion was dramatically reduced at pHs 6 and 8. It should be noted that under the conditions described by Haigh et al., high-level protein secretion was only induced under a  $\text{CO}_2$  atmosphere and was abolished when the growth medium was transferred to glass tubes or was not preequilibrated with  $\text{CO}_2$ . In contrast, under our conditions, EPEC secretion occurred in both air and  $\text{CO}_2$  atmospheres in glass tubes. This might be due to bacterially produced  $\text{CO}_2$  buffering the medium to generate a secretion-competent pH, but this might only be possible when the medium surface area is small and air- $\text{CO}_2$  diffusion is slow.

Temperature regulation is a mechanism frequently employed to modulate the expression of virulence genes, as pathogens often encounter temperature shifts upon entry into a host (27). We previously reported that EPEC protein secretion was reduced at low temperatures (18), and here we show that secretion is also decreased at higher temperatures. An interesting feature of the temperature regulation of EPEC secretion is that protein secretion appears maximal at normal human body temperatures and is inhibited at both lower and

higher temperatures, possibly aiding specificity of host infection. Rosenshine et al. (34) also recently reported that EPEC does not induce host signaling events when infected at  $28^\circ\text{C}$ . These EPEC-induced host signaling events, and protein secretion, have been correlated with epithelial cytoskeletal rearrangements and invasion in vitro (18, 19). Donnenberg et al. (4) have reported that enteroinvasive *E. coli*, but not EPEC, invasion into HEP-2 cells was inhibited at  $32^\circ\text{C}$ . However, we detected significant secretion at  $32^\circ\text{C}$  (about two- to threefold less than at  $36^\circ\text{C}$ ), which might result in reduced invasion levels.

We also observed that in contrast to growth in DMEM, protein secretion was not detectable after growth in M9 or LB medium. However, secretion could be induced if EPEC was transformed with CVD450, encoding the *per* positive regulon, probably due to the inability of the cells to regulate the expression of this regulon when it is present in multicopies. Thus, we assessed the contribution of the components comprising these media to secretion and tried to determine the factors in DMEM or M9 which stimulated or inhibited protein secretion. Several components of DMEM were required for maximal secretion, as their omission reduced secretion by two- to fourfold without any significant effect on bacterial growth. One of these components was calcium, a molecule implicated in the regulation of *Yersinia* virulence gene expression (44). In contrast to *Yersinia*, where millimolar concentrations (2.5 mM) of calcium inhibit virulence expression and protein secretion, EPEC required millimolar concentrations of calcium for maximal secretion. Another component known to regulate the expression of virulence determinants is iron (27), as host tissues are rich in iron binding proteins that efficiently sequester this essential element. We found that, in contrast to what was expected, secretion was reduced by omitting the  $0.25 \mu\text{M}$   $\text{Fe}(\text{NO}_3)_3$  from DMEM and was increased by the addition of  $\text{Fe}(\text{NO}_3)_3$  to 1 mM concentrations.

EPEC protein secretion was reduced by about fourfold if NaCl was omitted from DMEM, suggesting that secretion might be osmoregulated. Osmoregulation controls the expression of known virulence proteins (27), such as the *Yersinia* enterotoxin Yst, whose expression is also regulated by temperature and pH (28). To test this, we added increasing concentrations of NaCl or KCl to DMEM and observed a gradual inhibition of secretion, supporting the hypothesis.

We also investigated the relationship between bacterial growth phase and secretion in DMEM and found that secretion is induced by early log phase ( $A_{600} = 0.2$ ), with maximal

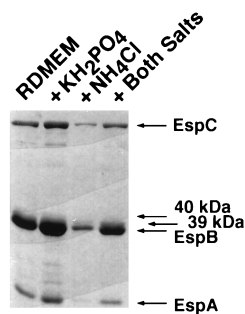


FIG. 7.  $\text{NH}_4\text{Cl}$  decreases EPEC protein secretion.  $\text{KH}_2\text{PO}_4$  and  $\text{NH}_4\text{Cl}$  were added to DMEM in the concentration present in M9 medium (see Materials and Methods). EPEC was diluted 1:100 in the medium, and cultures were grown to an optical density of 1 in the 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ . One-milliliter supernatant samples were isolated, and the proteins were precipitated by the addition of TCA (see Materials and Methods). Samples were resolved by SDS-PAGE (12%) and visualized by Coomassie blue.

secretion occurring by mid-log phase ( $A_{600} = 0.4$ ). Secretion appears to remain constant up to early stationary phase, as doubling the number of bacteria only doubled the detectable secreted EspB. The high levels of EPEC protein secretion at early growth phase contrasts with the expression of some virulence proteins of invasive pathogens, such as the *Yersinia* Inv and Yst proteins, which are induced in early stationary phase (28, 30). *Salmonella* is another invasive pathogen whose invasive ability is sensitive to aeration, growth phase, and osmolarity (reviewed in reference 27). Recently, *Salmonella* has been shown to secrete several proteins implicated in virulence (reviewed in reference 11). Bajaj and coworkers (2) have recently shown that expression of several of these secreted proteins is correlated with conditions that induce maximal invasiveness, such as low oxygen, high osmolarity, and alkaline pH. This regulation differs from that observed here for EPEC and probably reflects different adaptations of invasive and noninvasive pathogens to survive in different niches.

EPEC secretion was also found to be dependent on the presence of sodium bicarbonate, as its omission, in the presence of alternative buffering agents, resulted in decreased secretion levels. Thus, it appears that EPEC might use this compound as a positive signal to trigger secretion. In vivo, the acidic nature of the stomach contents is rapidly neutralized upon its entry into the small intestine by high levels of sodium bicarbonate which are present in the pancreatic juices and released by the intestinal mucosa (40). Sodium bicarbonate would be an appropriate signaling molecule to indicate passage from the stomach into the small intestine, the site of EPEC-host cell interactions. However, the addition of this compound to M9 did not induce secretion in this medium, indicating that other compounds also influence secretion. It would appear that the composition of DMEM is compatible for EPEC growth and secretion, but that it takes only one negative signal to shut down protein secretion, such as growth at 42°C or an external pH of 8. It still remains a possibility that EPEC can release additional virulence-associated proteins but that we have not managed to mimic the correct conditions. In support of this is the observed secretion of additional proteins which is particularly evident when  $\text{CaCl}_2$  is omitted from DMEM (Fig. 5A).

Although this analysis only used ELISA to quantify the secretion of EspB, it is apparent from the protein gels that all five secreted proteins are regulated in a similar manner. The only exception was seen with the strain lacking *per*, JPN15, which maintained high levels of EspC secretion in the absence of the other four proteins when grown in air (Fig. 1C). The *per* locus encodes a positive regulator required for the expression of several EPEC virulence-associated genes, such as those encoding intimin and the bundle-forming pilus, and for maximal EPEC protein secretion (13, 18, 31, and this study). Whereas these four proteins are secreted by a type III secretory apparatus, EspC is released by another mechanism (18, 42). The ability of EspC to be secreted in the absence of the *per* regulon suggests that that gene may be responsive to additional regulatory factors.

Very recently, Puente et al. (31) reported the results of their studies into the regulation of the bundle-forming pilus gene (*bfpA*), which demonstrated that it is regulated at the transcriptional level by the *per* regulon. Several of the conditions these authors describe as necessary to induce *bfpA* expression also induce EPEC protein secretion, suggesting that the *per* regulon also regulates secretion at the transcriptional level. However, it is still possible that secretion is modulated by controlling the transcriptional expression of the genes encoding the dedicated export apparatus and/or its assembly. In addition, BfpA expression is repressed during growth in LB (5,

12, 31), a growth medium not conducive to EPEC secretion, while both processes are inhibited in the presence of ammonia (this paper and 31). Given the similarity of regulation of these different virulence-related proteins, it is likely that additional EPEC virulence genes will be regulated in a similar manner and thus respond to the same external stimuli described here. Indeed, the secretion of EspC from wild-type EPEC, though not from the plasmid-cured *per* mutant JPN15 strain, mirrored that of EspB. Our results also suggest that the expression of known EPEC virulence determinants, such as intimin, type III accessory proteins, and BfpA, will also be regulated in response to the external stimuli found to modulate protein secretion.

The regulation of EPEC virulence genes in response to the environmental conditions described in this paper can be rationalized if one follows EPEC's passage throughout the infection process. Following ingestion, EPEC encounters a temperature shift to 37°C followed by the acidic conditions of the stomach. Although 37°C is a positive signal for secretion, we have shown that secretion is inhibited at low pH. Upon entry of the chyme into the small intestine, its acidic nature is rapidly neutralized by a sodium bicarbonate-rich solution emanating from the pancreas and intestinal mucosa. Secretion of such fluids would also lead to the generation of an isotonic environment, while the nutrient-rich environment would be ideal for rapid growth. One can envisage that as EPEC travels further down the intestine a point is reached where the pH, osmolarity, and other environmental conditions are ideal for maximal virulence gene expression and protein secretion, which marks this site as the region of maximal bacterial-host interaction. It is apparent that many of the factors which influence protein secretion mimic conditions found in the small intestine where EPEC colonize (36, 37, 40). The high concentration of ammonia in the colon, a compound which reduces both BfpA expression (31) and protein secretion (this paper), might inform EPEC that it has been unsuccessful in obtaining its ideal habitat for survival and replication and thus suppress virulence gene expression so the bacteria can be released into the external environment to begin the infectious cycle once again.

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