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Expression of Attaching/Effacing Activity by Enteropathogenic *Escherichia coli* Depends on Growth Phase, Temperature, and Protein Synthesis upon Contact with Epithelial Cells

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Enteropathogenic *Escherichia coli* (EPEC) induces tyrosine phosphorylation of a 90-kDa protein (Hp90) in infected epithelial cells. This in turn facilitates intimate binding of EPEC via the outer membrane protein intimin, effacement of host cell microvilli, cytoskeletal rearrangement, and bacterial uptake. This phenotype has been commonly referred to as attaching/effacing (A/E). The ability of EPEC to induce A/E lesions was dependent on bacterial growth phase and temperature. Early-logarithmic-phase EPEC grown at 37°C elicits strong A/E activity within minutes after infection of HeLa epithelial cells. EPEC de novo protein synthesis during the first minutes of interaction with the host cell was required to elicit A/E lesions. However, once formed, bacterial viability was not needed to maintain A/E lesions. The type of growth media and partial O_2 pressure level do not seem to affect the ability of EPEC to cause A/E lesions. These results indicates that the A/E activity of EPEC is tightly regulated by environmental and host factors.

Enteropathogenic *Escherichia coli* (EPEC) is a human-specific pathogen that causes severe infantile diarrhea. EPEC forms small colonies on the surface of infected epithelial cells by a process termed localized adherence (28). This self aggregation and initial adherence is mediated by the bundle-forming pilus and possibly additional factors (4). Initial adherence is followed by intimate attachment of bacteria to host cells through the outer membrane protein intimin, the product of the *eaeA* gene (16). Upon adherence to the epithelial cell surface, EPEC elicits attaching/effacing (A/E) lesions (24). A/E activity involves degeneration of brush border microvilli and assembly of highly organized cytoskeletal structures in the epithelial cells beneath intimately attached bacteria (18). This structure is composed of cytoskeletal elements such as actin filaments, α-actinin, ezrin, and talin (9, 18). This cuplike cytoskeletal structure frequently evolves into elongated actin tails that mediate extended pseudopod projections (26). Actin rearrangement is also involved in internalization of a subpopulation of EPEC by epithelial cells (1, 6).

Signal transduction, including induction of tyrosine phosphorylation of a 90-kDa host protein (Hp90) by the attached EPEC, is the initial event that leads to formation of A/E lesions (25). Several EPEC genes, including *sepA*, *sepB*, and the *eaeB*, are involved in signal transduction (10, 24a, 25). The EaeB protein is secreted (14, 17), and the *sepA* and *sepB* genes encode subunits of the type III secretion system which mediates EaeB secretion (17, 22). Phosphorylated Hp90 is a membrane protein and is involved in nucleating the assembly of the underlying cytoskeletal structure (25, 26). Phosphorylation of Hp90 also creates a binding site on the cell surface for intimin (26). Hp90-intimin association results in bacterial intimate attachment and focusing of cytoskeletal elements along the EPEC-HeLa cell contact area (26). Tyrosine phosphorylation of Hp90 is also involved in activation of host phospholipase C (11), which may lead to an elevation of intracellular free calcium levels (3) and in turn to activation of calcium-dependent serine or threonine protein kinase(s) (2, 21). Tyrosine phosphorylation of Hp90 is necessary for EPEC invasion (25).

Several others pathogens, including enterohemorrhagic *E. coli*, RDEC-1 (a rabbit-specific EPEC), *Hafnia alvei*, and *Citrobacter freundii* biotype 4280, cause A/E lesions morphologically similar to those caused by EPEC (7). All of these pathogens possess a 35-kb chromosomal fragment known as the LEE element (22). This chromosomal region is unique to A/E-causing pathogens, and it contains all of the genes known to be involved in eliciting A/E lesions, including *eaeA*, *eaeB*, *sepA*, *sepB*, and others (22).

Expression of virulence factors in pathogenic bacteria is usually tightly and coordinately regulated. Growth phase and environmental conditions characteristic of the host, including temperature and partial O_2 pressure, are the stimulus for virulence factor expression in various gram-negative pathogens (8, 20). In EPEC, the *eaeA* and *eaeB* genes are positively regulated by the plasmid-encoded *per* loci (13). In this study, we characterized the environmental conditions that regulate the A/E activity of EPEC.

MATERIALS AND METHODS

Bacterial strains and tissue culture cells. EPEC E2348/69 (5) bacteria were grown in LB agar or LB broth at 37°C without shaking. HeLa cells (ATCC CCL2) were grown and assayed at 37°C, under 5% CO_2, in minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum.

Determination of protein concentration. Protein concentration was determined by the bicinchoninic acid protein assay (Sigma) as described in the manufacturer instructions.

Invasion assay. Bacteria were added to HeLa cells that were seeded the previous day in a 24-well plate at a density of 10^5 cells per well, and the cells were then incubated (37°C, 5% CO_2) for the indicated period of time. The cells were then washed twice and incubated for an additional 90 min in medium containing 100 μg of gentamicin per ml. Monolayers were washed, lysed with 1% Triton X-100, and plated to determined the number of viable intracellular bacteria. Assays were carried out in duplicate or triplicate.

Sensitivity to chloramphenicol and gentamicin. Chloramphenicol or gentamicin was added to activated EPEC in MEM to a final concentration of 100 μg/ml.

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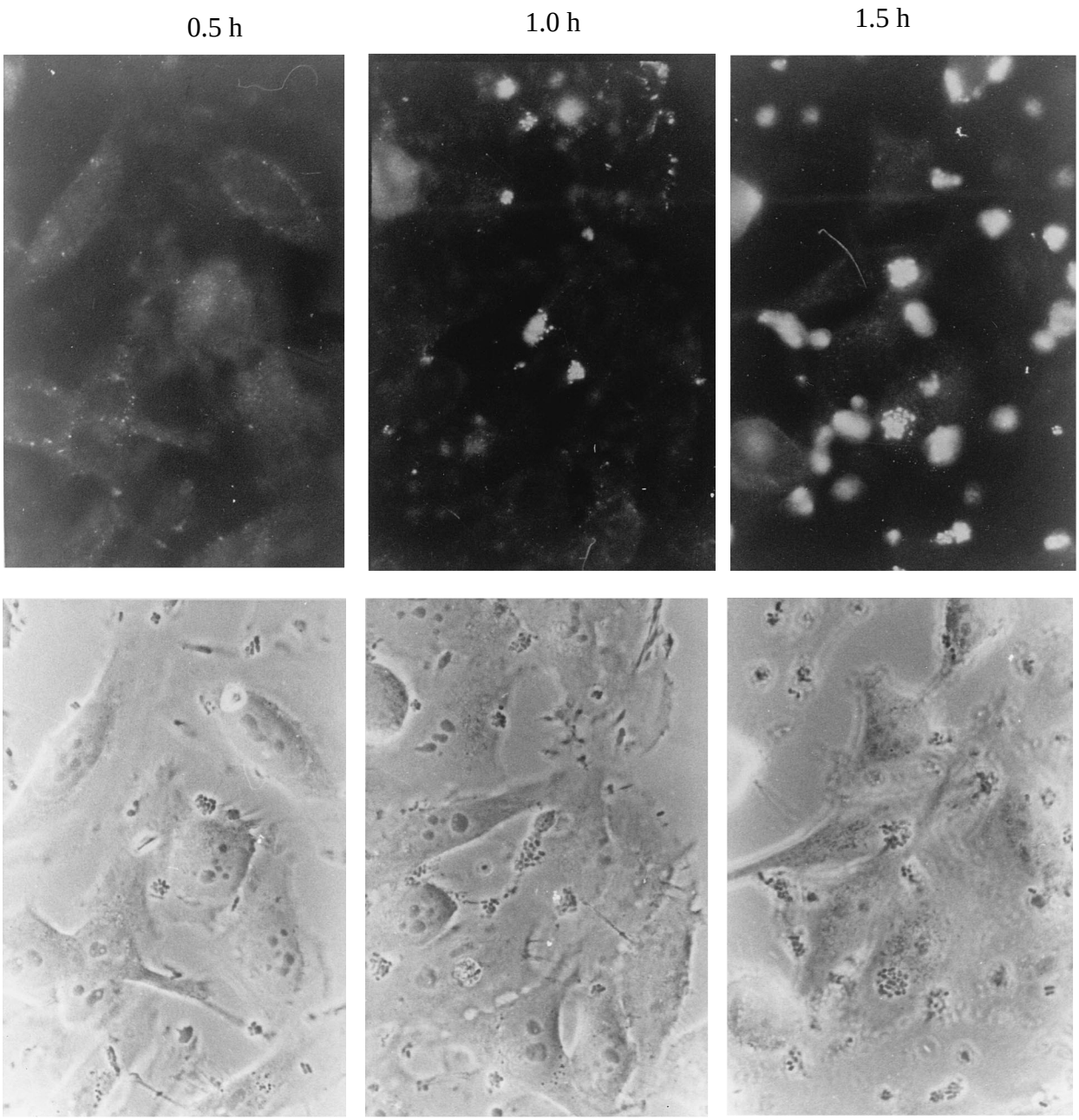

FIG. 1. Activation of EPEC in MEM. An early-stationary-phase EPEC culture in LB broth was diluted 1:50 in MEM and grown at 37°C, under 5% CO_2, without agitating. After 0.5, 1, and 1.5 h, 1 ml of culture was removed and centrifuged for 4 min at 1,000 × *g* onto HeLa cells, and the infected cells were incubated for 30 min at 37°C, under 5% CO_2. Infected cells were fixed and labeled with antiphosphotyrosine antibody (4G10). The immunofluorescent (top) and the corresponding phase-contrast (bottom) images are shown.

The treated culture was incubated at 37°C, and at various times, 1-ml aliquots were removed and immediately washed twice with phosphate-buffered saline (PBS). The washed bacteria were diluted and plated to determine viability.

Immunofluorescent microscopy. Cells were seeded and grown overnight on glass coverslips. After infection and/or treatment, the cells were washed with PBS and fixed for 30 min with 2% paraformaldehyde in PBS. The cells were washed three times with PBS, permeabilized for 5 min with 0.1% Triton X-100 in PBS, and washed as before. To stain tyrosine-phosphorylated proteins, the cells were incubated with 20 μl of monoclonal antiphosphotyrosine antibody (4G10 from UBI Inc. or PT66 from Sigma) for 60 min, washed, and incubated with 20 μl of secondary antibody anti-mouse immunoglobulin G, fluorescein isothiocyanate or Texas red conjugated (both from ICN Inc.). Actin filaments were stained with phalloidin rhodamine (Molecular Probes Inc.) as described by the manufacturer.

Protein extraction and fractionation. HeLa cells were seeded at a density of 2×10^6 cells per 100-mm-diameter tissue culture petri dish. The next day, the cells were infected and incubated for the indicated time at 37°C under 5% CO_2. The infected cells were washed with 10 ml of cold PBS and then scraped into 1.5 ml of PBS, centrifuged (2 min at 3,000 × *g*), and lysed in 0.2 ml of lysis solution (1% Triton X-100, 50 mM Tris-HCl [pH 7.6], 0.4 mM $NaVO_4$, 0.1 mg of phenylmethylsulfonyl fluoride per ml, 10 μg of leupeptin per ml). The lysate was spun (2 min, 23,000 × *g*), and the supernatant (1% Triton X-100-soluble fraction) was mixed with 50 μl of 5× loading buffer, boiled for 7 min, and cleared (5 min, 23,000 × *g*) prior to resolution by gel electrophoresis.

Western blotting (immunoblotting). Extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (AB-S 83; Schleicher & Schuell, Inc.) with a NovaBlot electrophoretic transfer unit (LKB) as described in the manufacturer's recommendations. Monoclonal antiphosphotyrosine (PT66 from Sigma or 4G10 from

TABLE 1. Kinetics of EPEC activation and formation of A/E lesions[a]

Time postinfection (min)	A/E lesion formation at[b]:						
	0 h and 0.045[c]	0.5 h and 0.046	1.0 h and 0.044	1.5 h and 0.079	2.0 h and 0.116	2.5 h and 0.178	3 h and 0.269
5	−	−	−	+	+	+	+
10	−	−	−	++	++	++	++
20	−	−	±	+++	++++	++++	++++
30	−	−	+	++++	++++	++++	++++

[a] An early-stationary-phase EPEC culture was diluted 1:50 in MEM and grown at 37°C, under 5% CO_2. The ability of EPEC, in different growth stages, to elicit A/E lesions was examined. Formation of A/E lesions was estimated by immunofluorescence microscopy after staining with antiphosphotyrosine antibody (4G10). For EPEC, the detection of tyrosine-phosphorylated proteins correlates spatially with the formation of A/E lesions.

[b] Formation of A/E lesions was scored from − to ++++, with − being no A/E at all and ++++ being intense formation of A/E lesions.

[c] Growth time and optical density at 562 nm (OD_{562}) are given. Bacterial growth was determined by protein extraction of 1 ml of culture and determination of protein concentration (OD_{562} by the bicinchoninic acid method). An OD of 0.26 is equivalent to a protein concentration of 0.45 mg/ml or about 10^8 CFU/ml.

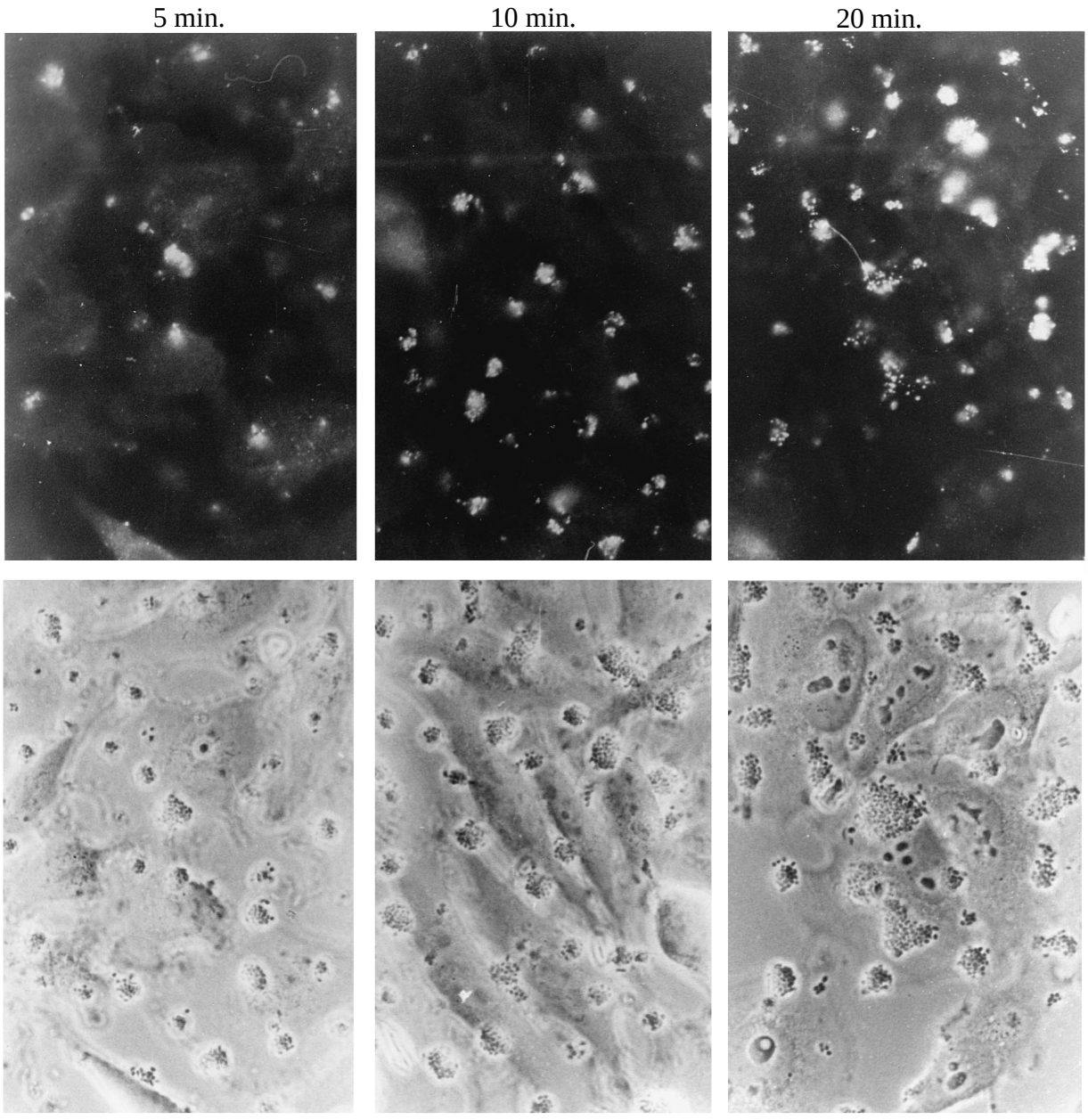

FIG. 2. Rapid induction of A/E lesions by activated EPEC. An early-stationary-phase EPEC culture in LB broth was diluted 1:50 in MEM and grown at 37°C, under 5% CO_2, without agitating for 2.5 h. Activated EPEC culture (1 ml) was spun for 4 min, at 1,000 × *g* on top of HeLa cells and incubated at 37°C under 5% CO_2. After 5, 10, and 20 min of incubation, the infected cells were fixed and labeled with antiphosphotyrosine antibody (4G10). The immunofluorescent (top) and corresponding phase-contrast (bottom) images are shown.

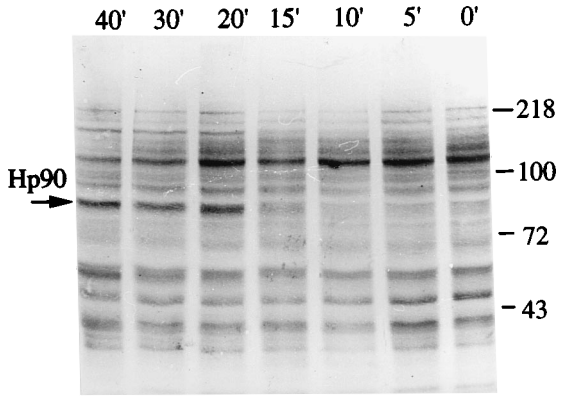

FIG. 3. Kinetics of induction of tyrosine phosphorylation of Hp90 by activated EPEC. EPEC was diluted 1:50 and grown in MEM at 37°C for 3.5 h, and 15 ml of activated EPEC was added to 3×10^6 HeLa cells in a 10-cm-diameter petri dish. Protein from infected cells was extracted at 0, 5, 10, 15, 20, 30, and 40 min postinfection and used for immunoblot analysis with antiphosphotyrosine antibody (PT66). Molecular weight markers are indicated in kilodaltons.

UBI Inc.) antibodies were diluted in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl [pH 7.5]) containing 1% bovine serum albumin (Sigma). Binding of secondary anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G (Amersham)-horseradish peroxidase conjugate antibody was detected with the enhanced chemiluminescence system (Amersham).

RESULTS

Expression of A/E activity is growth phase dependent. EPEC was grown for about 16 h without shaking in LB medium, at 37°C, to reach the early stationary phase (density of about 2×10^8 CFU/ml). This culture was diluted 1:50 in tissue culture MEM and grown at 37°C and under 5% CO_2. At various times, the ability of the growing culture to induce tyrosine phosphorylation of Hp90 in infected HeLa cells was examined by immunofluorescence microscopy with antiphosphotyrosine antibody. To synchronize the infection, EPEC bacteria were centrifuged onto the HeLa cells. EPEC cultures in the logarithmic growth phase were much more active than cultures in other phases of growth (Fig. 1 and Table 1). In the early to mid-logarithmic phase, induction of tyrosine phosphorylation and accumulation of tyrosine-phosphorylated proteins beneath the attached bacteria could be detected as early as 5 min after infection (Fig. 2 and Table 1). With short infection periods (less than 10 min), identification of A/E lesions with antiphosphotyrosine antibody was easier than it was with rhodamine-phalloidin staining. This is due to the very low background of tyrosine-phosphorylated proteins and the higher background of actin filaments and stress fibers seen with rhodamine-phalloidin staining.

In cells infected with activated (early- to mid-logarithmic-phase) EPEC, tyrosine phosphorylation of Hp90 could be detected by immunoblotting 20 min postinfection (Fig. 3). In contrast, with EPEC in the early stationary phase, tyrosine phosphorylation of Hp90 could be detected only 1.5 to 2 h postinfection (reference 25 and data not shown). Hp90 could not be extracted from activated EPEC with 1% Triton X-100 (data not shown). This together with other evidence (25) indicates that Hp90 is of HeLa origin.

Activated EPEC started to invade HeLa cells immediately following bacterial addition. Invasion was rapid in the first 20 min, reaching saturation about 30 min postinfection (Fig. 4).

EPEC activation appears equally potent either in MEM and under 5% CO_2 or in LB medium agitated or not agitated under normal atmospheric conditions (data not shown). Moreover, washing the activated culture, before infection, with fresh MEM did not reduce the activation. This indicates that the activation is intrinsic to the bacteria and not due to interaction of an EPEC-secreted product with the host cells. Agitating the EPEC culture did not inhibit activation or change the activation pattern with respect to the growth phase. Shaking the culture, however, enhanced the deactivation of EPEC at the late logarithmic growth phase. This is probably due to the higher growth rate and higher cell density in the shaken culture.

EPEC, activated in MEM, bound to the host cells in a localized adherence manner 5 min after infection (Fig. 2 and data not shown). EPEC grown in LB medium, agitated or not agitated, also bound to the host cell in a localized adherence manner (data not shown). Vuopio-Varkila and Schoolnik reported that EPEC spontaneously aggregates in MEM (30). This together with the pattern of EPEC binding to the HeLa cells indicates that EPEC may aggregate and then disaggregate during growth in LB medium or MEM. This growth pattern might interfere with determination of culture density by affecting culture turbidity or CFU. We therefore used the increase in protein concentration to measure growth.

Expression of A/E activity is temperature regulated. In contrast to growth at 37°C, EPEC activation could not be detected at 28°C at any of the growth phases. The ability of EPEC to aggregate and bind to the host cells was also strongly attenuated. Induction of tyrosine phosphorylation of Hp90 by EPEC that was activated at 28°C could not be detected by immunofluorescence microscopy or immunoblotting analysis with antiphosphotyrosine antibody (data not shown). To examine whether the A/E process can occur at 28°C, we activated EPEC in MEM at either 28 or 37°C and then infected at either 28 or 37°C. The EPEC cultures that were grown at 28°C adhered to the HeLa cells at a very low rate, and the attached bacteria were not capable of inducing tyrosine phosphorylation of Hp90 and recruiting actin filaments (Table 2). The EPEC cultures that were grown at 37°C induced tyrosine phosphorylation of Hp90 and A/E lesions, whether they were used for infection at 37 or 28°C (Table 2). This indicates that the expression of some

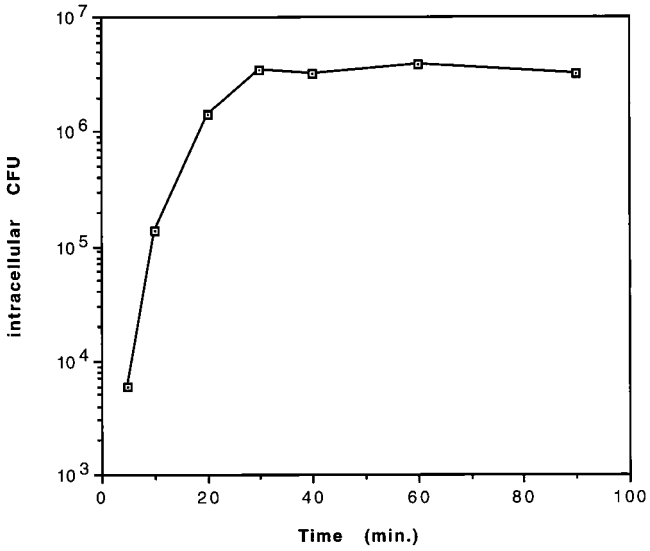

FIG. 4. Kinetics of invasion by activated EPEC. EPEC was activated in MEM for 3 h and used to infect HeLa cells. Invasion was stopped at various time points by washing away free bacteria and adding gentamicin (100 μg/ml). At this concentration, gentamicin kills rapidly exposed EPEC.

TABLE 2. Induction of A/E lesion formation by activated EPEC[a]

Activation temp (°C)	Infection temp (°C)	A/E lesion formation[b]
37	37	+++
37	28	++
28	37	−
28	28	−

[a] EPEC was activated in MEM at either 37 or 28°C. EPEC bacteria grow slower at 28°C than at 37°C and therefore were grown for 4.5 h to reach the same density as that of activated EPEC bacteria that were grown at 37°C. The activated EPEC bacteria were used to infect HeLa cells for 30 min at either 37 or 28°C. The infected cells were fixed and stained with phalloidin or antiphosphotyrosine antibody.

[b] Formation of A/E lesions was estimated by immunofluorescence microscopy and scored between − and +++, with − being no A/E at all and +++ being intense formation of A/E lesions.

A/E genes in EPEC is temperature regulated, but activated EPEC can induce A/E lesions at both temperatures. EPEC grown at 28°C also did not invade HeLa cells (data not shown).

EPEC de novo protein synthesis following contact with the host cell is required for induction of A/E lesions but not for maintenance and maturation of these lesions. We have determined that under the conditions used in the experiments described above, gentamicin reduces the viability of exposed activated EPEC by 10^3 within 5 min and by more than 10^4 with 10 min of exposure. In contrast, chloramphenicol did not reduce the viability of the activated EPEC over a 1-h exposure (data not shown).

Gentamicin added 5 min after infection of HeLa cells with activated EPEC completely inhibited invasion (Fig. 5). This indicates that at 5 min postinfection, most bacteria were still extracellular. Chloramphenicol was added either immediately after infection of HeLa cells with activated EPEC or 5 min postinfection and incubated with chloramphenicol in the medium for an additional 60 min. Invasion was stopped by replacing the medium with MEM containing 100 μg of gentamicin per ml to kill extracellular bacteria, and the number of intracellular bacteria was determined. Chloramphenicol completely inhibited invasion when added immediately after infection. However, if chloramphenicol was added at 5 min postinfection, a clear increase in protection against gentamicin compared with that of the two other cultures was evident (Fig. 5). Presumably, the 5-min incubation without chloramphenicol was sufficient for EPEC to trigger the uptake process which continued during the incubation with chloramphenicol. Thus, it appears that de novo protein synthesis in EPEC during the first minutes of host-bacterium interaction is required to trigger the uptake process, but once the process is triggered, it is no longer dependent on EPEC protein synthesis.

Gentamicin added immediately after infection with activated EPEC inhibited tyrosine phosphorylation of Hp90 and actin rearrangement. This was detected by immunofluorescence microscopy with antiphosphotyrosine antibody and phalloidin (Fig. 6 and data not shown). However, if gentamicin was added 5 to 10 min postinfection and incubation was continued for an additional 5 h, the A/E process (manifested by extensive bacterial binding, accumulation of tyrosine-phosphorylated proteins beneath the attached EPEC, pseudopod formation, and rounding up of the host cells) was nearly normal (Fig. 6 and data not shown). In comparison, at the time when bacterial protein synthesis ceased upon gentamicin addition (i.e., 5 to 10 min postinfection), A/E lesions were present only in very initial stages of formation (Fig. 6). Similar results were obtained when chloramphenicol instead of gentamicin was used.

Inhibition of tyrosine phosphorylation of Hp90 by chloramphenicol was also documented by immunoblotting (Fig. 7). If chloramphenicol was added at 15 to 20 min postinfection and incubation was continued for an additional 60 min with chloramphenicol in the medium, tyrosine phosphorylation of Hp90 occurred after the drug was added (Fig. 7). This indicates that induction of A/E lesions and tyrosine phosphorylation of Hp90 by activated EPEC is dependent on EPEC de novo protein synthesis during the few first minutes of the encounter between the bacteria and the host cells. Gentamicin kills EPEC rapidly; thus, once the process of formation of A/E lesions is triggered, the viability of EPEC (including protein synthesis) is not needed for maintenance and further maturation of the A/E lesions.

DISCUSSION

Many virulence genes of enteropathogenic bacteria are growth phase regulated. Expression of the *Salmonella spv* operon and *Yersinia mif* and *yst* genes is controlled by the stationary-phase-specific sigma factor σ^s (15, 19). *Salmonella* invasiveness is specifically expressed in the late logarithmic phase (20). In EPEC, the ability to induce A/E lesions could be detected only during the early to mid-logarithmic growth phase. At this stage, the activated EPEC bacteria induce rapid formation of A/E lesions and invade host cells within minutes after infection.

Tyrosine phosphorylation of Hp90 was detected by immunofluorescence microscopy 5 min postinfection. By immunoblot analysis using the same antiphosphotyrosine monoclonal antibody, tyrosine phosphorylation of Hp90 was detected only 20 min postinfection. Immunofluorescence microscopy appears to be a more sensitive assay because of the ability to detect the activation of a single epithelial cell by a single EPEC bacterium. Moreover, tyrosine-phosphorylated Hp90 is, in part, insoluble in 1% Triton X-100 because it interacts with the cytoskeleton (25, 26). We speculate that tyrosine-phosphorylated Hp90 first saturates its binding site in the cytoskeleton and only

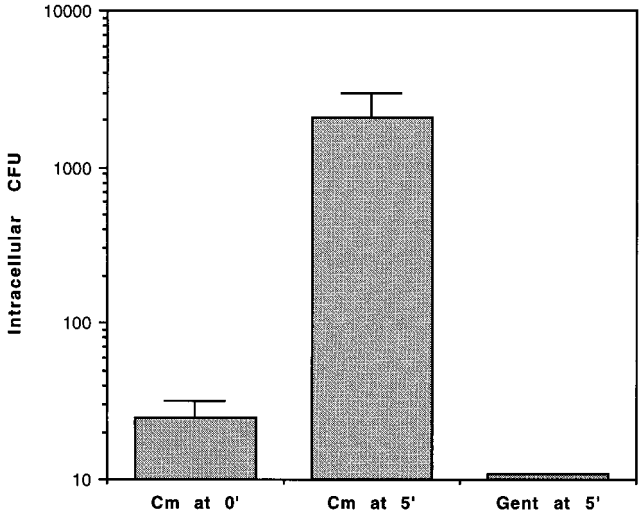

FIG. 5. De novo protein synthesis requirement for invasion of activated EPEC. HeLa cells were infected with activated EPEC culture in MEM. Chloramphenicol (Cm) was added immediately after infection or 5 min after infection, and gentamicin (Gent) was added 5 min after infection. The gentamicin-treated cells were processed for an invasion assay after 90 min. Infection of the chloramphenicol-treated cells included an additional 60 min of incubation with chloramphenicol in the medium. Invasion was stopped by adding gentamicin (100 μg/ml), and after 90 min, the cells were lysed with Triton X-100 and the number of intracellular bacteria was determined by plating.

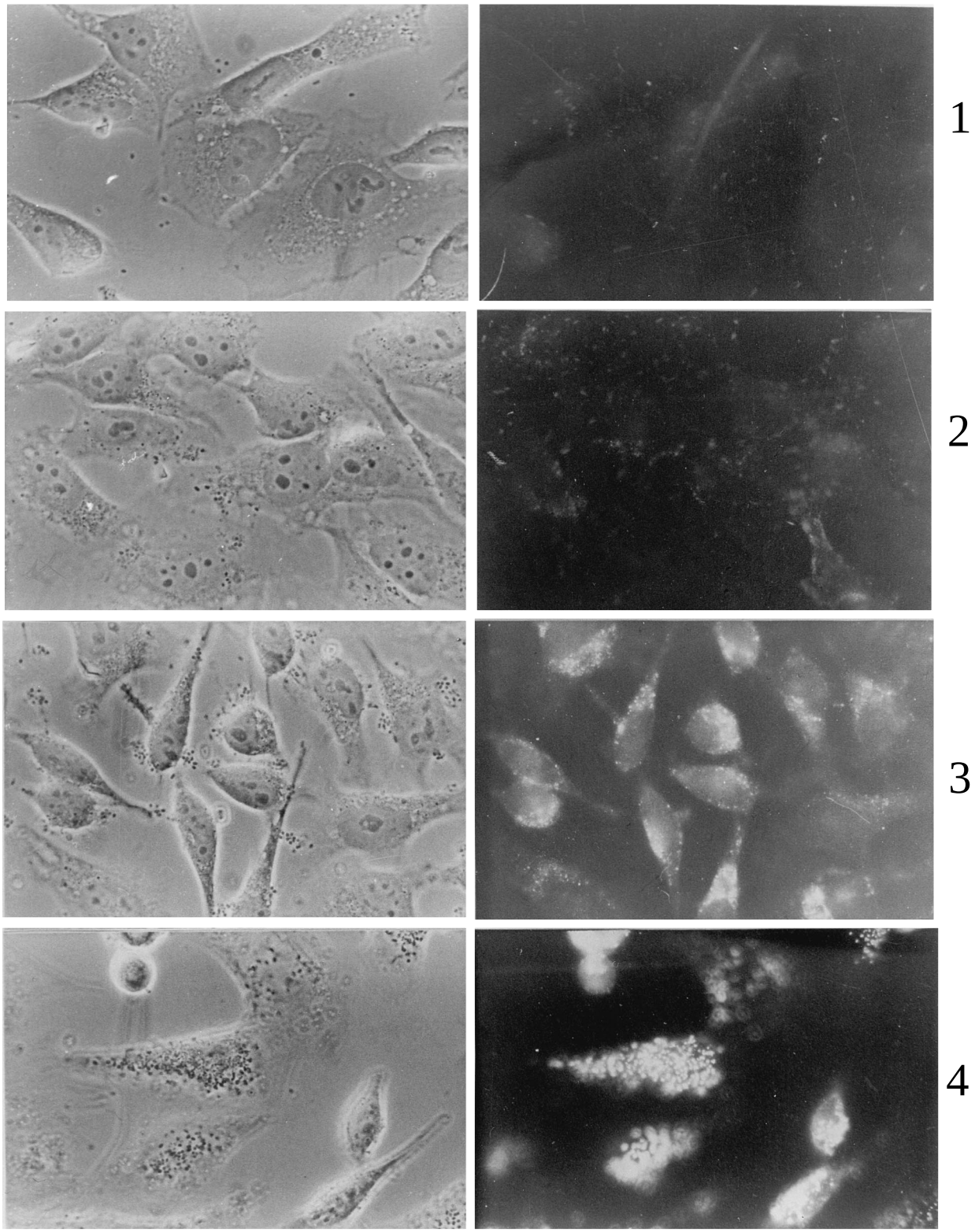

FIG. 6. Protein synthesis and induction of A/E lesions by activated EPEC. HeLa cells were infected with activated EPEC. Cells were fixed with 2% paraformaldehyde immediately (row 1) or after 10 min of infection (row 3). Alternatively, bacterial translation was blocked by adding gentamicin (100 μg/ml) immediately (row 2) or after 10 min of infection (row 4), and the cells were incubated for an additional 5 h in MEM plus gentamicin, at 37°C and under 5% CO_2, and the infected cells were fixed. The fixed cells were labeled with antiphosphotyrosine antibody (4G10) and examined by immunofluorescence microscopy. The immunofluorescent (right) and corresponding phase-contrast (left) images are shown.

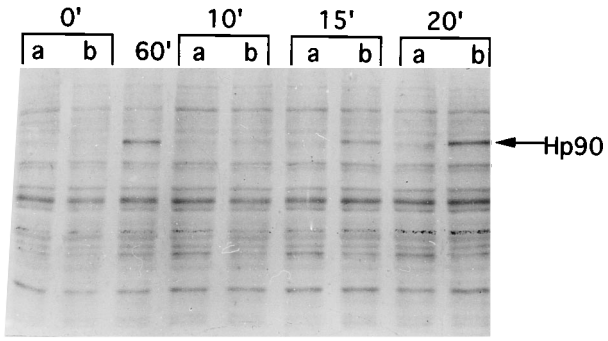

FIG. 7. Protein synthesis and induction of tyrosine phosphorylation of Hp90 by activated EPEC. Cells were infected with activated EPEC. Proteins of the infected cells were extracted at 0, 10, 15, 20, and 60 min postinfection (lanes a and 60′). Alternatively, at the same time points, bacterial protein synthesis was stopped by adding chloramphenicol (100 μg/ml), the cells were incubated for 60 min, and then proteins of the infected cells were extracted (lanes b). The extracted proteins were used for immunoblotting with antiphosphotyrosine antibody (PT66).

the excess of tyrosine-phosphorylated Hp90 becomes soluble in Triton X-100. This might be another reason for the delay in the appearance of tyrosine-phosphorylated Hp90 in the immunoblots of the Triton X-100-soluble fraction. The ability to induce A/E lesions and to invade is also dependent on the temperature during the bacterial growth. It was expressed at 37°C but not at 28°C. We termed EPEC that was grown to the early to mid-log phase at 37°C activated EPEC. Activated EPEC that was used for infection at 28°C still induced A/E lesions. This indicates that activation but not the A/E process itself is temperature regulated. The expression of many virulence genes in several enteropathogenic bacteria is temperature regulated. This includes the expression of the *cfa* gene (encoding CFA/I adhesin) in enterotoxigenic *E. coli*, of *ipa* and *mxi* genes (encoding invasion factors) in enteroinvasive *E. coli* and *Shigella* spp., the *pap* operon and *hly* gene (encoding the Pap pili and hemolysin) in uropathogenic *E. coli*, and the *yop* genes in *Yersinia enterocolitica* (see reference 8 and references therein). In most of these cases, the regulation has two loops, one of positive regulation by an AraC-like positive regulator and the other of negative regulation involving the H-NS DNA binding protein or closely related proteins (see reference 8 and references therein). Recently, an AraC-like homolog which positively regulates the expression of the *eaeA* and *eaeB* genes was described for EPEC (13).

Contact with the host cells appears to trigger invasiveness of *Salmonella* spp. (12), secretion of Ipa proteins by *Shigella* spp. (23), and injection of YopE into the host cytoplasm by *Yersinia* spp. (27, 29). Moreover, contact with the host cell also appears to stimulate YopE expression (27). The finding that EPEC can be activated to cause immediate A/E lesions enabled us to determine that de novo protein synthesis during the initial contact of EPEC with host cells is also needed for induction of A/E lesions. However, continuous protein synthesis in EPEC is not required for maintenance and maturation of A/E lesions. This can be interpreted in two ways. Contact with the host cell might induce expression of a stable factor that is needed for induction of A/E activity. Another possibility is that one of the factors involved in induction of A/E lesions is very labile, but once A/E activity is triggered, the activity of the labile factors is not needed for A/E maturation. For example, it is possible that a bacterial labile factor activates host tyrosine protein kinase, and once the host enzyme is activated, the bacterial factor is not needed. More work is needed to clarify the role of de novo protein synthesis, during the first minutes of host cell-pathogen interaction, in triggering A/E activity.

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