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Construction of an *eae* Deletion Mutant of Enteropathogenic *Escherichia coli* by Using a Positive-Selection Suicide Vector

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The ability to attach to epithelial cells, efface the microvillus surface, and disrupt the underlying cytoskeleton is characteristic of enteropathogenic *Escherichia coli* (EPEC). Recently, *eae*, a gene necessary for this phenomenon, was described (A. E. Jerse, J. Yu, B. D. Tall, and J. B. Kaper, Proc. Natl. Acad. Sci. USA 87:7839–7843, 1990). We report the use of a novel suicide vector containing the *pir*-dependent R6K replicon and the *sacB* gene of *Bacillus subtilis* to construct an *eae* deletion mutant of EPEC. This system enables positive selection for the loss of vector sequences. The resulting mutant, CVD206, is indistinguishable from the wild-type strain except for the loss of a 94-kDa outer membrane protein and attaching and effacing ability. Both the 94-kDa outer membrane protein and attaching and effacing ability are restored upon reintroduction of the *eae* gene on a plasmid. These results confirm the role of the *eae* gene in the attaching and effacing activity of EPEC and establish the utility of a new system for the construction of deletion mutations.

Enteropathogenic *Escherichia coli* (EPEC) strains of classical serotypes were first implicated as a cause of infantile diarrhea in the 1940s (2). Recent reports from Asia (20), eastern Europe (4), South America (13), Central America (7), and Africa (38) attest to the continued importance of these pathogens on a global scale.

Although the pathogenesis of EPEC infections has been poorly understood, several significant advances have recently been made. Classical EPEC strains adhere to tissue culture cells in a characteristic pattern termed localized adherence (37). This pattern is associated with the presence of a high-molecular-weight EPEC adherence factor plasmid (30) but may involve chromosomal genes as well (7). In addition, EPEC strains, as well as enterohemorrhagic *E. coli* strains (10), are capable of close attachment to the epithelial cell membrane, with destruction of microvilli and disruption of the underlying cytoskeleton (31, 34, 41, 43, 44). This effect, termed attaching and effacing activity (29), can be detected in the fluorescence actin staining (FAS) test with fluorescein isothiocyanate-labeled phalloidin to highlight the intense accumulation of filamentous actin underneath organisms attached to epithelial cells (22). Attaching and effacing activity is augmented by but is not dependent on the presence of the EPEC adherence factor plasmid (21, 23).

Recently, a chromosomal locus, termed *eae*, for *E. coli* attaching and effacing, was identified by Tn*phoA* mutagenesis (19). Sequences homologous to *eae* have been detected in all FAS$^+$ EPEC strains and in most enterohemorrhagic *E. coli* strains tested. Tn*phoA* mutants with mutations at this locus adhere to the brush border of tissue culture cells but fail to cause the ultrastructural attaching and effacing changes. Such mutants are also defective in their ability to invade HEp-2 cells (7). When such mutants are examined in the FAS test, faint fluorescence underneath adherent organisms can be detected, suggesting that *eae* mutants retain some ability to influence the epithelial cell cytoskeleton (7, 19). The *eae* gene consists of a 2,817-bp open reading frame, the predicted product of which shares 31% identical and 50% conserved amino acids with that of the *Yersinia pseudotuberculosis inv* gene (19). The product of the *eae* gene is a 94-kDa outer membrane protein recognized by convalescent-phase antisera from volunteers given EPEC (18).

While the results achieved with Tn*phoA* mutagenesis implicate *eae* in the attaching and effacing process, the possibility that the loss of attaching and effacing ability is due to the interruption of transcription of downstream genes has not been excluded. Therefore, it was desirable to construct a mutant with an internal deletion within *eae*. Such a mutant would be incapable of reversion to the wild type, obviating the need to apply selective pressure to avoid transposon loss, and would be immune to the acquisition of new mutations by replicative insertion of the transposon into other loci. A deletion mutant would therefore be more suitable for use in animal and human studies and might be an appropriate vaccine candidate.

To construct an EPEC *eae* deletion mutant, we combined two strategies that have been proved useful for allelic exchange mutagenesis. We used a suicide vector with a *pir*-dependent R6K replicon (28) and included the *sacB* gene of *Bacillus subtilis* (32). This system allowed positive selection for the loss of vector sequences after homologous recombination. We believe that this strategy greatly simplifies the construction of deletion mutations at specific loci.

MATERIALS AND METHODS

Bacterial strains and media. Bacteria were stored in LB broth plus 50% glycerol at −70°C and grown in LB broth, in M9 salts plus glucose (0.5%), or on LB agar. E2348/69 is a prototypic O127:H6 EPEC strain shown to be virulent in human challenge experiments (25). A nalidixic acid-resistant mutant, also proven virulent (26), was used in this work. SY327λ*pir*, a lysogen of SY327 [Δ(*lac pro*) *argE*(Am) *rif nalA recA56*] (27) containing the *pir* gene of plasmid R6K, was the host for transformation of plasmids containing the R6K replicon (28), and SM10λ*pir* (*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km) (39) was used for conjugal transfer of such plasmids. DH5α [*supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Bethesda Re-

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search Laboratories, Gaithersburg, Md.) was the host strain for transformation of other plasmids. Ampicillin (200 μg/ml), chloramphenicol (20 μg/ml), nalidixic acid (50 μg/ml), and kanamycin (50 μg/ml) were added as appropriate. Standard genetic techniques were performed as described previously (36).

Construction of pCVD441. Plasmid pCVD438 (kindly provided by Jun Yu) contains the *eae* gene on a 3,456-bp fragment cloned into the *Eco*RV and *Sph*I sites of pACY C184 (33). The cloned fragment includes 519 and 120 bp of DNA 5′ and 3′ of the *eae* gene, respectively, and interrupts a 467-bp open reading frame of unknown significance upstream of *eae*. No significant open reading frames are included within the 120 bp downstream of *eae*. The construction of pCVD441 containing the *eae* gene with a deletion of an internal 1,847-bp *Bst*EII fragment (nucleotides 585 to 2432, representing approximately 66% of the *eae* gene) is depicted in Fig. 1. SM10λ*pir* was grown to the mid-logarithmic phase, washed four times with distilled water, resuspended in 10% glycerol, and electrotransformed with pCVD441 in 0.2-cm cuvettes in a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) at 2.5 kV and 25 μF. Transformants were selected with ampicillin.

Construction of an *eae* deletion mutant of E2348/69. Plasmid pCVD441 was introduced into E2348/69 from SM10λ*pir* by filter mating, with selection for nalidixic acid and ampicillin resistance. Resulting exconjugants were confirmed to be resistant to kanamycin as well. A single such colony was purified and grown to the late logarithmic phase in LB broth without selection. Serial dilutions were inoculated onto LB plates containing 5% sucrose. Sucrose-resistant colonies were picked and tested for ampicillin and kanamycin sensitivity, indicative of the loss of suicide vector sequences. Such colonies were tested for the loss of the intact *eae* gene by colony hybridization with an *eae* DNA probe (19) entirely internal to the deleted *Bst*EII fragment.

PCR. An ampicillin-sensitive, kanamycin-sensitive, sucrose-resistant clone found negative in colony hybridization with the *eae* probe was tested for the loss of *eae* sequences by the polymerase chain reaction (PCR). E2348/69 and SM10λ*pir*(pCVD441) served as controls for full-length and shortened *eae* gene products. DNA was extracted as described previously (11) from colonies picked from fresh plates and suspended in phosphate-buffered saline (PBS). Two sets of primers were used. K38 (5′-GGG GAT CCG TGG TGG AGC CCA AAC ATG AT-3′) and K37 (5′-GGG GAT CCT TTT ACA CAA GTG GCA TAA GC-3′) were derived from sequences overlapping the 5′ end and complementary to the 3′ end of the *eae* gene, respectively, and were constructed to include the hexanucleotide recognition sequence for *Bam*HI to facilitate cloning of amplified *eae* genes. Yu4 (5′-TGA ACG CAG TAC GCA GAA GAT-3′) represents nucleotides 1394 to 1414 and K3 (5′-GCT GGA TTT GCT GAG CGC CAT G-3′) is complementary to nucleotides 2379 to 2401 of the *eae* gene. Both Yu4 and K3 are internal to the deleted *Bst*EII fragment. The reactions were performed with a commercially available kit (Perkin Elmer Cetus, Norwalk, Conn.) and a TwinBlock System temperature cycler (Ericomp, Inc., San Diego, Calif.), with 30 cycles of denaturation (1 min at 94°C), annealing (2 min at 50°C), and elongation (5 min at 72°C).

Southern hybridization. Total genomic DNA was isolated by sodium dodecyl sulfate (SDS) lysis and subjected to proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Approximately 2 μg of DNA was digested with excess *Bgl*II, separated on a 0.7% agarose gel, and transferred to nitrocellulose (40). Bacteriophage λ DNA digested with *Hin*dIII was included in adjacent lanes as a molecular weight standard. Plasmid pCVD438 was labeled by the random primer method (9) with [α-^{32}P]dATP. Hybrid-

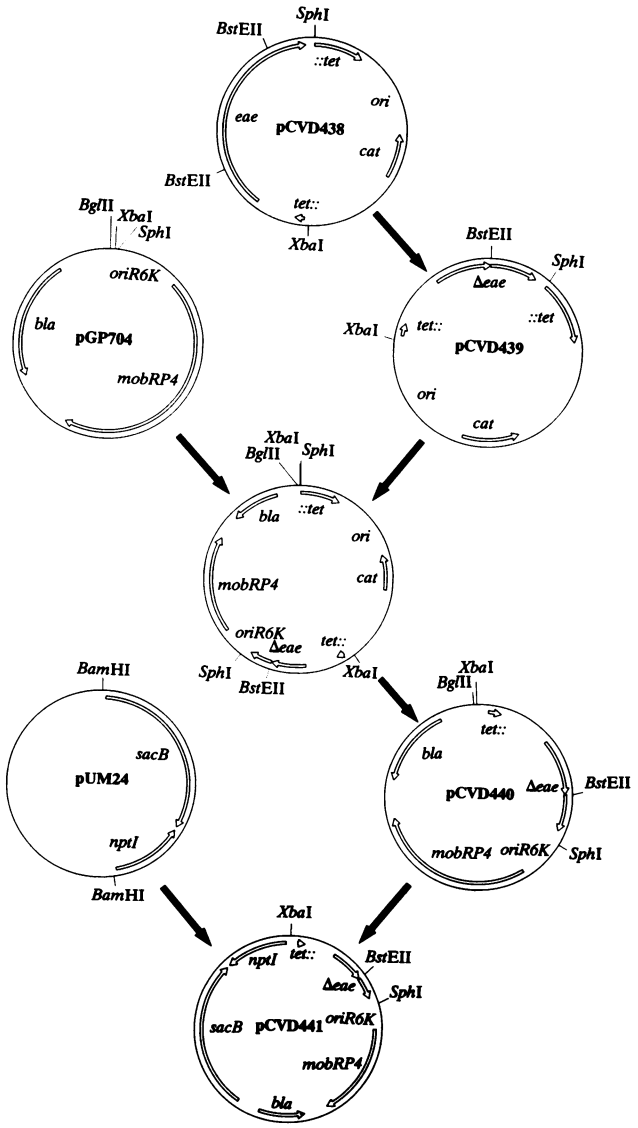

FIG. 1. Scheme for the construction of suicide vector pCVD441. Plasmid pCVD439 was created by digesting pCVD438 with *Bst*EII, rejoining the larger fragment with T4 DNA ligase, and selecting for chloramphenicol-resistant colonies in DH5α. This procedure was predicted to result in the loss of an internal 1,847-bp fragment. Suicide vector pGP704 (28) and pCVD439 were joined after digestion of both with *Sph*I, and the resulting plasmid was selected with ampicillin in DH5α. Plasmid pCVD440 was created by excision of a 3.6-kb *Xba*I fragment, religation, and selection for ampicillin-resistant colonies in SY327λ*pir*. This procedure resulted in the loss of the pACYC184 origin of replication and the chloramphenicol acetyltransferase gene. Plasmid pUM24 (32), which contains the *sacB* gene of *B. subtilis* and the *nptI* gene encoding resistance to kanamycin on a single 3.8-kb *Bam*HI fragment, was cut with *Bam*HI, and the products were ligated to *Bgl*II-digested pCVD440. The resulting plasmid, pCVD441, was selected on plates containing kanamycin after transformation of SY327λ*pir*. Plasmids are not drawn to scale.

ization was performed under stringent conditions (50% formamide, 37°C, 750 mM NaCl; 65°C wash with 750 mM NaCl). Bands were visualized by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.). Following initial film exposure, the blot was incubated with α-^{32}P-labeled λ DNA for visualization of molecular weight standards.

Nucleotide sequence analysis. Single-stranded template DNA was produced from CVD206 by PCR with unequal primer concentrations as described previously (8). Primer K61 (5′-TAG CCA GCT TCA GTC GCG-3′), representing nucleotides 521 to 538, was provided at 0.2 pM, and primer K37 was provided at 20 pM. The amplified product was purified by ethanol precipitation for nucleotide sequence determination by the dideoxy chain termination method with the K61 primer, a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio), and [α-^{35}S]dATP (800 to 1,000 Ci/mmol) (42). Bands were visualized by autoradiography of sequencing gels and compared with the published *eae* gene sequence.

Phenotypic characterization. The presence of the O127 surface antigen was assayed by slide agglutination with a specific antiserum (Difco Laboratories, Detroit, Mich.). Biotyping was performed with the API 20E system (Analytab Products, Plainview, N.Y.). Antibiotic resistance was tested on Trypticase soy agar plates by the Kirby-Bauer disk diffusion method. Growth rates in rich (LB) and minimal (M9 salts plus 0.5% glucose) media were tested by measuring the A_{600} with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Wild-type and mutant strains were tested in triplicate and compared by linear regression analysis.

Outer membrane proteins. Triton X-100-insoluble membrane material was purified as described previously (19) and separated on 7% SDS-polyacrylamide electrophoresis gels (14). After electrotransfer to nitrocellulose paper, separated proteins were exposed to rabbit antisera raised against the Eae-alkaline phosphatase fusion product of an *eae*::Tn*phoA* mutant (18). Goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was used as the second antibody, and the reaction was developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (15).

Adherence and FAS assays. Assays for localized adherence to HEp-2 cells were carried out in the presence of 1% D-mannose for 3 h as described previously (6). The FAS assay for filamentous actin accumulation was performed on HEp-2 cells for 3 and 6 h as described previously (22).

Electron microscopy. Caco-2 human colonic carcinoma cells (35) were cultivated in Eagle's minimal essential medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) on Transwell filters (Costar, Cambridge, Mass.). The medium was changed every 2 to 3 days, and the culture was grown until a fully developed brush border was apparent (14 days). Cells were washed with PBS, the medium was replaced with the same medium without additives, and 0.020 ml of an overnight LB broth culture of bacteria was added to the apical surface. Cells were incubated at 37°C for 8 h, with medium changes at 3 and 6 h, washed five times with PBS, and fixed overnight in 2% formaldehyde–1% glutaraldehyde. Specimens were postfixed in 1% osmium tetroxide. Thin sections were examined with a JEOL JEM 1200-EX transmission electron microscope.

RESULTS

Construction of CVD206. We constructed pCVD441, a suicide vector bearing an internal deletion within the *eae* gene, to create an EPEC mutant with a mutation at this locus by allelic exchange. This plasmid was designed to allow selection of a merodiploid strain in which the plasmid had been integrated into the *eae* gene by homologous recombination and to allow positive selection of a second recombination event resulting in resolution of the merodiploid state. After conjugative transfer of pCVD441 from SM10λ*pir*, a single colony was isolated and found to be resistant to nalidixic acid, ampicillin, and kanamycin and agglutinable with O127 antiserum. This isolate, E2348/69 *eae*::pCVD441, was grown without selection to the late logarithmic phase and spread on plates containing 5% sucrose. Of 21 sucrose-resistant colonies, 18 were found to be sensitive to ampicillin and kanamycin, suggesting that the loss of vector sequences had occurred. In other experiments, the percentages of ampicillin- and kanamycin-sensitive colonies obtained were found to vary considerably, suggesting that different mutational events could give rise to sucrose-resistant progeny. Results of Southern hybridization suggested that E2348/69 *eae*::pCVD441 may have a tandem repeat of vector sequences that may explain this variability (see below). One of the 18 ampicillin-sensitive, kanamycin-sensitive, sucrose-resistant colonies was found negative for internal *eae* sequences in colony hybridization. This isolate is referred to as CVD206.

Genetic analysis. We used several methods to verify the genetic construction of CVD206. We first confirmed that the size of the *eae* region had decreased as a result of the deletion of 1,847 bp from within the gene. Using primers overlapping the 5′ and 3′ ends of the *eae* gene, we amplified a 2.8-kb product from E2348/69. In contrast, the product obtained from CVD206 was 1.0 kb, consistent with the loss of a 1.8-kb internal fragment. Using primers internal to the *eae* *Bst*EII fragment, we amplified a 1.0-kb product from E2348/69, but no product could be amplified from CVD206 (data not shown). Results of Southern hybridization confirmed these findings (Fig. 2). Consistent with restriction endonuclease mapping data (not shown), a 7.2-kb *Bgl*II fragment from E2348/69 was found to hybridize to pCVD438. In contrast, the hybridizing fragment from CVD206 was 5.4 kb. Surprisingly, the hybridizing fragment from the merodiploid strain, E2348/69 *eae*::pCVD441, was estimated to be approximately 28 kb. Since pCVD441 is 9.2 kb and has no *Bgl*II sites, this result indicates that additional DNA had been inserted in this region. This result was most likely due to a tandem duplication arising by homologous recombination of adjacent native and mutated *eae* sequences under selective pressure for vector-encoded ampicillin resistance. Finally, we used direct sequence analysis of the PCR product from CVD206 to confirm the deletion. The sequence obtained was precisely as predicted, demonstrating the loss of an internal *Bst*EII fragment representing nucleotides 585 to 2432 of the gene (data not shown).

Comparisons of E2348/69 and CVD206. The wild-type and mutant strains were indistinguishable by colony morphology, O-antigen serotype, biotype, resistance to 15 antibiotics, growth rates in rich and minimal media, plasmid profile, genomic DNA restriction endonuclease digestion pattern, and ability to adhere to HEp-2 cells in a localized pattern (data not shown). Analysis of outer membrane proteins (Fig. 3) revealed similar patterns in the two strains, with the

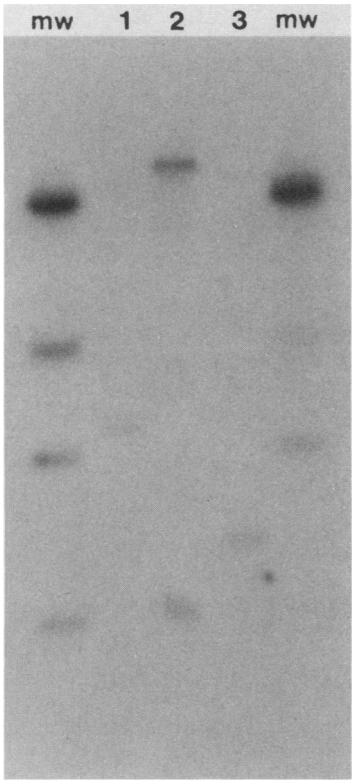

FIG. 2. Southern hybridization of genomic DNAs digested with *Bgl*II and hybridized with [α-^{32}P]dATP-labeled pCVD438. Lanes: mw, λ digested with *Hin*dIII (bands of 23.1, 9.4, 6.6, and 4.4 kb are visible); 1, E2348/69; 2, E2348/69 *eae*::pCVD441; 3, CVD206.

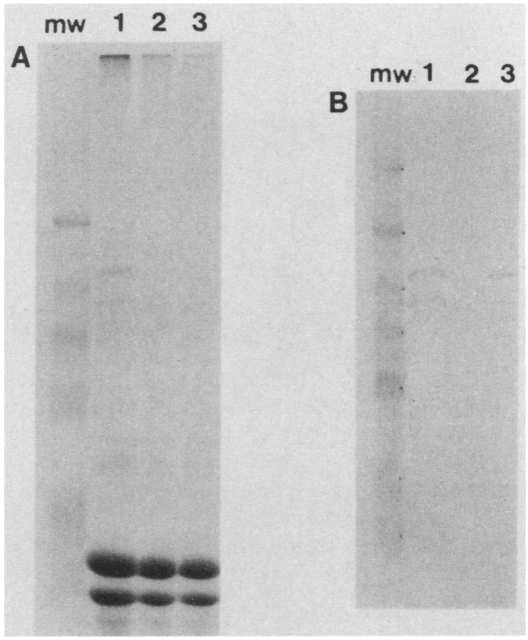

FIG. 3. Outer membrane proteins (50 μg per lane) separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (A) and transferred to nitrocellulose for Western blotting with antisera raised against an Eae-alkaline phosphatase fusion protein (B). Lanes: mw, SDS-7B (Sigma) molecular weight standards (α_2-macroglobulin, ca. 180,000; β-galactosidase, ca. 116,000; fructose-6-phosphate kinase, ca. 84,000; pyruvate kinase, ca. 58,000; fumurase, ca. 48,500; lactic dehydrogenase, ca. 36,500; and triosephosphate isomerase, ca. 26,600); 1, E2348/69; 2, CVD206; 3, CVD206(pCVD438).

exception of more intense staining of a 94-kDa band from E2348/69. In Western blotting (immunoblotting) with specific antiserum raised against an Eae-alkaline phosphatase fusion protein (18), bands with apparent molecular masses of 94 and 75 kDa could be demonstrated in outer membrane proteins from E2348/69 and CVD206 transformed with pCVD438 but not from CVD206. Thus, the Eae protein is absent from CVD206 but is restored when the *eae* gene is supplied on a plasmid. The smaller antigenically related fragment seen probably represents a degradation product of the 94-kDa outer membrane protein (3).

When strains were tested at 3 and 6 h in the FAS assay, intense fluorescence was observed in HEp-2 cells underneath adherent E2348/69 and CVD206(pCVD438), indicating high concentrations of filamentous actin. In contrast, only a faint shadow of fluorescence could be detected at 6 h in HEp-2 cells underneath adherent CVD206 or CVD206 transformed with pACYC184. Electron microscopy of Caco-2 cells infected with E2348/69 or CVD206(pCVD438) revealed typical attaching and effacing lesions throughout the specimens, characterized by a loss of microvilli, intimate attachment to the epithelial cell membrane, and the accumulation of filamentous material beneath adherent organisms (Fig. 4). Abundant microcolonies of CVD206 or CVD206(pACYC184) were observed adherent to Caco-2 cells. However, no intimate attachment to the epithelial cell membrane or accumulation of filamentous material beneath adherent organisms was observed with these strains.

DISCUSSION

Jerse et al. identified the *eae* locus of EPEC by screening Tn*phoA* mutants for the loss of the ability to induce filamentous actin accumulation (19). In similar studies, by screening EPEC Tn*phoA* mutants for the loss of the ability to invade HEp-2 cells, we found five noninvasive mutants with insertions at this locus and two noninvasive mutants with insertions just downstream of *eae* (7). No attaching and effacing lesions were detected when Caco-2 cells infected with *eae*::Tn*phoA* mutants were examined by electron microscopy. Subsequent DNA sequencing revealed that the *eae* gene is closely related to the *inv* gene of *Y. pseudotuberculosis*. Jerse et al. reported that restoration of the attaching and effacing phenotype was achieved with pCVD437, which contains a 7.2-kb *Bgl*II fragment that includes the *eae* gene as well as 4.1 kb of DNA 3′ to this locus (19). Taken together, these observations suggest that the *eae* gene may be necessary for EPEC attaching and effacing and invasion of epithelial cells. However, these data do not exclude the possibility that Tn*phoA* inactivated not only the *eae* gene but cistrons downstream of this locus as well. Such inactivation might result in the incorrect assignment to the *eae* locus of properties encoded by downstream genes. The restoration of wild-type activity by pCVD437 may have been due to complementation of downstream loci. We sought more conclusive evidence of the role of the *eae* gene in the attaching and effacing activity of EPEC by constructing a mutant with a deletion at this locus and testing its properties in tissue culture models.

To create a derivative of E2348/69 with a deletion in the

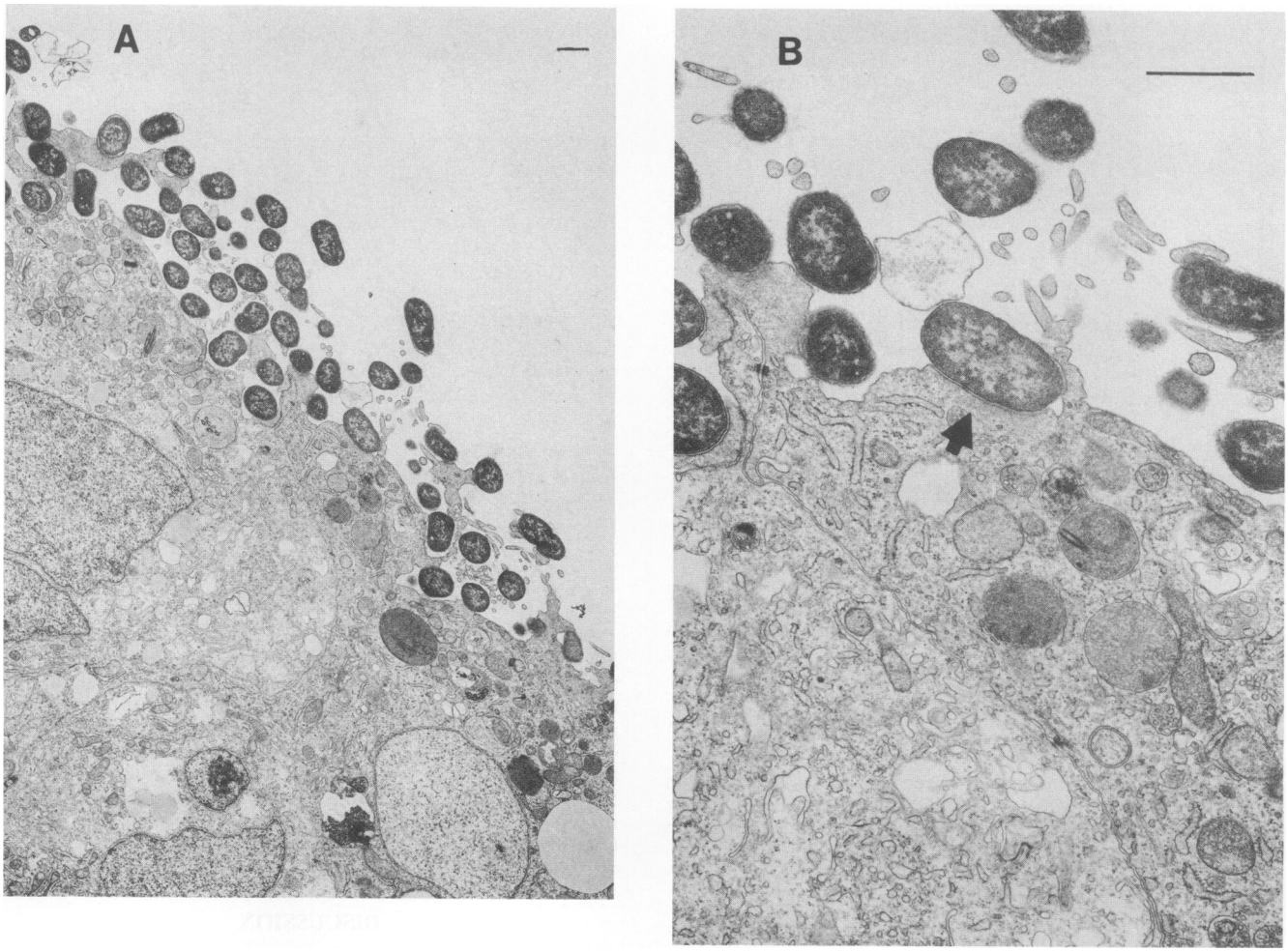

FIG. 4. Transmission electron micrographs of Caco-2 cells incubated with E2348/69 (A and B) or CVD206 (C and D). Lower magnification (A and C) demonstrates comparable avid adherence of both strains to the epithelial cells. At higher power, typical attaching and effacing lesions with the loss of microvilli, intimate attachment to the epithelial cell membrane, and disruption of the cytoskeleton (arrow) are evident throughout the specimen containing E2348/69 (B); similar findings were evident in electron micrographs of CVD206(pCVD438) (data not shown). CVD206 (D) and CVD206(pACYC184) (data not shown) were exclusively observed adhering nonintimately to the epithelial cells without associated cytoskeletal changes. Bars, 1 μm.

eae gene, we adapted a suicide vector system described by Miller and Mekalanos (28). This strategy relies on the fact that replication at the origin of plasmid R6K is dependant on π, the *pir* gene product (24). An altered version of a target gene can be cloned into a plasmid that contains the R6K origin but lacks the *pir* gene only in hosts that provide π in *trans*. After introduction of such a plasmid into the nonpermissive target strain by conjugation, selection for plasmid-encoded antibiotic resistance allows the isolation of merodiploid strains in which the plasmid has been integrated into the target gene by homologous recombination. To isolate a deletion mutant by using this strategy, one would then have to grow the merodiploid strains under nonselective conditions to allow a second recombination event to occur, resulting in the loss of vector sequences and the intact copy of the gene. Such an event is detected by the laborious screening of tens of thousands of colonies for sensitivity to the antibiotic originally used in selection. Sensitive colonies are then tested for the replacement of the wild-type allele with the deletion allele.

We developed a strategy that allows direct selection for the loss of suicide vector sequences, thereby obviating the need for screening large numbers of colonies and thus reducing the effort required for creating deletion mutants. We added the *sacB* gene of *B. subtilis* to the suicide vector to provide a conditionally lethal phenotype. The *sacB* locus encodes the enzyme levan sucrase, which is toxic for gram-negative organisms only in the presence of sucrose (12). The merodiploid strain, which contains the suicide vector and the mutated version of the *eae* gene integrated into the *eae* locus, was exposed to sucrose to directly select for organisms that had undergone a second recombination event resulting in the loss of the suicide vector. A similar strategy with a temperature-sensitive replicon and the *sacB* gene was recently reported for the allelic replacement of genes of the type 1 fimbrial cluster in *E. coli* (1). In addition to plasmid pCVD441, containing the *eae* deletion allele, we developed a *sacB*-containing suicide vector for general use (data not shown). Plasmid pCVD442 is composed of the *mob*, *ori*, and *bla* regions from pGP704 (28) and the *sacB* gene. Five unique

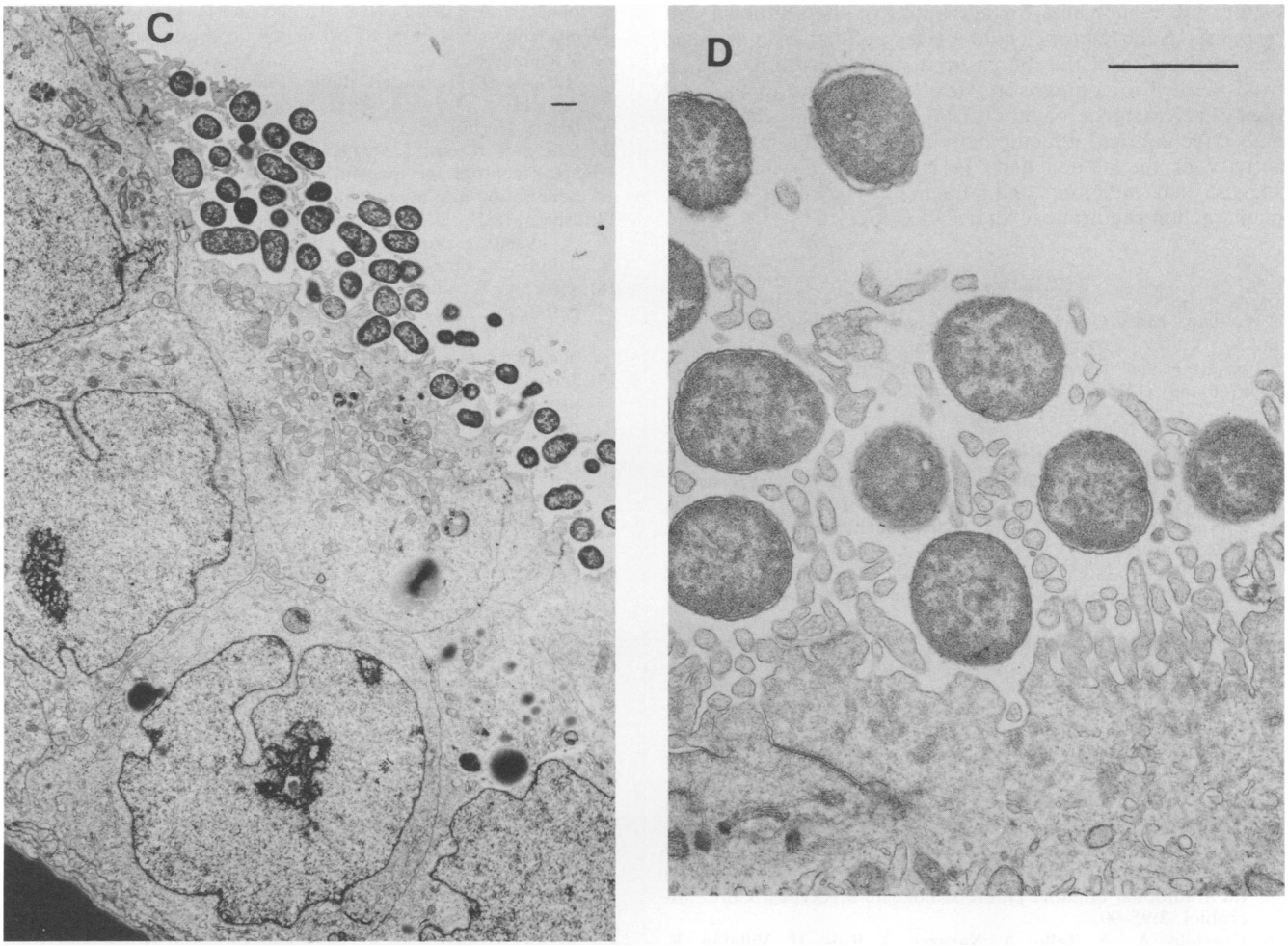

FIG. 4—*Continued.*

restriction endonuclease sites, including a blunt-end *Sma*I site, are available on this vector for cloning.

Using this strategy, we were able to introduce pCVD441 into wild-type EPEC strain E2348/69. The results of subsequent Southern analysis were consistent with integration of the vector into the *eae* gene; unexpectedly, however, the size of the resulting restriction fragment suggested a tandem duplication in this region. Such an event could occur through homologous recombination of daughter chromatids at the intact and mutated alleles of the *eae* locus during DNA replication under ampicillin selection pressure. Nevertheless, we were able to select for the loss of all vector sequences by growing the merodiploid strain in the presence of sucrose. One such ampicillin-sensitive, sucrose-resistant isolate, CVD206, was shown by colony hybridization, PCR, Southern blotting, and DNA sequencing to be the desired *eae* deletion mutant.

CVD206 was indistinguishable from the parent strain in most respects. However, the mutant was deficient in the ability to induce the accumulation of filamentous actin in HEp-2 cells and the ability to cause the attaching and effacing effect on Caco-2 cells. Unlike the parent strain, which attached directly and intimately to the membrane of the epithelial cells and induced a disruption of cytoskeletal elements, CVD206 adhered at a greater distance from the membrane. Wild-type attaching and effacing ability was restored to the mutant by the introduction of a plasmid that contains the cloned *eae* gene without other significant EPEC sequences. Furthermore, CVD206 was found to lack a 94-kDa outer membrane protein recognized by antisera raised against an Eae-alkaline phosphatase fusion protein. This result is consistent with the recent demonstration that this protein is in fact the product of the *eae* gene (18). These results confirm the work of Jerse et al. (19), who demonstrated the loss of attaching and effacing ability in a Tn*phoA* mutant of EPEC, and provide evidence that the *eae* gene itself is required for attaching and effacing.

What is the role of *eae* in EPEC attaching and effacing and invasion? The striking similarity between the *eae* gene of EPEC and the *inv* gene of *Y. pseudotuberculosis* suggests the possibility of functional correspondence and evolutionary relatedness. Invasin, the product of *inv*, binds with a high affinity to members of the β_1 family of integrins (16) and allows efficient invasion of epithelial cells (17). Although *eae* is necessary for attaching and effacing and invasion by EPEC, unlike *inv*, *eae* is not sufficient to confer these properties on laboratory *E. coli* strains, suggesting that additional EPEC loci are required. The residual effects of the *eae* mutant on cellular actin (the faint shadow of fluorescence seen in the FAS assay) may indicate that other EPEC

factors are responsible for activation of the epithelial cell response. Such factors could be less effective in the *eae* mutant because of the increased distance of the bacterium from the cell in comparison with the proximity of the wild type. The existence of other Tn*phoA* mutations that eliminate attaching and effacing activity (7) and the absence of activity of the cloned gene in laboratory *E. coli* strains suggest that attaching and effacing represent a complex phenomenon requiring several EPEC loci.

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