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Temperature- and Medium-Dependent Secretion of Proteins by Shiga Toxin-Producing *Escherichia coli*

FRANK EBEL,[1] CHRISTINA DEIBEL,[1] ANDREAS U. KRESSE,[2] CARLOS A. GUZMÁN,[2] AND TRINAD CHAKRABORTY[1]*

Institut für Medizinische Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen,[1] and National Center for Biotechnology, Department of Microbiology, D-38124 Braunschweig,[2] Germany

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Infections due to Shiga toxin-producing *Escherichia coli* (STEC) are responsible for severe diarrheal disease in humans and livestock, and these bacteria have recently emerged as a leading cause of renal failure in children. In this study, we have examined medium- and temperature-dependent production of secreted proteins from a STEC O26 serotype strain. Growth of bacteria in Luria broth led to the detection of secreted polypeptides of 104, 55, 54, and 37 kDa (p104, p55, p54, and p37, respectively). When grown in serum-free tissue culture medium, only p104, p37 and two additional polypeptides of 25 and 22 kDa (p25 and p22) were present in supernatant fluids. Production of these polypeptides was growth temperature dependent and induced in cultures grown at 37°C. N-terminal amino acid sequencing revealed that p104 was homologous to the secreted p110 of enteropathogenic *Escherichia coli* (EPEC), and both proteins belong to a family of secreted proteins in pathogenic bacteria of which the immunoglobulin A protease of *Neisseria gonorrhoeae* is the prototype. The N-terminal amino acid sequences of p55 and p54 were unique to the STEC strain, while p37 and p25 were found to be highly homologous to the similarly sized EspA and EspB proteins, previously detected in culture supernatants of EPEC. Molecular cloning and sequencing of STEC *espB* alleles from two different serotypes showed that the encoded polypeptides were about 80% homologous. A monoclonal antibody raised against STEC EspB also cross-reacted with its EPEC analog and allowed us to demonstrate medium- and temperature-dependent production of this important virulence factor in STEC and EPEC strains of differing serotypes.

Bacteria of the species *Escherichia coli* are the major constituent of the human aerobic flora. Different groups of pathogenic *E. coli* are important pathogens of the gut, resulting in different forms of diarrheagenic diseases. Shiga toxin-producing *E. coli* (STEC), also synonymously called verotoxin-producing *E. coli*, are a major public health concern in the Western world. STEC infection results in a hemorrhagic form of colitis which sometimes causes a severe complication called hemolytic-uremic syndrome, leading to acute renal failure in children and death (14). STEC, isolated from patients suffering from hemorrhagic colitis, are also referred to as enterohemorrhagic *E. coli* (EHEC). These clinical isolates are restricted to a few serotypes, of which O157:H7 seems to be of particular importance. However, other serotypes, including O111 and O26, have also recently been recognized as being associated with hemolytic-uremic syndrome (6).

Contaminated food is the major source of infection by STEC, and a dose lower than 10^3 bacteria was reported to be sufficient for productive infection (13). A characteristic feature of STEC bacteria is their ability to adhere closely to the gut epithelium, efface the surrounding microvilli, and induce actin-based pedestals underneath the eukaryotic membrane. The resulting histopathological phenotype called attaching/effacing (A/E) lesion is also characteristic in infection caused by enteropathogenic *E. coli* (EPEC) and some strains of *Citrobacter freundii* (32) and *Hafnia alvei* (1). The underlying mechanisms that lead to A/E lesions appear to be highly similar and can be genetically pinpointed to the presence of a distinct chromosomal 35-kb segment, called the LEE locus (locus of enterocyte effacement), that is present in these strains but absent from both the laboratory *E. coli* K-12 and other wild-type *E. coli* strains (23). To date, a number of genes present in this region have been sequenced. The *eaeA* (*E. coli* attaching and effacing) gene, which encodes a 97-kDa surface protein, was cloned and sequenced for both EPEC and STEC (4, 17, 36) and appears to act as an adhesin mediating tight contact of the bacterium with the eukaryotic membrane. Two additional genes, designated *espA* and *espB*, are probably involved in signal transduction events necessary for the formation of actin-based pedestals in infected cells (10, 30). The *espB* gene, previously designated *eaeB*, has to date been cloned and sequenced only for EPEC (9), but hybridization studies indicate that it is also present in STEC strains of serotype O157:H7 (22). These studies have furthermore revealed that the EspB polypeptide is secreted despite the absence of a signal petide, suggesting that it may be transported by proteins constituting a type III secretion system. Indeed, sequencing data have recently provided evidence for the existence of such a secretion system, designated *sep* (for secretion of *E. coli* proteins), present within the 35-kb LEE segment of an EPEC strain (16). This has been corroborated and extended by studies demonstrating that the EspB is at least one of two proteins secreted by EPEC bacteria following contact with eukaryotic cells (19).

Secretion of virulence factors is a common feature of many pathogenic bacteria. We and others have recently demonstrated that many STEC bacteria secrete, in addition to the Shiga toxin, small quantities of a hemolysin (Hly_{EHEC}) which is a member of the superfamily of RTX toxins (33, 35). In this study, we have performed a detailed analysis of the other

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie, Frankfurter Str. 107, 35390 Giessen, Germany. Phone: 49 641 702 7388. Fax: 49 641 702-7387. Electronic mail address: trinad.chakraborty@mikrobio.med.uni-giessen.de.

TABLE 1. Strains used in this study

Strain	Group	Serotype	Source	Reference
413/89-1	STEC	O26:H$^-$	Calf	35
EDL933	STEC	O157:H7	Human	24
HUS-2	STEC	O111:H$^-$	Human	18
H-19	STEC	O26:H11	Human	15
S102-9	STEC	O5	Calf	7
E2348/69	EPEC	O126:H6	Human	8
CB207	A/E positive	O55	Calf	35

polypeptides present in the supernatant fluids of STEC at different growth temperatures. We found temperature-regulated expression of three secreted proteins with molecular masses of 104, 37, and 25 kDa. N-terminal sequencing of these proteins revealed that the 37-kDa protein is the EspB protein of STEC and permitted the cloning and sequencing of this gene from two different STEC serotypes. A monoclonal antibody was produced against the STEC EspB and allowed us to demonstrate that production of this protein in supernatants in both EPEC and STEC strains is temperature dependent. N-terminal sequencing of the 25-kDa polypeptide indicated that it was highly homologous to a similar-sized protein detected in supernatants of EPEC strains. However, a search for sequence homologies to the N-terminal 26 amino acid residues of the 104-kDa polypeptide indicated that it is a novel protein that may be specific for STEC strains.

MATERIALS AND METHODS

Bacterial strains and growth media. The STEC and EPEC strains used in this study are summarized in Table 1. Bacteria were grown at 37°C with shaking in either Luria-Bertani (LB) broth or serum-free tissue culture medium (minimal essential medium [MEM], RPMI 1640, or Dulbecco's modified Eagle medium [DMEM]; GIBCO, Eggenstein, Germany), each supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4).

SDS-PAGE, immunoblotting, and sequence analysis. Bacteria were grown in LB broth or MEM to an optical density at 600 nm of 0.9. Determination of bacterial counts was performed by plating diluted aliquots of culture fluid with a spiral plater (Autoplate 3000; Biosys, Karben, Germany). Suspensions were centrifuged at 3,500 × *g* for 15 min, and the supernatant proteins were precipitated by the addition of 10% (vol/vol) trichloroacetic acid, overnight incubation at 4°C, and subsequent centrifugation at 4,000 × *g* for 30 min. The dry pellet was resuspended in 1.5 M Tris (pH 8.8), and after the addition of sample buffer, the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie brilliant blue staining. For N-terminal sequencing, the proteins were separated by SDS-PAGE and blotted onto ProBlott membranes (Applied Biosystems, Weiterstadt, Germany) with a semidry device. After visualization of the protein bands with 0.1% amido black in 45% methanol and 7% acetic acid, bands of interest were cut out and analyzed with a phase sequencer (model A470; Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid analyzer.

Database searches for homologous proteins were performed with the BLASTP and FASTA algorithms (2, 25).

Generation of monoclonal antibodies specific for the EspB protein. Proteins secreted by strain 413/89-1 in MEM were separated on SDS–10% PAGE gels and stained with Coomassie brilliant blue. The band with an apparent molecular mass of 37 kDa was excised, and electroelution was performed in 25 mM Tris base–192 mM glycine–0.025% SDS directly into dialysis tubes. The material was lyophilized after extensive dialysis against 0.1 M $(NH_4)_2CO_3$, dissolved in phosphate-buffered saline, analyzed by SDS-PAGE, and stored at −80°C. N-terminal sequencing of the eluted polypeptide revealed its identity with EspB. Immunization and generation of monoclonal antibodies were performed by standard protocols with P3X63Ag8.653 (ATCC CRL 1580) myeloma cells. Subcloning was performed by limited dilution with peritoneal macrophages as feeder cells, and hybridomas were maintained in OPTI-MEM (GIBCO) supplemented with 5% fetal calf serum. Screening was performed with concentrated supernatants of strain 413/89-1 grown in MEM that were separated on SDS-PAGE and blotted onto Immobilon P membranes (Millipore, Eschborn, Germany). Several hybridoma supernatants were tested simultaneously for specific antibodies with a miniblotting apparatus (Biometra, Göttingen, Germany). The subclass of the antibody was determined with specific antibodies (Dianova, Hamburg, Germany).

PCR, cloning, and sequencing of *espB* genes. Routine techniques were performed by use of standard procedures (31). Two oligonucleotides were derived for the amplification of the *espB* genes of STEC 413/89-1 and EDL933. The sense primer EspB1 (5′-ATGAATACTATCGATAATAA-3′) was deduced from the N-terminal sequence of the EspB protein secreted by STEC 413/89-1, while the antisense primer EspB4 (5′-AACGTATCGACCATGATCAA-3′) was homologous to a region immediately downstream of the *espB* gene of EPEC E2348/69 (9). The sense primer EspB5 (5′-AACCAGGCGAATTATATACA-3′) that was used to amplify the complete *espB* gene of strain EDL933 was homologous to a region about 100 bp upstream of the *espB* gene of strain E2348/69 (9).

Material from one bacterial colony grown overnight on an LB plate was resuspended in 100 μl of sterile water and heated to 95°C for 10 min. Five microliters of this supension was used as a template for PCR amplification. The amplified products were separated on an agarose gel, and the fragment with the expected size was eluted from the gel with the JETSORB kit (Genomed, Bad Oeynhausen, Germany) and subsequently cloned into the pCRII vector as described in the instructions of the manufacturer (TA-cloning kit; INVITROGEN, Leek, The Netherlands). The clone was sequenced by the Sanger dideoxynucleotide chain termination method with universal vector primers and an automatic sequencing apparatus (Applied Biosystems).

Nucleotide sequence accession number. The nucleotide sequences reported here have been submitted to the GenBank-EMBL database under accession numbers X96953 and X99670.

RESULTS

Overexpression of secreted proteins in tissue culture medium. We first examined culture supernatants for proteins secreted by the STEC strain 413/89-1, a serotype O26 strain isolated from the diarrheal stool of a weaning calf. Bacteria were grown either in LB broth or in serum-free tissue culture medium (MEM) to an optical density at 600 nm of 0.9. The secreted proteins were concentrated by trichloroacetic acid precipitation of supernatants, separated by SDS-PAGE, and stained with Coomassie brilliant blue (Fig. 1). Four dominant protein bands with apparent molecular masses of 104, 55, 54, and 37 kDa (p104, p55, p54, and p37, respectively) as well as a variety of minor bands were detected in the supernatants of bacteria grown in LB broth. Growth in tissue culture medium, however, led to the expression of four proteins, namely, p104, p37, and additional proteins of approximately 25 and 22 kDa (p25 and p22, respectively). The amount of the p104 polypeptide was slightly reduced in MEM, while the p55 polypeptide was not seen in most preparations performed with MEM. The observed overexpression of p37, p25, and p22 was also found when bacteria were grown in other tissue culture media (DMEM and RPMI 1640). Surprisingly, expression of the Hly_{EHEC} in MEM was not increased to a level detectable by Coomassie staining.

N-terminal sequence analysis of proteins secreted by STEC 413/89-1. To characterize the proteins secreted in either LB broth or MEM, trichloroacetic acid-precipitated samples were separated by SDS-PAGE, transferred onto ProBlott membranes, and further analyzed by N-terminal sequence analysis. Sequences were obtained from p104, p55, p54, and p37 secreted in LB broth and from p104, p37, p25, and p22 secreted in MEM (Fig. 1, arrows).

The N termini of the p104 polypeptides secreted in LB broth and MEM were identical and had significant similarities to five proteins from four different gram-negative organisms, i.e., the Hap protein (*Haemophilus* adherence and penetration) (12) and the immunoglobulin A1 (IgA1) protease (27) from *Haemophilus influenzae*, the SepA protein from *Shigella flexneri* (*Shigella* extracellular protein) (5), the Tsh protein from the avian pathogenic *E. coli* strain χ7122 (temperature-sensitive hemagglutination) (28), and the IgA1 protease from *Neisseria gonorrhoeae* (26) (Fig. 2A). A motif, DFAENKG, that was strongly conserved in all of these proteins was also detected in the N-terminal sequence of a 110-kDa protein that has recently been identified in the supernatant of the EPEC strain

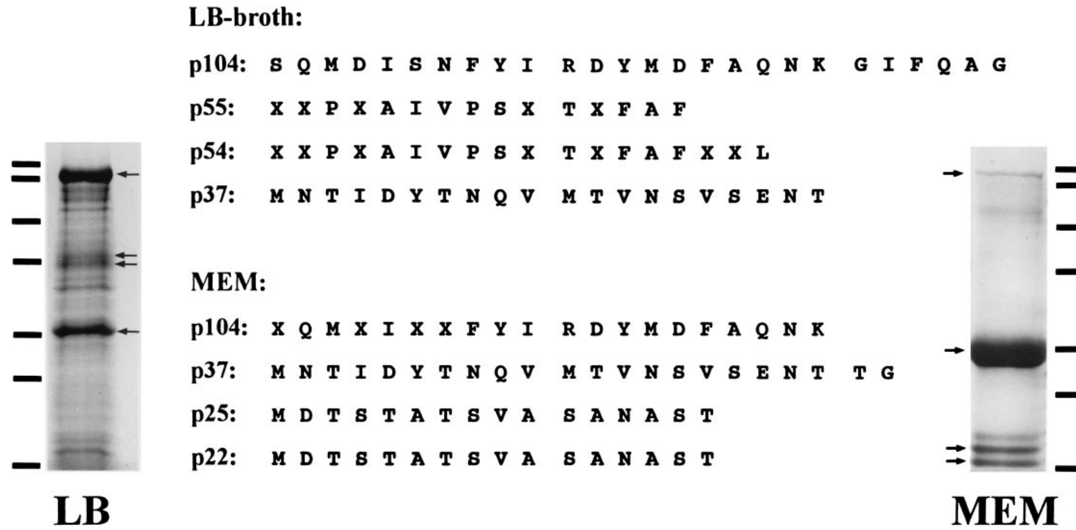

FIG. 1. Supernatant proteins of STEC 413/89-1 grown in LB broth or MEM were stained with Coomassie brilliant blue. Proteins that were further analyzed by N-terminal sequencing are indicated by arrows. Amino-terminal sequence data are presented in a one-letter code; X represents an amino acid that has not been identified. Standard molecular mass markers are indicated (top to bottom, 116, 97, 66, 55, 36, 31, and 21 kDa).

E2348/69 (19). Indeed, 10 of 21 amino acids from this sequence were identical to those observed for p104, suggesting that these proteins are functionally related.

Sequencing of polypeptides p54 and p55 led to the unambiguous identification of only 9 of the 15 N-terminal amino acids sequenced. From these data, the N termini of p54 and p55 were indistinguishable, indicating that p54 is probably a slightly truncated form of p55. Comparison with known sequences in databases revealed no obvious homologies to other proteins. Immunoblot experiments performed with a monoclonal antibody specific for p55 and p54 revealed that these proteins are present in the supernatant but absent from the cellular fraction, demonstrating that p55 and p54 are secreted by an efficient but yet-unknown mechanism (data not shown).

The N-terminal sequence of p37 showed a high degree of homology to the sequence of the EspB protein from EPEC (Fig. 2). From the 22 amino acids obtained from the N terminus of p37, amino acids at 12 positions were identical, with an overall similarity of 68.1% to the EspB protein of the EPEC strain E2348/69. Proteins p25 and p22 showed identical N-terminal sequences and were in turn almost identical to the N-terminal sequence of the EspA protein present in the su-

A:

p104	S Q M D I S N F Y I	R D Y M **D F A** Q **N K**	**G** I **F** Q A **G A** T
p110	A G L N I D N V W A	R D Y L **D** L **A** Q **N K**	**G**
Hap	A G H T Y F G I D Y	Q Y Y R **D F A** E **N K**	**G** K **F** T V **G A** Q
SepA	S A T V S A E I P Y	Q I F R **D F A** E **N K**	**G** Q **F** T P **G A** T
Tsh	A G T V N N E L G Y	Q L F R **D F A** E **N K**	**G** M **F** R P **G A** T
IgA-n[1]	A A L V R D D V D Y	Q I F R **D F A** E **N K**	**G** K **F** F V **G A** T
IgA-h[2]	A A L V R D D V D Y	Q I F R **D F A** E **N K**	**G** K **F** T V **G A** Q

B:

EspB$_{STEC}$	**M N T I D** Y T **N** Q V	M T **V N S V** S E N **T**	**T** G
EspB$_{EPEC}$	**M N T I D** N N **N** A A	I A **V N S V** L S S **T**	**T** D

C:

EspA$_{STEC}$	**M D T S T** A T **S V A**	**S A N A S T**
EspA$_{EPEC}$	**M D T S T** T A **S V A**	**S A N A S T**

FIG. 2. Sequence homologies of the secreted proteins of STEC 413/89-1 to known proteins from the Swiss-Prot database. (A) p104; (B) EspB; (C) EspA. Residues which are conserved are shown in bold type. IgA-n[1], IgA protease of *N. gonorrhoeae* (26); IgA-n[2], IgA protease of *H. influenzae* (27).

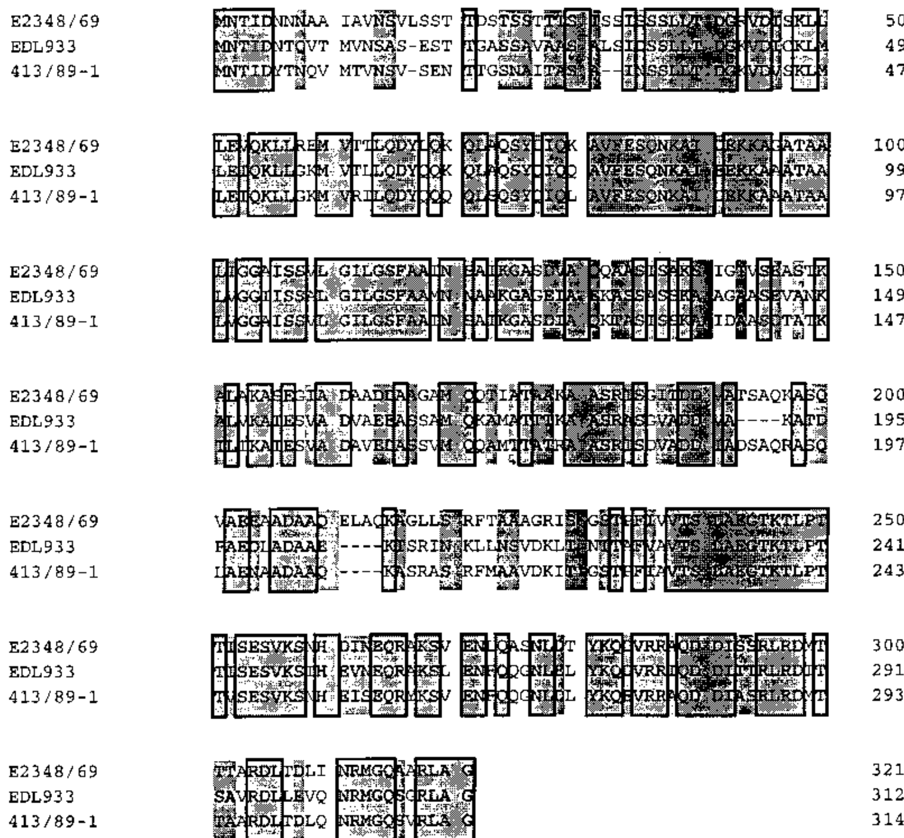

FIG. 3. Comparison of amino acid sequences derived from the sequences of the *espB* genes from EPEC E2348/69 (9), O26:H⁻ STEC 413/89-1, and O157:H7 STEC EDL933. Identical amino acid residues are boxed, similar residues are shaded.

pernatant of EPEC E2348/69 (19, 20). No homologies to other proteins in databases were detected for p25 of EPEC and STEC.

Cloning and sequencing of the *espB* gene from STEC 413/89-1 and EHEC EDL933. To date, no sequence data are available for the *espB* gene of STEC strains. To clone the respective gene, oligonucleotides derived from the N terminus of the EspB polypeptide secreted by strain 413/89-1 and the published sequence 3′ to the *espB* gene from EPEC E2348/69 (9) were used to amplify the corresponding genes from strains 413/89-1 and EDL933. The complete sequence of the *espB* gene of strain EDL933 was also amplified by using a primer derived from the upstream region of the *espB* gene of EPEC E2348/69. The resulting PCR products were cloned in the pCRII vector and subsequently sequenced with vector-derived primers. The amino acid sequences encoded by either genes are presented in Fig. 3.

The two open reading frames predicted polypeptides of 314 amino acids, corresponding to 33.2 kDa for strain 413/89-1, and 312 amino acids, corresponding to 32.6 kDa for strain EDL933. The sequences of the EspB proteins from STEC 413/89-1 and EDL933 and EPEC E2348/69 exhibited overall homologies of between 84 and 76.9%. The unusual stretch of 18 serine or threonine residues at amino acids 18 to 38 of the EspB polypeptide from EPEC E2348/69 (9) was not highly conserved in the EspB polypeptide of either STEC strain. No striking sequence homologies to any other protein in current databases were observed.

Production of a monoclonal antibody specific for the EspB protein. To generate specific reagents for immunodetection, the EspB protein secreted by the STEC strain 413/89-1 was electroeluted and used for immunization of BALB/c mice to generate monoclonal antibodies. The monoclonal antibody produced by hybridoma clone A182/22 reacted specifically with the EspB protein in concentrated supernatants of STEC 413/89-1. Apart from the full-length protein of 37 kDa, a bona fide truncated form of 33 kDa was also detected in small amounts in culture supernatants of this strain (Fig. 4, lane c). The antibody was characterized as a IgG1 subclass molecule and enabled us to further investigate the expression of the EspB polypeptide by immunoblot analysis.

The expression of EspB is thermoregulated. Since the expression of many bacterial virulence factors is thermoregulated, we analyzed the expression of EspB during growth at 20 and 37°C in either LB broth or MEM. The pattern of supernatant proteins and the corresponding EspB-specific immunoblot are shown in Fig. 5. In the supernatant of bacteria grown in LB broth, many minor protein bands were detectable after Coomassie staining, indicating that even during exponential growth a certain percentage of bacteria were lysed. Proteins with molecular masses of 104, 55, 54, and 37 kDa were present in higher amounts in the culture fluid, suggesting an efficient secretion mechanism (Fig. 5A, lanes A and B), and their production was clearly temperature dependent. Proteins with molecular masses of 55 and 54 kDa were expressed at much lower levels at 37°C than at 20°C, whereas the expression of proteins p104, p37, and p25 was significantly increased when bacteria were grown at 37°C. When analysis of the secreted proteins was performed after growth in MEM, no background due to cell lysis was detectable. At a growth temperature of 20°C,

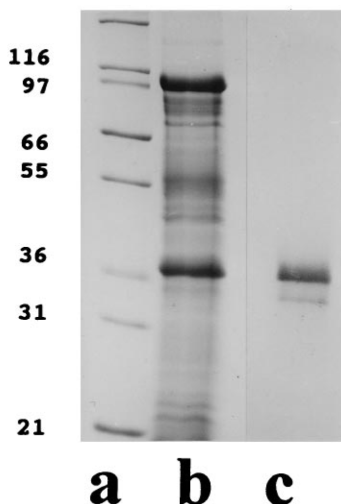

FIG. 4. Detection of the EspB polypeptide in supernatant proteins from STEC 413/89-1 grown in LB broth. Proteins were separated onto an SDS–10% PAGE gel and either stained with Coomassie brilliant blue (lane b) or reacted with monoclonal EspB-specific antibody A182/22 on a corresponding immunoblot (lane c). Molecular mass markers are shown (lane a; top to bottom, 200, 116, 97, 66, 55, 36, 31, and 21 kDa).

several minor protein species were observed (Fig. 5A, lane C), while at 37°C, production of p104, p37, p25, and p22 was induced (Fig. 5A, lane D).

The analysis of the EspB expression was performed in more detail with immunoblots with the monoclonal antibody A182/22 (Fig. 5B). In LB broth and MEM, expression of EspB was low (LB broth) or not detectable (MEM) at 20°C (Fig. 5B, lanes A and C) but was dramatically increased when bacteria were grown at 37°C (Fig. 5B, lanes B and D). A similar mode of regulation of the EspB expression was also observed in total extracts of bacterial cells. No difference in the apparent molecular weights of the cytoplasmic and secreted forms of EspB was obvious, suggesting that it is not proteolytically processed during transport (data not shown).

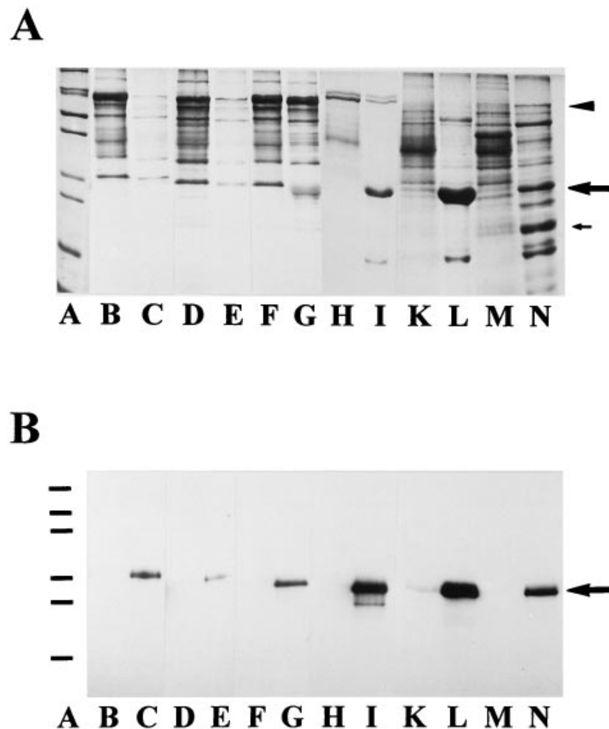

FIG. 6. Detection of EspB in the supernatant of different STEC and EPEC strains. Five STEC strains (lanes B to I), one bovine A/E-positive isolate (lanes K and L), and one EPEC strain (lanes M and N) were grown either in LB broth (lanes B, D, F, H, K, and M) or MEM tissue culture medium (lanes C, E, G, I, L, and N). Supernatant proteins were separated on an SDS–10% PAGE gel and subsequently stained with Coomassie brilliant blue (panel A), and a corresponding immunoblot was reacted with the EspB-specific monoclonal antibody A182/22 (panel B). The following strains were used: STEC EDL933 (lanes B and C), STEC HUS-2 (lanes D and E), STEC H-19 (lanes F and G), STEC S102-9 (lanes H and I), the bovine A/E-positive strain CB207 (lanes K and L), and EPEC E2348/69 (lanes M and N). For serotypes, see Table 1. Molecular mass markers are indicated in panel B (top to bottom, 97, 66, 55, 36, 31, and 21 kDa). The EspB polypeptide is indicated by a large arrow in both panels, and the corresponding degradation product in the supernatant of EPEC strain E2348/69 is indicated by a small arrow in panel A. The E2348/69 polypeptide whose N-terminal sequence is identical to that of the p110 protein described by Kenny and Finlay (19) is indicated by an arrowhead.

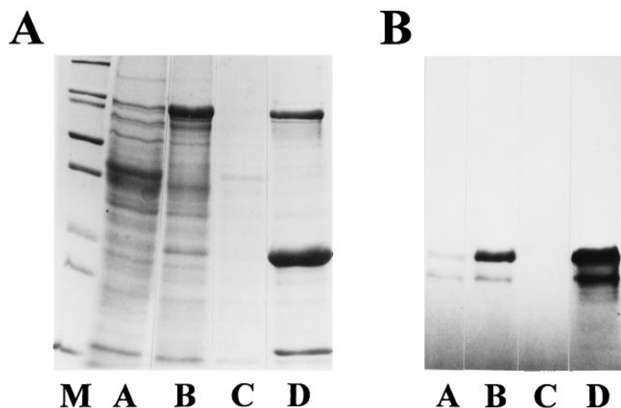

FIG. 5. The expression of EspB is thermoregulated and strongly enhanced by growth in tissue culture medium. STEC 413/89-1 was grown either in LB broth (lanes A and B) or MEM tissue culture medium (lanes C and D) at either 20°C (lanes A and C) or 37°C (lanes B and D). Supernatant proteins were stained with Coomassie brilliant blue (panel A), and a corresponding immunoblot was reacted with the EspB-specific monoclonal antibody A182/22 (panel B). Molecular mass markers are indicated (top to bottom, 200, 116, 97, 66, 55, 36, 31, and 21 kDa).

Detection of EspB secreted by different pathogenic *E. coli* strains. The difference in the expression of the EspB polypeptide in STEC 413/89-1 grown either in LB broth or in MEM prompted us to analyze other pathogenic *E. coli* strains for medium-dependent production. Six strains with differing serotypes, all of which induced A/E lesions, were investigated. One isolate (EDL933) represented the classical EHEC serotype, O157:H7, three strains represented other STEC serotypes (O26, O111, O5), the O55 strain is an A/E-positive bovine isolate, and EPEC was represented by strain E2348/69 (Table 1). Production of EspB was investigated by immunoblotting with concentrated supernatant proteins derived from equal numbers of bacteria. Expression of EspB was dramatically increased in all strains through growth in MEM, in comparison with growth in LB broth, where no or very low amounts of EspB were detectable in the supernatants (Fig. 5B, lanes B, D, F, H, K, and M). The EspB polypeptide was already detectable by Coomassie staining in three of the six strains grown in MEM (Fig. 6A, lanes I, L, and N, large arrow). However, immunoblot analysis with monoclonal antibody A182/22 enabled us to detect the EspB polypeptide in the supernatants of all six strains grown in MEM (Fig. 6, lanes C, E, G, I, L, and N). Thus, the

monoclonal antibody A182/22 recognizes an epitope that is conserved in the STEC and EPEC strains examined. Sequencing of the N-terminal 15 amino acids of the 28-kDa protein additionally present in the supernatant of EPEC E2348/69 grown in MEM (Fig. 6A, lane N, small arrow, and 6B, lane N, arrow) revealed that it was identical to the N-terminal sequence of EspB (data not shown). This bona fide degradation product of EspB was not recognized by the antibody A182/22, indicating that the epitope of A182/22 is located within the C-terminal 10 kDa of the EspB polypeptide.

In all of the STEC strains examined in this study, a protein band in the range of 104 kDa was observed. In most strains, these polypeptides were expressed at significantly higher levels during growth in LB broth than in MEM (Fig. 6, lanes B to I). This regulation and their apparently similar molecular weights suggested that these polypeptides may be related to the p104 protein of strain 413/89-1. In supernatants of EPEC strains, only one polypeptide was detectable in this molecular weight range (Fig. 6A, lane N, arrowhead). However, the N-terminal amino acid sequence of this polypeptide was different from the one obtained from p104 but identical to the sequence of a 110-kDa protein recently described by Kenny and Finlay (19).

Polypeptides with molecular masses of 25 kDa were only detectable in the MEM supernatants of strains S102-9, CB207, and E2348/69 (Fig. 6A, lanes I, L, and N). Since the same three strains also express high levels of the EspB protein, expression of these two proteins may be coregulated in these strains.

DISCUSSION

STEC and EPEC are able to bind and interact with eukaryotic enterocytes in a complex way, resulting in the appearance of a specific kind of histopathological phenotype, called A/E lesion. In this study, we have examined in detail the proteins secreted by a Shiga toxin-producing strain under different growth conditions. When grown in LB broth, these bacteria produce several dominant polypeptides. The expression of the p37 polypeptide was clearly enhanced when bacteria were grown in tissue culture medium (MEM), while the amount of one polypeptide (p104) was reduced in MEM. A protein doublet of about 55 kDa was obtained only after growth in LB broth, while two polypeptides (p25 and p22) were detectable only in MEM-derived supernatants. Expression of all four polypeptides in MEM was temperature dependent and detected only in supernatants of bacteria grown at 37°C. N-terminal amino acid sequencing led to the identification of five different polypeptides. The 37-kDa polypeptide was identified as the EspB homolog of the STEC strain. The amino acid sequences obtained from two STEC strains revealed that they had an overall homology of 68.1% to the sequence of the EPEC EspB protein. A monoclonal antibody produced against EspB of STEC 413/89-1 allowed us to show that expression of the EspB protein in EPEC and STEC strains was variable and medium dependent.

Recently, Kenny and Finlay (19) have reported that EPEC E2348/69 secretes five polypeptides with molecular masses of 25, 37, 39, 40, and 110 kDa to the supernatant when grown in tissue culture medium (DMEM), while no secretion of proteins was observed after growth in LB broth. Comparison of the N-terminal sequencing data obtained by Kenny and Finlay (19) with the data presented here revealed similarities but also significant differences between the patterns of secreted proteins in STEC and EPEC. Hence, while the p25 and p37 proteins of STEC and EPEC were highly homologous in their N termini, proteins p39, p40, and p110 from EPEC were not detectable in the supernatant of STEC; instead, a protein with a molecular mass of 104 kDa and two novel polypeptides of about 55 kDa were also observed.

The motif DFAXNKGXFXXG located between amino acids 15 and 26 of the N terminus of p104 is homologous to the N-terminal sequences of p110 of EPEC E2348/69 and the secreted proteins of the IgA protease family. These include the Hap protein and the IgA protease of *H. influenzae* (12, 27), the IgA protease of *N. gonorrhoeae* (26), the SepA protein of *S. flexneri* (5), and the Tsh protein of the avian pathogenic *E. coli* strain χ7122 (28). The most interesting feature of these proteins is their self-directed transport through the outer membrane (21). These proteins are synthesized in the cytoplasm as precursors with molecular masses of 130 to 170 kDa, which contain N-terminal signal peptides necessary for transport across the cytoplasmic membrane, by use of the Sec-dependent machinery (29). The remaining polypeptide is subsequently transported over the outer membrane by a unique mechanism that is not dependent on any other accessory proteins, resulting in the cleavage of the protein, insertion of a C-terminal fragment in the outer membrane, and the secretion of mature polypeptides that range between 100 and 110 kDa into the supernatant. Homologies were found between the N termini of these mature, secreted polypeptides and the N termini of p104 and p110, revealing the conserved motif DFAXNKGXFXXG (Fig. 2). It has been recently shown that secretion of p110 in EPEC strains is not dependent on the *sep* transport genes located in the LEE locus (16, 19), a finding that supports the notion that the molecules are self-secreted. Since several proteins of this family such as Tsh and Hap have been implicated in adhesive processes, it is tempting to speculate that the p104 and p110 proteins may facilitate the mediation of adhesion of these bacteria to eukaryotic cells. Current studies aim at cloning and characterizing the gene encoding p104 as a prelude to understanding its function in STEC pathogenesis.

A 55-kDa double band was detected in some but not all supernatants of strain 413/89-1 grown in LB broth at either 20 or 37°C. N-terminal sequences were obtained for both polypeptides by automated Edman degradation. The identity of both sequences indicated that the double band resulted from a limited proteolytic digestion of the polypeptide present in the upper band. No obvious homologies to sequences present in current databases were detectable. Immunoblots performed with a specific monoclonal antibody raised against p55 demonstrated that this protein is present only in the supernatant and is absent from the cellular fraction, indicating that p55 is not released by partial lysis of bacteria.

The p37 protein secreted by strain 413/89-1 was identified as the product of the hitherto-unidentified *espB* gene of STEC. Identity of 54.5% and similarity of 68.1% were obtained for the N-terminal 22 amino acids of EspB from strains 413/89-1 and E2348/69, with a cluster of five identical amino acids at the extreme N terminus (8) (Fig. 2). Molecular cloning of the *espB* genes from STEC 413/89-1 and EDL933 revealed that these proteins showed a homology of approximately 80% to the known sequence of the EspB protein from EPEC. The unusual enrichment of serine and threonine residues in an N-terminal 20-amino-acid stretch, which was reminiscent of that of internalin A of *Listeria monocytogenes* (9), was not strongly conserved in the STEC *espB* genes nor were obvious motifs detected with the PROSITE algorithm (3). The pairwise comparison of the different EspB sequences revealed that the homology between the two STEC-derived sequences (84.1% similarity) was not significantly higher than their respective homologies to the EPEC EspB sequence (82.2 and 76.9% similarities). This observation is reminiscent of the greater variability in sequence homologies found within the C-terminal

280 amino acids of the EaeA polypeptide in EPEC and STEC strains (4, 17, 36), a region thought to be responsible for specific and intimate attachment of this protein with the eukaryotic membrane (11). These results could be interpreted to reflect unique interactions employed by these molecules in individual strains.

Since EspB seems to be a crucial factor involved in signal transduction events during the infection process of EPEC (10, 30), we have analyzed this protein in more detail. To investigate the expression of EspB under different growth conditions with more precision, we raised a monoclonal antibody against electrophoretically pure EspB protein from strain 413/89-1 grown in MEM. N-terminal sequence analysis of secreted proteins of EPEC E2348/69 revealed the presence of a 28-kDa degradation product of EspB apart from the full-length 37-kDa polypeptide. This C-terminal degraded form was not recognized by antibody A182/22, indicating that the epitope of this monoclonal antibody is located within the C-terminal 10 kDa of EspB.

A comparison of the amounts of secreted EspB for strain 413/89-1 when grown in MEM or LB broth showed that its expression is drastically increased in the tissue culture medium. Furthermore, expression of EspB is clearly thermoregulated like many other virulence factors of pathogenic bacteria (23). A dramatic increase in the expression of EspB in strain 413/89-1 was observed when the growth temperature was shifted from 20 to 37°C. Temperature-regulated expression has also been previously reported for several proteins secreted by EPEC E2348/69 (19). Immunoblot analysis of cytoplasmic extracts from bacteria grown in either LB broth or MEM at either 20 or 37°C revealed the existence of an intracellular pool of EspB that was detectable in cytoplasmic extracts mainly after growth at 37°C, indicating that the temperature dependence of secretion is regulated at the level of transcription or translation and is not due to a release of preformed cytoplasmic polypeptides through a temperature-dependent activation of the secretion system. The apparent molecular weights of the EspB polypeptide in the bacterial cytoplasm and in the supernatant appeared to be identical, indicating that EspB in STEC is exported by a signal sequence-independent process. The *espA* and *espB* genes from EPEC, which have been sequenced by Donnenberg et al. (9) and Kenny et al. (20), also contain no signal peptides, and it has been shown recently that they are part of the locus for enterocyte effacement (LEE) gene cluster. Interestingly, the same cluster codes for a set of proteins showing strong homology to type III transport systems of other pathogenic bacteria (16, 22) that are involved in the secretion of the EspA and EspB polypeptides (16, 19, 20).

The overexpression of the EspB polypeptide in the STEC strain 413/89-1 was not restricted to growth in MEM but was also observed after growth in a different kind of tissue culture medium. Careful studies must be performed to analyze the basis of this regulation and to verify whether the composition of tissue culture medium is an inductive stimulus. The observed overexpression was a general phenomenon since strains of six different serotypes from both EPEC and STEC produced higher levels of EspB in MEM than in LB broth. However, the dramatically increased amounts of EspB that were secreted by strain 413/89-1 were not observed for all strains investigated except the EPEC strain E2348/69, the bovine A/E strain CB207, and the serotype O5 STEC strain S102-9.

The polypeptide p25 was homologous in its N terminus to the recently described EspA protein secreted by EPEC E2348/69 (19, 20) (Fig. 2). This protein was demonstrated to be essential for the induction of eukaryotic signal transduction cascades, and the corresponding gene was recently sequenced and localized within the LEE locus between the *eaeA* and *espB* genes (20). In our study, the secreted proteins EspA (p25) and EspB (p37) from several different strains showed similar expression patterns as revealed by Coomassie staining, and production of both proteins was especially enhanced during growth in MEM at 37°C. In several other strains, neither EspA nor EspB was visible by Coomassie staining. Nevertheless, the production of EspB in these strains was demonstrated by using a EspB-specific monoclonal antibody. Since EspA is also required for induction of signal transduction pathways required to generate the A/E lesion, demonstration of EspA production in these strains would be of great importance. Since the genes coding for both proteins are located in the LEE locus, coordinate expression of these bona fide virulence factors seems to be reasonable. The cloning and identification of the *espA* gene from STEC will permit its detailed examination.

In conclusion, our study demonstrates that there are strong similarities in proteins secreted by EPEC and STEC strains. Additionally, production of these proteins in both types of strains is medium and temperature dependent and the proteins appear to be secreted by at least two independent transport mechanisms. Evidence that EspA and EspB are secreted by a type III secretion pathway whose components are encoded by the family of *sep* genes present in the LEE locus has already been presented (16). In contrast, the novel p104 protein of STEC and the p110 protein of EPEC may be secreted by an autocatalytic transport mechanism across the outer membrane. Further studies are aimed at genetically identifying the proteins described in this study and biochemically characterizing their gene products. In addition, the mechanisms leading to medium- and temperature-dependent expression of these proteins promise to be a fascinating area of study and may provide important clues as to how virulence expression in these bacteria is controlled during the infection process.

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