APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2009, p. 1796–1799 Vol. 75, No. 6
0099-2240/09/$08.00+0 doi:10.1128/AEM.02232-08

Comparative Analysis of Attachment of Shiga-Toxigenic *Escherichia coli* and *Salmonella* Strains to Cultured HT-29 and Caco-2 Cell Lines[▽]

Glen E. Mellor,[1] Rebecca M. Goulter,[1,2] T. W. Raymond Chia,[1,3] and Gary A. Dykes[1]*

Food Science Australia, Brisbane, Queensland, Australia[1]; School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Queensland, Australia[2]; and School of Agricultural Science, University of Tasmania, Hobart, Tasmania, Australia[3]

Received 28 September 2008/Accepted 12 January 2009

The ability of *Escherichia coli* and *Salmonella* isolates to attach to Caco-2 and HT-29 cell monolayers was measured. All isolates displayed a greater ability to attach to Caco-2 cells than HT-29 cells, and overall *E. coli* isolates attached better to both cell lines than *Salmonella* isolates. Bacteria that were considered to be pathogenic displayed no greater ability to attach to cell lines than those that were not considered to be pathogenic. Additionally, no correlation was found between cell line attachment and previously determined hydrophobicity results.

Cultured intestinal cell lines are often used in attachment assays as indicators of the pathogenic potential of bacteria (9, 15). The ability of bacteria to attach to the intestinal epithelium may help explain the differences in pathogenicity among strains (4). It has also been suggested that bacterial physicochemical properties, such as cellular surface charge and hydrophobicity, can influence bacterial attachment to surfaces, including human intestinal cell lines (7, 12, 20).

The usefulness of attachment assays for food-borne bacterial pathogens and the influence of physicochemical properties on attachment are not always clear from the literature. Previous studies investigating the attachment of bacteria to cell lines have used either a small number of isolates (15) or several strains from a number of different species (9). This study was undertaken to investigate how a large number of closely related bacterial isolates with different physicochemical properties, encompassing those commonly associated with human disease (e.g., *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Virchow, *S. enterica* serovar Infantis, and Shiga-toxigenic *Escherichia coli* [STEC] serotype O157) and those that are not (e.g., *S. enterica* serovar Sofia, and non-STEC) differ in the degree of attachment to intestinal cell lines.

Twenty strains of *E. coli* previously used in other studies (18) investigating attachment to stainless steel (Table 1), along with 25 *Salmonella* strains previously used in other studies (T. W. R. Chia, R. M. Goulter, T. McMeekin, G. A. Dykes, and N. Fegan, submitted for publication) investigating attachment to stainless steel, glass, nitrile butyl-rubber, polyurethane, and Teflon (Table 1), were selected for the cell attachment assay. Isolates were cultured on tryptic yeast soya glucose agar (TYSG) from Protect beads (Technical Service Consultants, Lancashire, United Kingdom) and stored at 4°C. Cultures were grown in 10 ml tryptic soya broth (TSB; Oxoid, Basingstoke, United Kingdom) and incubated at 37°C under aerobic conditions for 18 ± 2 h. A 1-ml aliquot of each overnight culture was added to 9 ml of phosphate-buffered saline (PBS; 2.67 mM KCl, 137 mM NaCl, 8.1 mM Na_2HPO_4, 0.74 mM KH_2PO_4, pH 7.4) centrifuged at 4,500 × *g* for 10 min, and washed once with 10 ml PBS. In order to achieve a bacterial suspension of ~1.5×10^8 cells/ml, the pellet was resuspended in Dulbecco's modified minimal essential medium (DMEM; 1× high glucose) containing 25 mM D-glucose, 146 mM L-glutamine, and 110 mM sodium pyruvate (Gibco, Invitrogen, CA).

The HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-37) intestinal cell lines were used for the attachment assays. Cells were maintained at 37°C in 5% CO_2 and 95% air in T-75 flasks (Sarstedt, Nümbrecht, Germany) containing 10 ml of complete growth media for each of the HT-29 and Caco-2 cell lines. For HT-29 cells, this comprised DMEM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Invitrogen) and antibiotics to a final concentration of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). For Caco-2 cells, this comprised DMEM supplemented with 20% (vol/vol) FBS, 1% (vol/vol) nonessential amino acids, and antibiotics to final concentrations of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Medium was changed every second day, and cells were subcultured using a 1:6 split ratio every 2 to 3 days. At ~80% confluence, cells were split by adding trypsin-EDTA solution (Invitrogen) (0.25% [wt/vol] trypsin–1 mM EDTA) and counted in a Neubauer counting chamber before seeding at a concentration of 1×10^5 cells/well in 24-well tissue culture plates (Sarstedt) containing 2 ml of complete growth medium/well. Plates were incubated at 37°C in 5% CO_2 and 95% air. Culture medium was changed on alternate days until cells reached confluence. At this stage, complete growth medium minus antibiotics was added and the assay was performed the following day.

Prior to the attachment assay, HT-29 and Caco-2 cell monolayers in each well were washed once with 2 ml DMEM to remove residual culture medium. Aliquots of 2 ml of bacterial suspensions were added to the tissue culture wells and incubated for 1 h at 37°C in 5% CO_2 and 95% air. Following incubation, the unattached bacteria were removed by washing

* Corresponding author. Mailing address: Food Science Australia, P.O. Box 3312, Tingalpa DC, Queensland 4173, Australia. Phone: 61 7 3214 2037. Fax: 61 7 3214 2150. E-mail: gary.dykes@csiro.au.

▽ Published ahead of print on 23 January 2009.

TABLE 1. *S. enterica* and *E. coli* isolate numbers, serovars or serotypes, and sources

Isolate no.	Serovar or serotype	Source
S. enterica		
ATCC[a] 14028	Typhimurium	Control
S1380a	Typhimurium	Chicken rinse
S1567a	Typhimurium	Chicken rinse
S1672	Typhimurium	Chicken
S1673	Typhimurium	Chicken
S1674	Typhimurium	Chicken
S1297a	Virchow	Chicken rinse
S1680	Virchow	Chicken
S1457b	Infantis	Chicken rinse
S1677	Infantis	Chicken
S1679	Infantis	Chicken
S1296a	Sofia	Chicken rinse
S1309a	Sofia	Chicken rinse
S1540b	Sofia	Chicken rinse
S1628	Sofia	Chicken
S1629	Sofia	Chicken litter
S1630	Sofia	Chicken
S1634	Sofia	Chicken meat
S1635	Sofia	Human
S1636[f]	Sofia	Lab generated
S1637	Sofia	Human
S1638	Sofia	Chicken
S1640	Sofia	Chicken
S1663	Sofia	Chicken
S1667	Sofia	Chicken
E. coli		
EC1812[b]	O157:H7	Human bloody diarrhea[c]
EC1820[b]	O157:H7	Human death[c]
EC1596[b]	O157:H7	EDL933[c,d]
EC119[b]	O157:H7	Beef carcass
EC200[b]	O157:H7	Beef carcass
EC514[b]	O157:H7	Boneless beef
EC623[b]	O157:H7	Beef cattle hide
EC1814[b]	O157:H−	Human bloody diarrhea[c]
EC183[b]	O157:H−	Sheep feces
EC329[b]	O157:H−	Beef cattle feces
EC516[b]	O157:H−	Calf feces
EC26[b]	O111:H−	Calf feces
EC473[b]	O26:H111	Dairy cattle feces
EC2141[b]	O91:H21	Cattle feces
EC2170[b]	O165:H25	Cattle feces
EC2177[b]	O113:H21	Cattle feces
EC2214[b]	O174:H21	Cattle feces
EC1598	O13rel:H4[e]	Unknown
EC1858	O1:H7	ATCC[a] 11775
EC614	O157:HR[g]	Beef cattle feces

[a] ATCC, American Type Culture Collection.
[b] Contains at least one of the stx_1, stx_2, *eae*, or *ehxA* genes as previously reported by Rivas et al. (19).
[c] Strains received from R. Robins-Browne, University of Melbourne, Australia.
[d] Strain implicated in outbreak.
[e] Isolate is related (rel) to the O13 serotype and produced a weak titer with the O13 serotype only.
[f] Mutant strain of S1635.
[g] H antigen is rough.

TABLE 2. *S. enterica* and *E. coli* attachment per 100 Caco-2 or HT-29 intestinal cells

Isolate no.	Mean (SD) attachment (CFU/100 cells) of isolate to:	
	Caco-2	HT-29
S. enterica		
ATCC[a] 14028	1,174 (307)	182 (89)
S1380a	764 (50)	43 (5)
S1567a	547 (158)	32 (2)
S1672	628 (231)	33 (6)
S1673	588 (93)	20 (1)
S1674	448 (136)	17 (7)
S1297a	826 (145)	36 (19)
S1680	1,002 (41)	37 (16)
S1457b	661 (49)	33 (4)
S1677	1,381 (159)	25 (7)
S1679	762 (221)	25 (5)
S1296a	660 (77)	47 (12)
S1309a	1,079 (506)	83 (77)
S1540b	915 (223)	42 (20)
S1628	668 (147)	45 (37)
S1629	1,487 (814)	49 (21)
S1630	805 (171)	36 (8)
S1634	591 (114)	36 (8)
S1635	991 (325)	38 (28)
S1636[f]	992 (243)	33 (12)
S1637	828 (64)	35 (7)
S1638	1,045 (113)	36 (7)
S1640	1,301 (771)	36 (17)
S1663	632 (317)	62 (38)
S1667	647 (124)	40 (19)
E. coli		
EC1812[b,c]	1,739 (762)	218 (69)
EC1820[b,c]	1,229 (578)	266 (101)
EC1596[b,c,d]	1,472 (244)	257 (71)
EC119[b]	1,131 (386)	327 (212)
EC200[b]	888 (350)	151 (84)
EC514[b]	874 (115)	140 (60)
EC623[b]	1,988 (437)	253 (93)
EC1814[b]	1,821 (565)	240 (123)
EC183[b]	1,911 (1,147)	277 (131)
EC329[b]	864 (146)	272 (149)
EC516[b]	1,595 (357)	401 (252)
EC26[b]	1,276 (277)	242 (172)
EC473[b]	923 (521)	289 (116)
EC2141[b]	2,015 (755)	315 (115)
EC2170[b]	1,143 (152)	296 (230)
EC2177[b]	1,185 (272)	147 (136)
EC2214[b]	863 (219)	258 (137)
EC1598[e]	817 (32)	142 (82)
EC1858[a]	1,927 (691)	181 (104)
EC614[g]	3,336 (479)	620 (158)

[a] ATCC, American Type Culture Collection.
[b] Contains at least one of the stx_1, stx_2, *eae*, or *ehxA* genes as previously reported by Rivas et al. (19).
[c] Strains received from R. Robins-Browne, University of Melbourne, Australia.
[d] Strain implicated in outbreak.
[e] Isolate is related (rel) to the O13 serotype and produced a weak titer with the O13 serotype only.
[f] Mutant strain of S1635.
[g] H antigen is rough.

the monolayers with 2 ml of sterile PBS four times. Attached cell lines and bacteria were then detached from the wells and cell lines, respectively, by incubating the monolayer in 200 μl trypsin-EDTA per well for 10 min at 37°C followed by vigorous pipetting. Following detachment, serial 10-fold dilutions were prepared using 0.85% saline and 100-μl aliquots were plated onto TYSG and incubated at 37°C for 18 h in air. Spread plates were counted to quantify bacteria before and after attachment. As shown in Table 2, the attachment results are presented as CFU/100 intestinal cells, as obtained by dividing the final count of detached bacteria by the average intestinal cell count/well (determined using a Neubauer chamber). The assay was per-

formed in triplicate with independently grown bacterial cultures.

Statistical analysis of results was performed with MINI TAB15 software (Minitab Inc., Minneapolis, MN) using analysis of variance and Tukey's method at a 95% confidence level. Microsoft Excel (Microsoft Corporation, Redmond, WA) was used to determine R^2 values for correlating physicochemical results with cell line attachment results.

The average of relative standard deviations (SDs) for Caco-2 cell attachment (26.10%) was lower than that for HT-29 attachment (42.89%), suggesting, in this instance, that Caco-2 attachment assays have a lower level of variability. The mean of triplicate values for *E. coli* and *Salmonella* attachment numbers was determined and expressed as CFU/100 cells for each cell line. *Salmonella* and *E. coli* cell attachment to Caco-2 cells was significantly greater than their respective mean attachment to HT-29 cells ($P \leq 0.05$). The mean attachment of *E. coli* strains was greater for Caco-2 cells (1,449.7 bacteria/100 Caco-2 cells) and HT-29 cells (264.6 bacteria/100 HT-29 cells) than the mean attachment of *Salmonella* cells (856.89/100 and 44.04/100, respectively). Variation between individual triplicate counts was relatively high, making it difficult to identify significant differences between isolates of the same genus. Reported variations in SDs of up to 50% of the mean are not uncommon in the literature (1, 8, 13). This finding is not unexpected as assays that involve two independent biological systems would be naturally more variable than those that involve one. In addition, individual strains showing high variability are more likely to occur in our study due to the large number of strains. Another issue that may have influenced the variability observed in our study is that previous attachment studies have used low incubation temperatures (4°C) to prevent bacterial invasion of Caco-2 cells during attachment (2), while others have used 37°C (1, 10). An incubation temperature of 37°C was used for this study, being more representative of bacterial attachment in a human system than 4°C. Consequently, the present study did not account for those bacterial cells which had invaded, as intestinal cells were not lysed before bacterial enumeration, a feature which may vary between strains and result in greater variability. While the use of trypsin-EDTA in detaching bacteria from cell lines may not be as effective as methods that lyse cells totally, it is nonetheless an accepted and widely used method (10, 13, 17). In addition, a comparative adhesion study by Le Blay et al. (10) demonstrated little difference between plate count enumeration (using trypsin-EDTA), enzyme-linked immunosorbent assay, and radiolabeling in detecting adherent bacteria.

In spite of the limitation produced by the variability discussed above, some clear and statistically significant conclusions could be drawn and the assay regarded as useful. Specifically, it was apparent that EC614 (a sorbitol-fermenting, Shiga toxin-negative *E. coli* O157:HR strain) attached to Caco-2 cells at significantly higher levels than all other *E. coli* isolates, except EC623, -1814, -183, -2141, and -1858 ($P \leq 0.05$), and it's attachment to HT-29 cells was notably greater than other *E. coli* isolates. In the HT-29 assays, *S. enterica* serovar Typhimurium ATCC 14028 attached at significantly higher levels than all other *Salmonella* isolates ($P \leq 0.05$), although no significant difference ($P \geq 0.05$) was seen between *Salmonella* isolates in the Caco-2 assays.

Hydrophobicity is suggested to play a role in attachment of bacteria to surfaces (21); however, evidence for correlation between hydrophobicity and attachment is contradictory (11, 14, 16). Attachment data from this study were compared with previously reported physiochemical and attachment data from the same isolates. No correlation was observed between the results of this study and hydrophobicity results obtained using hydrophobic interaction chromatography (R^2 = 0.0004 to 0.0089), contact angle measurement (R^2 = 0.0158 to 0.0347), bacterial attachment to hydrocarbons (for hexadecane, R^2 = 0.0055 to 0.0839; for xylene, R^2 = 0.0021 to 0.0769), or previously described stainless steel attachment results (R^2 = 0.000006 to 0.1630) (3, 18, 19; Chia et al., submitted). It should be noted in these previous studies no direct correlation was observed between attachment of *E. coli* to stainless steel and cell surface hydrophobicity (18). While at a strain level, hydrophobicity did not appear to influence bacterial adhesion capacity, when analyzed at a species level, the mean values for hydrophobicity measurements (using bacterial attachment to hydrocarbons for hexadecane and hydrophobic interaction chromatograpy) showed *E. coli* to be significantly more hydrophobic than *Salmonella* ($P \leq 0.05$), which may be linked to the greater attachment of the former species to the cell lines.

Previous studies have shown that *Salmonella* attachment to stainless steel was greater than that of *E. coli* (Chia et al., submitted), a finding which is inconsistent with the cell line attachment data demonstrating *E. coli* attachment to cell lines is greater than that of *Salmonella* ($P \leq 0.05$). *E. coli* O157:H7 has, however, been shown to attach to bovine primary cell lines significantly better than *S. enterica* serovar Typhimurium (5).

Isolates commonly associated with human disease did not appear to attach to cell lines in greater numbers than those that were not. According to Harington et al. (6), *Salmonella enterica* subsp. II serovars (such as *S. enterica* serovar Sofia) may be considered to have low virulence for humans in comparison to *Salmonella enterica* subsp. I serovars. There was no obvious difference in bacterial attachment between subspecies I serovars (*S. enterica* serovar Typhimurium, *S. enterica* serovar Infantis, and *S. enterica* serovar Virchow) and subspecies II serovars (*S. enterica* serovar Sofia). Additionally, STEC strains carrying combinations of virulent marker genes (stx_1, stx_2, *eae*, and *ehxA*) did not attach better than non-STEC strains (lacking virulence marker genes).

Caco-2 cell lines grow and differentiate differently from HT-29 cell lines. While the cells used in our assays were not grown for long enough to allow significant and obvious differentiation, the expression of various levels of differentiation-related features could not be ruled out and may be responsible for the dissimilar levels of bacterial attachment observed in this study.

In summary, this study found that Caco-2 and HT-29 cells appear to differ as a model for measuring bacterial attachment to human intestinal cells, with *E. coli* and *Salmonella* demonstrating a superior ability to colonize confluent Caco-2 cells. The study provides evidence to support the lack of correlation between hydrophobicity and bacterial attachment to cultured cell lines and shows isolates considered pathogenic to humans do not display a greater ability to attach to either cell line than those that are not.

We thank Roy Robins-Brownes, Department of Microbiology and Immunology, University of Melbourne, for supplying O157:H7 strains from his collection. We thank M. W. Heuzenroeder and I. L. Ross, Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, South Australia, for supplying *S.* Sofia isolates S1628, S1629, S1630, S1634, S1635, S1636, S1637, S1638, and S1640 and J. Bates, Queensland Health, for donating *Salmonella* isolates S1672, S1673, S1674, S1677, S1679, S1680, S1663, and S1667.

REFERENCES

1. **Candela, M., G. Seibold, B. Vitali, S. Lachenmaier, B. J. Eikmanns, and P. Brigidi.** 2005. Real-time PCR quantification of bacterial adhesion to Caco-2 cells: competition between bifidobacteria and enteropathogens. Res. Microbiol. **156:**887–895.
2. **Chessa, D., M. G. Winter, S. P. Nuccio, C. Tukel, and A. J. Baumler.** 2008. RosE represses Std fimbrial expression in *Salmonella enterica* serotype Typhimurium. Mol. Microbiol. **68:**573–587.
3. **Chia, T. W. R., N. Fegan, T. A. McMeekin, and G. A. Dykes.** 2008. *Salmonella* Sofia differs from other poultry-associated *Salmonella* serovars with respect to cell surface hydrophobicity J. Food Prot. **71:**2421–2428.
4. **de Luna, M. G., A. Scott-Tucker, M. Desvaux, P. Ferguson, N. P. Morin, E. G. Dudley, S. Turner, J. P. Nataro, P. Owen, and I. R. Henderson.** 2008. The *Escherichia coli* biofilm-promoting protein antigen 43 does not contribute to intestinal colonization. FEMS Microbiol. Lett. **284:**237–246.
5. **Dibb-Fuller, M. P., A. Best, D. A. Stagg, W. A. Cooley, and M. J. Woodward.** 2001. An in-vitro model for studying the interaction of *Escherichia coli* O157:H7 and other enteropathogens with bovine primary cell cultures. J. Med. Microbiol. **50:**759–769.
6. **Harrington, C. S., J. A. Lanser, P. A. Manning, and C. J. Murray.** 1991. Epidemiology of *Salmonella sofia* in Australia. Appl. Environ. Microbiol. **57:**223–227.
7. **Jacobson, S. H., K. Tullus, and A. Brauner.** 1989. Hydrophobic properties of *Escherichia coli* causing acute pyelonephritis. J. Infect. **19:**17–23.
8. **Jankowska, A., D. Laubitz, H. Antushevich, R. Zabielski, and E. Grzesiuk.** 2008. Competition of *Lactobacillus paracasei* with *Salmonella enterica* for adhesion to Caco-2 cells. J. Biomed. Biotechnol. **2008:**357964.
9. **Kim, S. H., and C. I. Wei.** 2007. Invasiveness and intracellular growth of multidrug-resistant *Salmonella* and other pathogens in Caco-2 cells. J. Food Sci. **72:**M72–M78.
10. **Le Blay, G. L., I. Fliss, and C. Lacroix.** 2004. Comparative detection of bacterial adhesion to Caco-2 cells with ELISA, radioactivity and plate count methods. J. Microbiol. Methods **59:**211–221.
11. **Li, J., and L. A. McLandsborough.** 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. Int. J. Food Microbiol. **53:**185–193.
12. **Liu, Y., S. F. Yang, Y. Li, H. Xu, L. Qin, and J. H. Tay.** 2004. The influence of cell and substratum surface hydrophobicities on microbial attachment. J. Biotechnol. **110:**251–256.
13. **Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth.** 1999. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. Am. J. Physiol. **276:**G941–G950.
14. **Marin, M. L., Y. Benito, C. Pin, M. F. Fernandez, M. L. Garcia, M. D. Selgas, and C. Casas.** 1997. Lactic acid bacteria: hydrophobicity and strength of attachment to meat surfaces. Lett. Appl. Microbiol. **24:**14–18.
15. **Musken, A., M. Bielaszewska, L. Greune, C. H. Schweppe, J. Muthing, H. Schmidt, M. A. Schmidt, H. Karch, and W. Zhang.** 2008. Anaerobic conditions promote expression of Sfp fimbriae and adherence of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:NM to human intestinal epithelial cells. Appl. Environ. Microbiol. **74:**1087–1093.
16. **Pan, W. H., P. L. Li, and Z. Y. Liu.** 2006. The correlation between surface hydrophobicity and adherence of *Bifidobacterium* strains from centenarians' faeces. Anaerobe **12:**148–152.
17. **Rhoades, J. R., G. R. Gibson, K. Formentin, M. Beer, N. Greenberg, and R. A. Rastall.** 2005. Caseinoglycomacropeptide inhibits adhesion of pathogenic *Escherichia coli* strains to human cells in culture. J. Dairy Sci. **88:**3455–3459.
18. **Rivas, L., N. Fegan, and G. A. Dykes.** 2007. Attachment of Shiga toxigenic *Escherichia coli* to stainless steel. Int. J. Food Microbiol. **115:**89–94.
19. **Rivas, L., N. Fegan, and G. A. Dykes.** 2005. Physicochemical properties of Shiga toxigenic *Escherichia coli*. J. Appl. Microbiol. **99:**716–727.
20. **Sinde, E., and J. Carballo.** 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microbiol. **17:**439–447.
21. **van Loosdrecht, M. C. M., J. Lyklema, W. Norde, G. Schraa, and A. J. B. Zehnder.** 1987. Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. Appl. Environ. Microbiol. **53:**1898–1901.