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Aggregation Substance Increases Adherence and Internalization, but Not Translocation, of *Enterococcus faecalis* through Different Intestinal Epithelial Cells In Vitro

S. SARTINGEN,[1] E. ROZDZINSKI,[1] A. MUSCHOLL-SILBERHORN,[2] AND R. MARRE[1]*

Department of Medical Microbiology and Hygiene, University Hospital of Ulm, 89081 Ulm,[1] and NWFIII-Microbiology, University of Regensburg, 93053 Regensburg,[2] Germany

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The aggregation substance of *Enterococcus faecalis* increased bacterial adherence to and internalization by epithelial cells originating from the colon and duodenum but not by cells derived from the ileum. However, enterococcal translocation through monolayers of intestinal epithelium was not observed.

Enterococcus faecalis, a gram-positive facultative anaerobic bacterium, belongs to the normal flora of the intestinal tract and is also found in the vaginal vault and the oral cavity. Enterococci have increasingly gained attention as pathogens, since they have become the fourth leading cause of nosocomial infections in the United States (8). *E. faecalis* frequently causes local or systemic infections, such as urinary tract and abdominal infections, wound infections, bacteremia, and endocarditis (15). It is assumed that many enterococcal infections are endogenous, resulting from bacterial translocation from the intestinal lumen to extraintestinal sites (16, 31).

Surprisingly little is known about the virulence factors of *E. faecalis*. One of the potential virulence factors is the aggregation substance (AS), an adhesin encoded by inducible sex pheromone plasmids (for a review, see reference 5). The AS, a signal peptide-containing protein, appears as a hair-like structure on the cell surface and is incorporated primarily into the "old" parts of the cell wall (30). It has been demonstrated that this adhesin is responsible for bacterium-bacterium contact during conjugative transfer of sex pheromone plasmids (5).

The AS contains two Arg-Gly-Asp motifs which are known to be recognized by integrins, a family of eukaryotic cell surface receptors (24). Since integrins have been reported to be expressed on intestinal epithelial cells (2), we hypothesized that the AS promotes adherence and possibly invasion into these cells, thereby enabling enterococci to translocate through the intestinal epithelial barrier.

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Three different *E. faecalis* strains (Table 1), kindly provided by R. Wirth (Institute of Microbiology, University of Regensburg, Regensburg, Germany), were used to study the effect of the AS on adherence, internalization, and translocation through intestinal epithelial cells. The bacteria were cultured in Todd-Hewitt medium (Difco, Augsburg, Germany) supplemented with erythromycin (20 μg/ml) for growth of the plasmid-containing strains (OG1X/pAM721 and OG1X/pAM944). Since *E. faecalis* strains expressing the AS spontaneously clump, all inocula were sonicated (Branson W-450 Sonifier; 80 W, 20 s, continuously) to obtain single-cell suspensions. *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Escherichia coli* HB101 grown in Luria-Bertani medium were used as positive and negative controls, respectively. For the experiments described below, the bacteria were harvested in mid-log phase and suspended in tissue culture medium without supplements, which served as the infection medium. Appropriate dilutions of the suspensions were plated on nutrient agar in order to determine the CFU per milliliter of infection medium.

Intestinal epithelial cell lines originating from the colon (HT 29 and T84), ileum (HCT-8), and duodenum (Hutu 80) were used at passages below 10 from a stock culture purchased from the American Type Culture Collection (Manassas, Va.). Hutu 80 cells were cultured in minimum essential Eagle medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 2 mM L-glutamine; HCT-8 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 mM sodium pyruvate; HT 29 cells were cultured in McCoy's 5a medium containing 10% fetal calf serum and 2 mM L-glutamine; and T64 cells were grown in a 1:1 mixture of Nutrient Mixture Ham's F12 medium and Dulbecco's modified Eagle medium containing 10% fetal calf serum and 2.5 mM L-glutamine. In order to select for HT 29 cells which expressed signs of structural and functional differentiation and polarization, including, e.g., apical microvilli, tight junctions, and enzyme expression, HT 29 cells were grown in glucose-free Dulbecco's modified Eagle medium supplemented with 15% fetal calf serum (dialyzed against 0.15 M NaCl). 4 mM L-glutamine, and 5 mM galactose (10, 21). These cells, which were termed HT 29/1, were used at postconfluence, after 18 to 24 days of incubation. Using transmission electron microscopy and biochemical assays, we could show that these mature HT 29/1 enterocytes had characteristics of polarized cells (data not shown). T84 cells are able to differentiate spontaneously and resemble adult colonic crypt cells. However, they do not form well-developed brush borders (6).

Unless otherwise stated, all tissue culture reagents (endotoxin-free media) were purchased from Sigma (Deisenhofen, Germany). The epithelial cell lines were tested continuously for the presence of mycoplasmas by 4′, 6′-diamidino-2-phenylindole (DAPI) (Roth, Karesruhe, Germany) staining (25) or by PCR (Stratagene, Amsterdam Zuidoost, The Netherlands). For in vitro assays, 6×10^4 intestinal epithelial cells/cm^2 were seeded into 24-well tissue culture trays (Nunc GmbH, Wiesbaden, Germany) and were grown for several days. Wells with confluent monolayers were shown to contain

* Corresponding author. Mailing address: Department of Medical Microbiology and Hygiene, University Hospital of Ulm, Robert Koch Straße 8, D-89081 Ulm/Donau, Germany. Phone: 49-731-50-24600. Fax: 49-731-50-24619. E-mail: reinhard.marre@medizin.uni-ulm.de.

TABLE 1. *E. faecalis* strains

Strain	Relevant characteristic	Reference
OG1X	Plasmid free	13
OG1X/pAM721	pAD1 derivative containing Tn*917* insertion within regulatory region of *asa-1* resulting in constitutive expression of AS	12
OG1X/pAM944	pAD1 derivative containing Tn*917* insertion in *asa-1* resulting in secretion of truncated AS	7, 13

1×10^6 to 2×10^6 cells/well. Cell viability was ≥95%, as determined by trypan blue staining (1).

To investigate whether the AS promotes adherence to intestinal epithelium, binding of the AS-positive *E. faecalis* strain (OG1X/pAM721) was compared with that of the AS-negative strain (OG1X) using a published protocol (20) with minor modifications. A bacterial suspension of 10^8 CFU/ml of infection medium was added to each tissue culture well, yielding a multiplicity of infection of 100:1. After incubation for 1 h at 37°C in 5% CO_2, the cells were washed four times with Hanks' balanced salt solution (HBSS) to remove nonadherent bacteria and were lysed by the addition of 1 ml of 1% Triton X-100 for 5 min. The total number of adherent bacteria was determined by plating aliquots of cell lysates on appropriate nutrient agar. The data are expressed as means and standard deviations for six wells and are representative of at least four independent assays.

The *E. faecalis* strain OG1X/pAM721, which constitutively expressed Asa1, bound to HT 29, HT 29/1, T84, and Hutu 80 cells significantly better (percentages attached: 8.4 ± 0.8, 2.7 ± 0.6, 8.8 ± 0.6, and 11.2 ± 0.9, respectively) than strain OG1X, lacking AS (percentages attached: 2.9 ± 0.6, 1.3 ± 0.4, 2.8 ± 0.7, and 2.7 ± 0.6, respectively) ($P < 0.001$) (Fig. 1). In contrast, the adherence of *E. faecalis* to cells originating from the ileum (HCT-8) was independent of AS. These results confirm and extend the observations of Olmsted et al. (19), who reported that the AS of *E. faecalis* contributes to adherence to HT 29 cells, and of our own group, who showed for the first time that the AS is involved in the attachment of *E. faecalis* to eukaryotic cells (17). It may thus be postulated that the AS mediates adherence to a wide variety but not to all epithelial cell types. The reason why AS does not play a role in attachment to the ileum-derived cell line HCT-8 is not clear.

It might be argued that factors other than the AS affected adherence, since the defective regulator of pAM721 could derepress additional genes which might code for attachment molecules. However, in another study (27), we showed that the adherence of OG1X/pAM721 (AS constitutively expressed) and the adherence of OG1X/pAM944/pWHH6 (AS not constitutively expressed) were similar, while deletions in the AS gene resulted in a partial loss of attachment to macrophages; these results suggested that it is not the derepressed regulatory gene but the AS which contributes to the interaction between *E. faecalis* and eukaryotic cells. That study also revealed that the N-terminal RGD motif and the adjacent N-terminal region were essential for binding to macrophages.

In order to investigate the influence of the AS on the internalization of *E. faecalis* by enterocytes, confluent epithelial cell monolayers were infected with bacterial suspensions of strains OG1X and OG1X/pAM721 containing approximately 2×10^8 CFU/ml. After 2 h of incubation at 37°C in 5% CO_2, the enterocytes were washed eight times with HBSS to remove nonadherent bacteria. Gentamicin sulfate (10 μg/ml) and penicillin G (100 μg/ml) were added to the infection medium to eliminate viable extracellular bacteria (19, 31). *E. coli* HB101 (negative control) and *Salmonella* serovar Typhimurium (positive control) were killed by gentamicin sulfate (100 μg/ml) only. After an additional 2.5 h of incubation, enterocytes were washed 12 times with HBSS and lysed for 5 min with 1% Triton X-100. Intracellular viable bacteria were quantified by plating aliquots of serial dilutions on appropriate nutrient agar. Each bacterial strain was tested in at least four independent assays with six wells each and performed on different days.

Both *E. faecalis* strains invaded all tested cell lines at a substantially higher level than the noninvasive strain *E. coli*

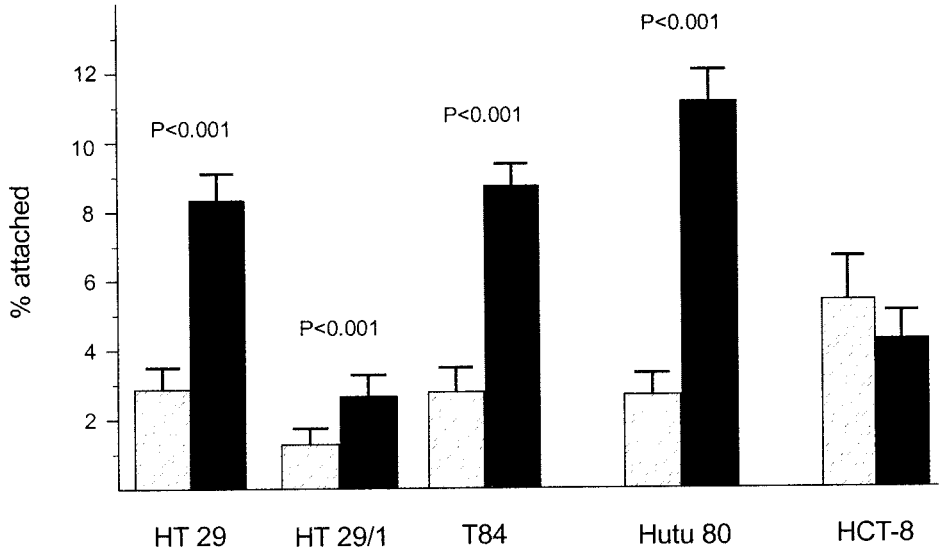

FIG. 1. Adherence of AS-negative *E. faecalis* strain OG1X (hatched bars) and AS-positive *E. faecalis* strain OG1X/ pAM721 (solid bars) to intestinal epithelial cells. The efficiency of adherence is expressed as the percentage of the inoculum remaining attached to eukaryotic cells and was calculated as follows: percentage attached = (number of adherent bacteria × 100)/number of bacteria in inoculum. The values are means and standard deviations for six wells and are representative of four independent assays. Statistical analysis was done with the *t* test.

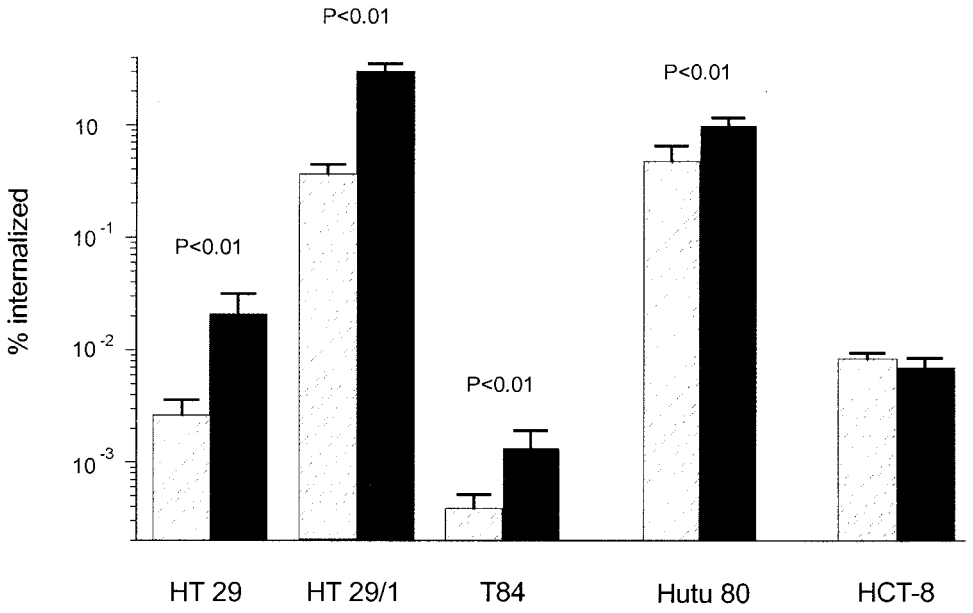

FIG. 2. Internalization of AS-negative *E. faecalis* strain OG1X (hatched bars) and AS-positive *E. faecalis* strain OG1X/pAM721 (solid bars) by intestinal epithelial cells. The efficiency of internalization is expressed as the percentage of the inoculum found intracellularly and was calculated as follows: percentage internalized = (number of intracellular bacteria × 100)/number of bacteria in inoculum. The values are means and standard deviations for six wells and are representative of four independent experiments. Statistical analysis was done with the Mann-Whitney U test.

HB101 and significantly less than the invasive *Salmonella* serovar Typhimurium strain (percentages internalized: 9.0×10^{-2} for HT 29 cells and 1.8×10^{-2} for T84 cells) without affecting the viability of the cells, as shown by the trypan blue exclusion assay. As shown in Fig. 2, the AS increased the internalization of *E. faecalis* by HT 29 and HT 29/1 cells >8-fold and that by T84 and Hutu 80 cells 4- and 3-fold, respectively ($P < 0.01$), whereas invasion into HCT-8 cells was not promoted by the AS. Polarization of the colonic cells, as tested with HT 29/1, had no detectable influence on the adherence and invasion processes.

Since the sex pheromone plasmid of *E. faecalis* OG1X/pAM721 also codes for cytolysin, it can be speculated that the invasion of colonic mucosa might have been affected by this virulence factor as well. However, the adhesion and invasion of the AS-negative and cytolysin-negative *E. faecalis* strain OG1X and the cytolysin-positive strain, which expresses AS lacking the membrane anchor, so that AS is not exposed on the bacterial surface (OG1X/pAM944), were similar. This result indicates that the increased invasion observed in this study is mediated by the AS but not by cytolysin.

In a previous study (17), we suggested that integrins might be the site of attachment for eukaryotic cells, since (i) the AS possesses the integrin binding motif RGDS and (ii) the synthetic peptide RGDS (Arg-Gly-Asp-Ser) competitively inhibited the binding of *E. faecalis* to cultured renal tubular cells. Similar results were obtained using human macrophages which, in addition, showed that the adherence of *E. faecalis* involves interactions of the AS with β2-integrins (22, 26, 27). However, in contrast to data concerning pathogens such as *Shigella* species (18), evidence is still weak. An alternative molecule which could serve as a mediator of attachment to epithelial cells is fibronectin, which is secreted by a wide range of cells, including intestinal epithelial cells (11), which binds to the AS (E. Rozdzinski, A. Muscholl, R. Wirth, and R. Marre, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. B-26, p. 26, 1996), and which is, in contrast to integrins, present at the luminal site of epithelial cells.

Since integrins can play a major role in AS-mediated adherence of enterococci to intestinal tract cells only if the bacteria are able to reach the integrin-bearing basolateral site of the epithelium by translocation across the mucosal surface, we tested if *E. faecalis* is able to translocate through the epithelial cell monolayer. Intestinal epithelial cells were cultured on filter units (Transwell 3415; Costar, Cambridge, Mass.) containing 0.33-cm^2 porous membranes and placed in the wells of a 24-well tissue culture tray. Bacterial suspensions were added to the apical surface of the cell monolayer. Viable bacteria in the underlying basolateral medium were quantified each hour for the duration of the experiment by plating dilutions on appropriate nutrient agar. *E. faecalis* was not detected in the basolateral medium after a maximum of 8 h of incubation. *Salmonella* serovar Typhimurium, the positive control, penetrated well through the cell layer, while *E. coli* HB101 did not, confirming the integrity of the tight junctions (data not shown). These results indicate that the intact epithelial cell layer serves as a barrier for enterococci. However, in vivo studies with mice demonstrated that enterococci could indeed migrate across intact intestinal mucosa and spread to the mesenteric lymph nodes, liver, and spleen when intestinal overgrowth with *E. faecalis* was induced (31). Since the observation period in those experiments lasted for 21 days, it is likely that the maximum possible incubation time of 8 h in our in vitro model was too short for the enterococci to cross the cell layer.

One of the main questions is if AS-mediated adherence and invasion or internalization matter in vivo. The published data are still controversial. The significance of the AS in the development of experimental endocarditis in rabbits and its association with an increased vegetation weight have been shown recently (4). Another study with the rabbit model of enterococcal endocarditis also documented that the presence of the AS in combination with the expression of the enterococcal

binding substance caused high mortality (26). In other animal models (experimental endocarditis and experimental endophthalmitis), the AS had no influence on the outcome of the infections (3, 14). These experimental models, however, do not reflect a situation where the enterococci have to adhere to intestinal epithelia and translocate through the mucosa. The clinical situation and animal experiments suggest that a loss of the functional integrity of the epithelial cell layer, e.g., by severe shock, stress, trauma, or colorectal cancer, facilitates the invasion of enterococci (9, 23, 29). The AS could contribute to this process, since it enables bacteria to attach to epithelial cells.

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