APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1999, p. 4234–4238 Vol. 65, No. 9
0099-2240/99/$04.00+0

Clostridium difficile Cell Attachment Is Modified by Environmental Factors

ANNE-JUDITH WALIGORA, MARIE-CLAUDE BARC, PIERRE BOURLIOUX, ANNE COLLIGNON, AND TUOMO KARJALAINEN*

Département de Microbiologie, Faculté de Pharmacie, Université de Paris-Sud, 92296 Châtenay-Malabry Cedex, France

Received 6 January 1999/Accepted 28 June 1999

Adherence of *Clostridium difficile* to Vero cells under anaerobic conditions was increased by a high sodium concentration, calcium-rich medium, an acidic pH, and iron starvation. The level of adhesion of nontoxigenic strains was comparable to that of toxigenic strains. Depending on the bacterial culture conditions, Vero cells could bind to one, two, or three bacterial surface proteins with molecular masses of 70, 50, and 40 kDa.

Clostridium difficile is recognized as the major causative agent of pseudomembranous and antibiotic-associated colitis (14). Its pathogenicity is mediated by two exotoxins, toxins A (308 kDa) and B (207 kDa), which both damage human colonic mucosa in vitro and are potent cytotoxic enzymes (24). Other potential virulence factors include the capsule (9), proteolytic enzymes (17, 18, 28, 29), flagella (32), fimbriae (4), and an adhesin(s) potentially involved in mucus and cell association (2, 5, 12, 16).

Proliferation of *C. difficile* in the colon results from the suppression of members of the normal microbiota (i.e., suppression of colonization resistance) during or after antibiotherapy. A colonic ecosystem in equilibrium is characterized by a pH at the luminal surface of around 6.2 to 6.8 (11); an osmolarity of 310 mosM (1); low oxygen tension (1); a temperature of 37°C; calcium and magnesium concentrations of 5 and 2 mM, respectively; (30), and a low level of free iron (7). Oral intake of antibiotics and the subsequent disturbance of this equilibrium can profoundly alter any of these parameters. In this study, we investigated whether environmental conditions deviating from physiological ones constitute a stimulus for *C. difficile* that could drive it to increased adherence and subsequent colonization.

The effect of environmental stresses on *C. difficile* adherence was investigated in vitro in the Vero cell adherence model (16) by using either different culture or adherence assay conditions. Toxigenic *C. difficile* isolate 79-685 was used as the reference strain in all of the adherence assays. Other isolates used are listed in Table 1. Before adherence assays, bacteria were subcultured twice for 24 h each time in an anaerobic chamber at 37°C in tryptone-glucose-yeast extract (TGY) broth (Difco) that had been prereduced prior to use.

All cell adherence assays were performed as previously described by us (16), in accordance with two protocols: (i) in an aerobic atmosphere enriched with 10% CO_2 (partially aerobic conditions) with bacteria exposed to a heat shock (20 min at 60°C) prior to contact with Vero cells (12) and (ii) under strict anaerobic conditions with no heat shock. Bacteria and cells were incubated together for 1 h or more at 37°C under aerobic (with CO_2) or strict anaerobic conditions. Nonadherent bacteria were eliminated by five washings in PBS (10 mM phosphate buffer, 150 mM NaCl), pH 7.0, and the cells were fixed and stained with May-Grünwald-Giemsa stain (Sigma). The adhesion index is given as the average number of adhering bacteria (counted at a magnification of ×1,000) per cell ± the standard deviation from at least three different assays. The significance of differences between various treatments was assessed by Student's *t* test.

When our assays were performed under partially aerobic conditions after a heat shock, the adherence level was slightly lower than that observed under strict anaerobic conditions with no heat shock, due to the death of some bacteria (Fig. 1A), but similar adherence profiles were always observed for all of the environmental factors tested. Only results obtained under strict anaerobic conditions with no heat shock are shown in the figures.

Toxins. To compare the kinetics of adherence of five nontoxigenic and two toxigenic *C. difficile* strains (Table 1), the bacteria were incubated with Vero cells for 1 to 4 h. All of the *C. difficile* strains tested were adherent, and there was no significant interstrain variability in adhesion levels ($P < 0.05$) (Fig. 1B). Thus, cell adherence, in contrast to mucus association, as shown by us (15) and Borriello et al. (3, 5), does not appear to be promoted by toxins.

TABLE 1. Serogroups, toxin production, and origins of *C. difficile* isolates studied

Strain	Serogroup[a]	Production of toxins A and B[b]	Origin	Source
79-685	S3	+	Adult with PMC[c]	VPI[b]
VPI-10463	G	+	Adult with wound abscess	Institut de Bactériologie[e]
90-111	D	0	Child carrier	Jean Verdier Hospital[f]
93-136	D	0	Child carrier	
93-226	D	0	Child carrier	
93-296	D	0	Child carrier	
93-379	D	0	Adult carrier	

[a] As defined by Delmee et al. (10).
[b] Toxin assay was performed as described in reference 18.
[c] PMC, pseudomembranous colitis.
[d] VPI, Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg.
[e] Strasbourg, France.
[f] Bondy, France.

* Corresponding author. Mailing address: Département de Microbiologie, Faculté de Pharmacie, Université de Paris-Sud, 92296 Châtenay-Malabry Cedex, France. Phone: (33) 1-46 83 55 49. Fax: (33) 1-46 83 58 83. E-mail: tuomo.karjalainen@cep.u-psud.fr.

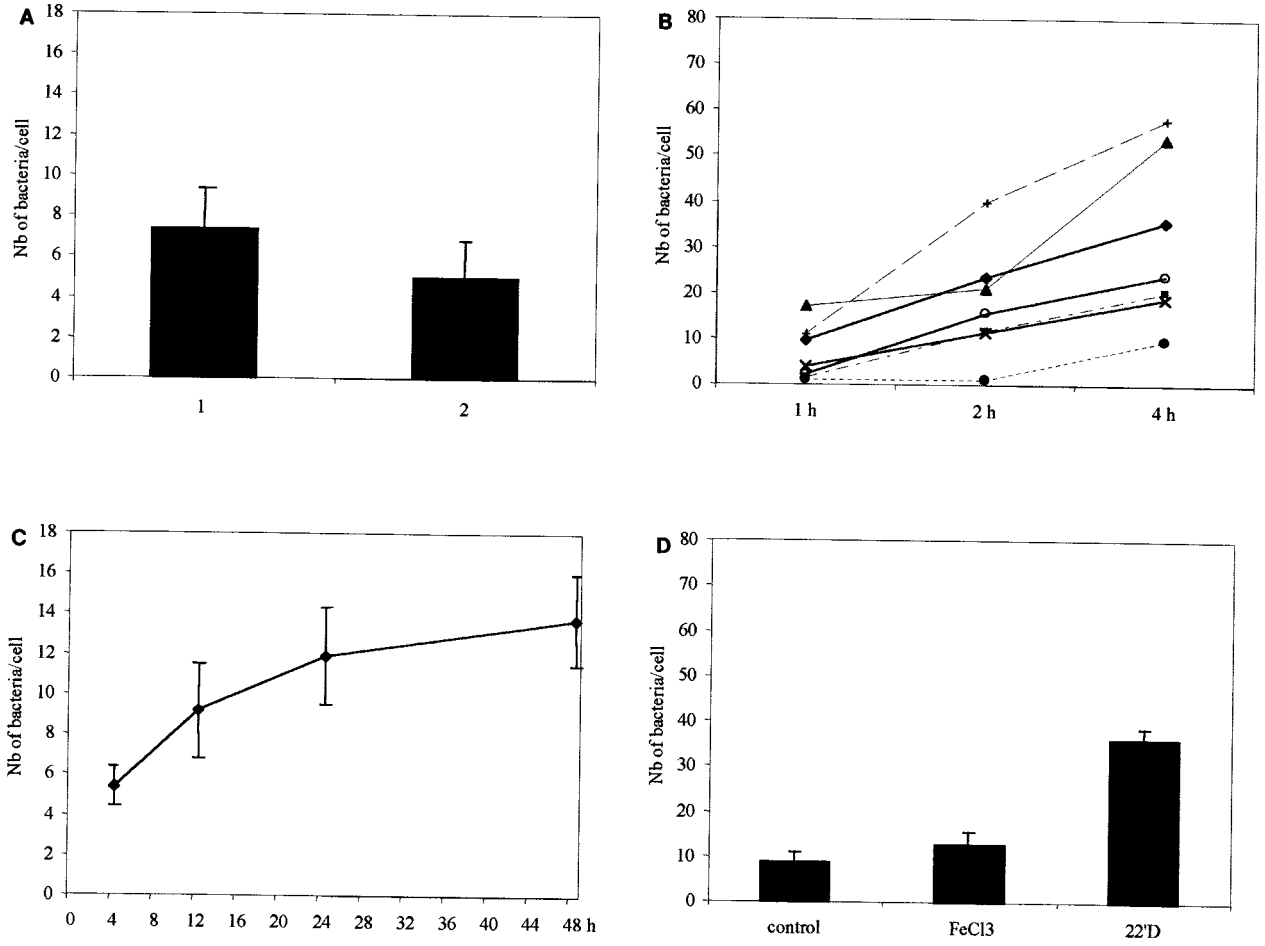

FIG. 1. (A) Adherence of *C. difficile* to Vero cells under anaerobic conditions (part 1) or after a heat shock under aerobic (with CO_2) conditions (part 2). (B) Adherence to Vero cells of seven *C. difficile* strains as a function of time. Symbols for toxigenic strains: ◆, 79-685; ■, VPI-10409. Symbols for nontoxigenic strains: ▲, 90-111; ×, 93-136; ○, 93-226; ●, 93-296; +, 93-379. (C) Effect of growth phase on the adherence of *C. difficile* to Vero cells. (D) Effect of iron in the culture medium on the adherence of *C. difficile* to Vero cells. The control was TGY, the concentration of $FeCl_3$ was 50 μM, and that of 2,2′-dipyridyl (22′D) was 200 μM. Nb, number.

Growth phase. To study the effect of the growth phase on *C. difficile* adherence, bacteria were cultivated under anaerobic conditions for 4 (early exponential phase), 12 (early stationary phase), 24, or 48 (stationary phase) h prior to the cell adherence assay. As shown in Fig. 1C, expression of adherence was growth phase dependent, reaching a maximum late in stationary phase. Stationary phase can be considered a stress that can trigger expression of pathogenic determinants (8).

Iron. To investigate the effect of iron starvation on the adherence capacity of *C. difficile*, bacteria were cultured (i) under iron-rich conditions created by adding 50 μM $FeCl_3$ (Sigma) to TGY broth or (ii) in iron-limited medium prepared by adding 200 μM 2,2′-dipyridyl (Sigma) to TGY broth. Bacteria were subsequently washed with PBS, and adherence assays were performed as described above. As shown in Fig. 1D, bacteria collected from iron-limited cultures displayed significantly higher Vero cell adherence than did those collected from iron-rich or control cultures ($P < 0.05$). An iron-poor environment could drive *C. difficile* toward colonization of target tissues, which should facilitate acquisition of iron from tissues later damaged by the pathogenic process.

Sodium chloride. Osmolarity is one environmental factor which regulates the interaction of enteric microorganisms with eucaryotic cells (6, 20, 23, 25–27, 31). To investigate whether *C. difficile* adherence to cells is osmoregulated, bacteria were cultivated in TGY broth containing 50 (low osmolarity), 150 (medium osmolarity), or 500 (hyperosmolarity) mM NaCl. As shown in Fig. 2A, part 1, adherence of bacteria grown in the presence of 500 mM NaCl was increased three- to fourfold, as opposed to 50 and 150 mM NaCl ($P < 0.05$). In contrast, adherence of *C. difficile* grown in the presence of 50 mM NaCl was not influenced by modifications of the sodium concentration of the adherence assay medium (Fig. 2A, part 2).

Interestingly, when bacteria were grown at high NaCl concentrations, morphological modifications were evident; i.e., they were short and surrounded by viscous material (data not shown). High osmolarity may induce physicochemical changes in the bacterial surface, especially hydrophobicity (19, 34), thus changing adherence properties. Alternatively, high osmolarity, along with other environmental stimuli (O_2, temperature, and

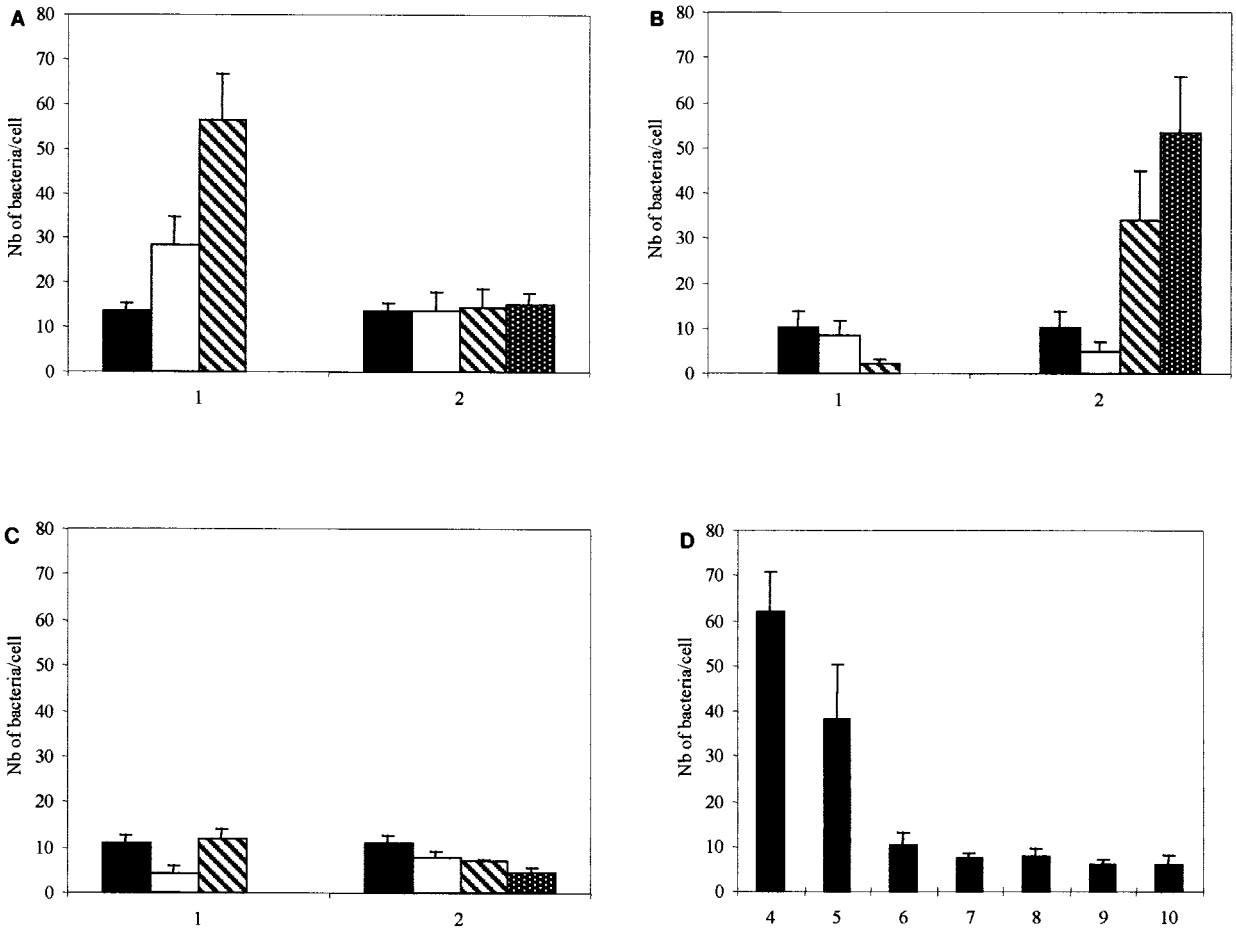

FIG. 2. Effects of cations and pH on *C. difficile* adherence to Vero cells. (A) Part 1, NaCl concentrations in growth medium: ■ (control [TGY broth]), 50 mM; □, 150 mM; ▧, 500 mM. Part 2, NaCl concentrations in adhesion medium: ■, control; □, 50 mM; ▧, 150 mM; ▩, 500 mM. (B) Part 1, $CaCl_2$ concentrations in growth medium: ■ (control [TGY broth]), 1 mM; □, 12.5 mM; ▧, 25 mM. Part 2, $CaCl_2$ concentration in adhesion medium: ■, control; □, 1 mM; ▧, 12.5 mM; ▩, 25 mM. (C) Part 1 $MgCl_2$ concentrations in growth medium: ■ (control [TGY broth]), 1 mM; □, 22.5 mM; ▧, 45 mM. Part 2, $MgCl_2$ concentrations in adhesion medium: ■, control; □, 1 mM; ▧, 22.5 mM; ▩, 45 mM. (D) Treatment of *C. difficile* at pHs 4 to 10 before adhesion assay. Nb, number.

starvation) can change DNA superhelicity, thus increasing transcription of adherence-related genes (13).

Calcium and magnesium. We examined whether concentrations of calcium and magnesium deviating from physiological values could influence *C. difficile* adherence to cells. Variations in the calcium concentration in the growth medium did not influence adherence (Fig. 2B, part 1). On the other hand, adherence was increased 13-fold when the adherence assay was performed in the presence of 12.5 mM $CaCl_2$ and 17-fold when it was performed in the presence of 25 mM $CaCl_2$, compared with the standard condition ($P < 0.05$) (Fig. 2B, part 2). This augmentation could be linked to modification of the net positive charge of the *C. difficile* surface (19); thus, calcium could simply play a nonspecific role in adherence.

Magnesium chloride did not modify adherence at any of the concentrations tested (1, 22.5, and 45 mM), whether present in the growth or adherence medium (Fig. 2C). The absence of an effect due to $MgCl_2$ suggests that the phenomenon observed with other chlorides is solely due to the cation part of the salt.

Acid shock. pH variations in the colon could occur due to alimentary variations, drugs (11), as a result of inflammation (21), or other, as yet unknown, factors. *C. difficile* spores or vegetative forms could therefore encounter acidic or basic pH in the intestine (1). To determine the effect of pH on *C. difficile* adherence, bacteria were washed and resuspended in PBS with pHs ranging from 4 to 10 and incubated for 45 min at 37°C. The low pH of the bacterial suspension was rapidly neutralized during the incubation with Vero cells, excluding the hypothesis that variations of *C. difficile* adherence could be due to modification of Vero cell receptors. Viability of Vero cells after incubation at pH extremes was confirmed with trypan blue dye (Sigma). As shown in Fig. 2D and 3B, maximal adhesion occurred at pH 4 with an 8.3-fold increase ($P < 0.05$) compared with standard conditions (pH 6.8; Fig. 2D). The increase in adherence caused by an acidic pH could result from (i) unmasking of a bacterial cell surface component with adhesin activity (22) or (ii) induction of expression of the adhesin itself.

Adhesins involved in cell attachment. We investigated whether expression of *C. difficile* adhesive proteins is modified under conditions that were found, as described above, to alter

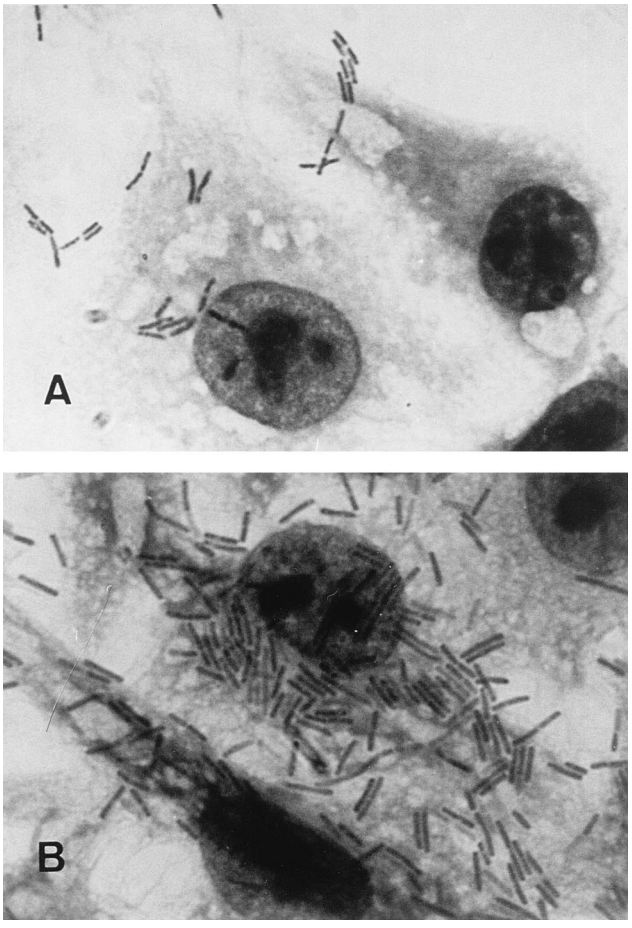

FIG. 3. Patterns of *C. difficile* adherence to Vero cells examined by light microscopy (magnification, ×1,000). Panels: A, control (*C. difficile* treated at pH 7.0 before adhesion assay); B, *C. difficile* treated at pH 4 before adhesion assay.

cell adherence. Extraction of surface proteins and separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described by Wexler et al. (33). Subsequently, proteins were electrically transferred to a polyvinylidene fluoride membrane (Millipore) which was blocked with 5% bovine serum albumin at 37°C for 4 h, washed with PBS, and incubated for 90 min at 37°C under 5% CO_2 with Vero cells metabolically labeled with L-[^{35}S]methionine (Amersham) for 4 h and resuspended in minimum essential medium (Life Technologies) (10^5 cells/cm^2 of membrane). After washing with PBS, protein-bound cells on the blotted membrane were detected by exposure of the membrane to Kodak Biomax photographic film.

Three surface proteins with molecular masses of 70, 50, and 40 kDa able to bind to Vero cells were observed (Fig. 4A and B). The binding of the 70-kDa protein to Vero cells varied little as a function of the environmental conditions, in contrast to that of the 40-kDa protein, whose expression was enhanced by acidic or osmotic shock and iron limitation. Bacteria cultured in NaCl-rich medium also overexpressed the 50-kDa protein (Fig. 4B and C).

We have identified several *C. difficile* genes encoding heat shock proteins (e.g., DnaK and GroEL), some of which may turn out to be identical to the three proteins described here.

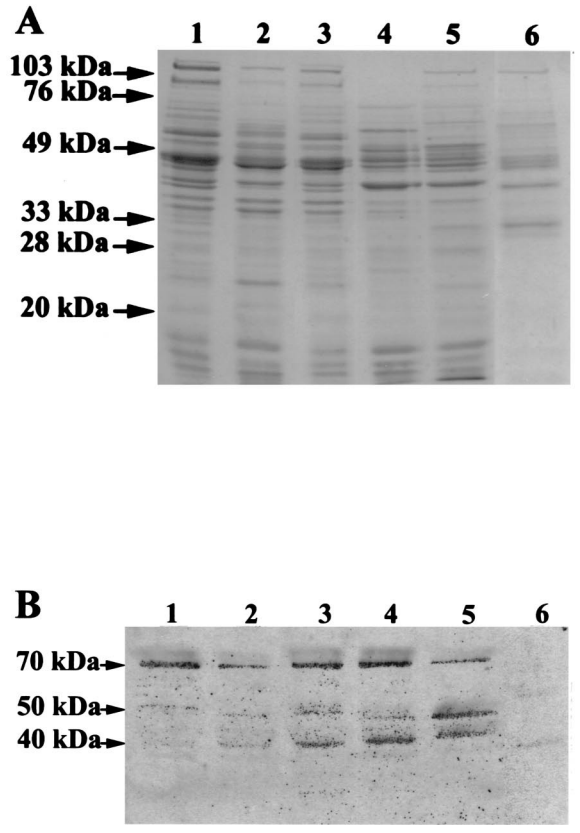

FIG. 4. Adhesins involved in cell attachment. (A) Surface proteins from *C. difficile* grown under various conditions (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). (B) Proteins on membrane which bound Vero cells (autoradiography). (C) Optical density determined by densitometric scanning of autoradiograms for the 70-kDa (■), 50-kDa (□), and 40-kDa (▨) proteins (expressed in comparison with the control). Lanes: 1, non-heat-shocked *C. difficile*; 2, *C. difficile* heat shocked under partial anaerobic conditions; 3, *C. difficile* treated at pH 4; 4, *C. difficile* grown in the presence of 200 μM 2,2′-dipyridyl; 5, *C. difficile* grown in the presence of 500 mM NaCl; 6, negative control (*Clostridium indolis* proteins [nonadherent, normal-flora bacterial strain]).

This work was supported in part by the FAIR Program of the European Union (CT95-0433) and by the ACC-SV6 program of the Ministère de l'Education Nationale, de l'Enseignement Supérieur, et de la Recherche of France.

REFERENCES

1. **Bermudez, L. E., M. Petrofsky, and J. Goodman.** 1997. Exposure to low oxygen tension and increased osmolarity enhance the ability of *Mycobacterium avium* to enter intestinal epithelia (HT-29) cells. Infect. Immun. **65:** 3768–3773.
2. **Borriello, S. P.** 1979. *Clostridium difficile* and its toxins in the gastrointestinal tract in health and disease. Res. Clin. Forums **1:**33–35.
3. **Borriello, S. P.** 1990. Pathogenesis of *Clostridium difficile* infection of the gut. J. Med. Microbiol. **33:**207–215.
4. **Borriello, S. P., H. A. Davies, and F. E. Barclay.** 1988. Detection of fimbriae amongst strains of *Clostridium difficile*. FEMS Microbiol. Lett. **49:**65–67.
5. **Borriello, S. P., A. R. Welch, F. E. Barclay, and M. A. Davies.** 1988. Mucosal association by *Clostridium difficile* in the hamster gastrointestinal tract. J. Med. Microbiol. **25:**191–196.
6. **Cacalano, G., M. Kays, L. Saiman, and A. Prince.** 1992. Production of the *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. J. Clin. Investig. **89:**1866–1874.
7. **Conte, M. P., C. Longhi, V. Buonfiglio, M. Polidoro, and L. Seganti.** 1994. The effect of iron on the invasiveness of *Escherichia coli* carrying the *inv* gene of *Yersinia pseudotuberculosis*. J. Med. Microbiol. **40:**236–240.
8. **Conte, M. P., C. Longhi, M. Polidoro, G. Petrone, V. Buonfiglio, S. di Santo, E. Papi, L. Seganti, P. Visca, and P. Valenti.** 1996. Iron availability affects entry of *Listeria monocytogenes* into the enterocytelike cell line Caco-2. Infect. Immun. **64:**3925–3929.
9. **Davies, H. A., and S. P. Borriello.** 1990. Detection of capsule in strains of *Clostridium difficile*. Microb. Pathog. **9:**141–146.
10. **Delmee, M., M. Homel, and G. Wauters.** 1985. Serogrouping of *Clostridium difficile* strains by slide agglutination. J. Clin. Microbiol. **21:**323–327.
11. **Evans, D. F., G. Pye, R. Bramley, A. G. Clark, T. J. Dyson, and J. D. Hardcastle.** 1988. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut **29:**1035–1041.
12. **Eveillard, M., V. Fourel, M.-C. Barc, S. Keineis, M.-H. Coconier, T. Karjalainen, P. Bourlioux, and A. Servin.** 1993. Identification and characterization of adhesive factors of Clostridium difficile involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secreting HT29 cells in culture. Mol. Microbiol. **7:**371–381.
13. **Galán, J. E., and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:**1879–1885.
14. **George, W. L.** 1984. Antimicrobial agent associated colitis and diarrhea: historical background and clinical aspects. Rev. Infect. Dis. **6:**208–213.
15. **Gomez-Trevino, M., H. Boureau, T. Karjalainen, and P. Bourlioux.** 1996. *Clostridium difficile* adherence to mucus: results of an *in vivo* and *ex vivo* assay. Microb. Ecol. Health Dis. **9:**329–334.
16. **Karjalainen, T., M.-C. Barc, A. Collignon, S. Trollé, H. Boureau, J. Cotte-Laffitte, and P. Bourlioux.** 1994. Cloning of a genetic determinant from *Clostridium difficile* involved in adherence to tissue culture cells and mucus. Infect. Immun. **62:**4347–4355.
17. **Karjalainen, T., A. Collignon, M.-C. Barc, and P. Bourlioux.** 1995. Molecular cloning of the collagenase gene of *Clostridium difficile*, p. 407–412. *In* P. I. Duerden et al. (ed.), Medical and environmental aspects of anaerobes. Science Reviews, Northwood, United Kingdom.
18. **Karjalainen, T., I. Poilane, A. Collignon, M.-C. Barc, M. Gomez-Trevino, H. Boureau, and P. Bourlioux.** 1995. *Clostridium difficile* virulence: correlation between toxigenicity, adherence, enzyme production and serogroup. Microecol. Ther. **25:**157–163.
19. **Krishna, M. M., N. B. L. Powell, and S. P. Borriello.** 1996. Cell surface properties of *Clostridium difficile*: haemagglutination, relative hydrophobicity and charge. J. Med. Microbiol. **44:**115–123.
20. **Kunin, C. M., T. H. Hua, R. L. Guerrant, and L. O. Bakaletz.** 1994. Effect of salicylate, bismuth, osmolytes, and tetracycline resistance on expression of fimbriae by *Escherichia coli*. Infect. Immun. **62:**2178–2186.
21. **Lee, J.-Y., and M. Caparon.** 1996. An oxygen-induced but protein F-independent fibronectin-binding pathway in *Streptococcus pyogenes*. Infect. Immun. **64:**413–421.
22. **Lee, S. G., C. Kim, and Y. C. Ha.** 1997. Successful cultivation of a potentially pathogenic coccoid organism with trophism for gastric mucin. Infect. Immun. **65:**49–54.
23. **Mai, G. T., J. G. McCormack, W. K. Seow, G. B. Pier, L. A. Jackson, and Y. H. Thong.** 1993. Inhibition of adherence of mucoid *Pseudomonas aeruginosa* by alginase, specific monoclonal antibodies, and antibiotics. Infect. Immun. **61:**4338–4343.
24. **Riegler, M., R. Sedivy, C. Pothoulakis, G. Hamilton, J. Zacherl, G. Bischof, E. Cosentini, W. Feil, R. Schiessel, J. T. LaMont, et al.** 1995. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. J. Clin. Investig. **95:**2004–2011.
25. **Saiman, L., G. Cacalano, D. Gruenert, and A. Prince.** 1992. Comparison of adherence of *Pseudomonas aeruginosa* to respiratory epithelial cells from cystic fibrosis patients and healthy subjects. Infect. Immun. **60:**2808–2814.
26. **Saiman, L., K. Ishimoto, S. Lory, and A. Prince.** 1990. The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. J. Infect. Dis. **161:**541–548.
27. **Sauvage, M., N. Eloumi, M. L. Capmau, and C. Hulen.** 1991. Inhibition de la biosynthèse des alginates chez les souches mucoides de *Pseudomonas aeruginosa*. Pathol. Biol. **39:**606–612.
28. **Seddon, S. V., and S. P. Borriello.** 1992. Proteolytic activity of *Clostridium difficile*. J. Med. Microbiol. **36:**307–311.
29. **Seddon, S. V., I. Hemingway, and S. P. Borriello.** 1990. Hydrolytic enzyme production by *Clostridium difficile* and its relationship to toxin production and virulence in the hamster model. J. Med. Microbiol. **31:**169–174.
30. **Tamura, G. S., J. M. Kuypers, S. Smith, H. Raff, and C. E. Rubens.** 1994. Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. Infect. Immun. **62:**2450–2458.
31. **Tartera, C., and E. S. Metcalf.** 1993. Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherence to and invasion of human intestinal cells. Infect. Immun. **61:**3084–3089.
32. **Tasteyre, A., M.-C. Barc, T. Karjalainen, P. Dodson, S. Hyde, N. Powell, S. Bulteau, P. Bourlioux, and P. Borriello.** Identification, characterization, and expression of a gene encoding flagellin of *Clostridium difficile*. Submitted for publication.
33. **Wexler, H., M. E. Mulligan, and S. M. Finegold.** 1984. Polyacrylamide gel electrophoresis patterns produced by *Clostridium difficile*. Rev. Infect. Dis. **6:**S229–S234.
34. **Wood-Helie, S. J., H. P. Dalton, and S. Shadomy.** 1986. Hydrophobic and adherence properties of *Clostridium difficile*. Eur. J. Clin. Microbiol. **5:**441–445.