Inducible Expression of *Enterococcus faecalis* Aggregation Substance Surface Protein Facilitates Bacterial Internalization by Cultured Enterocytes

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Aggregation substance (AS) is an *Enterococcus faecalis* surface protein that may contribute to virulence. Using a recently described system for controlled expression of AS in *E. faecalis* and the heterologous host *Lactococcus lactis*, experiments were designed to assess the effect of AS on bacterial internalization by HT-29 and Caco-2 enterocytes. AS expression was associated with increased internalization of *E. faecalis* by HT-29 enterocytes and of *L. lactis* by HT-29 and Caco-2 enterocytes. Compared to enterocytes cultivated under standard conditions, either cultivation in hypoxia or 1-h pretreatment of enterocytes with calcium-free medium resulted in increased internalization of both *E. faecalis* and *L. lactis* (with and without AS expression). Also, AS expression augmented these increases when *E. faecalis* was incubated with pretreated HT-29 enterocytes and when *L. lactis* was incubated with pretreated Caco-2 and HT-29 enterocytes. These data indicated that AS might facilitate *E. faecalis* internalization by cultured enterocytes.

Although *Enterococcus faecalis* is a component of the normal human intestinal flora, enterococci number among the top three nosocomial microbial pathogens (8), and strains resistant to all useful antimicrobial agents are increasingly involved in fatal infections (12). Thus, it is important to clarify the mechanisms involved in extraintestinal dissemination of enterococci. Aggregation substance (AS) protein may be involved in virulence and is expressed on the surface of *E. faecalis*. AS molecules are encoded by different pheromone-responsive plasmids; e.g., Asc10 is encoded by pCF10, and Asa1 is encoded by pAD1 (6). Pheromones produced by potential recipients induce expression of AS on the surfaces of plasmid-containing donor cells. AS facilitates aggregation of donor and recipient bacteria and aids conjugative plasmid transfer (6).

The gene for Asc10 was recently cloned in a vector containing a nisin-inducible promoter, resulting in surface expression of Asc10 on *E. faecalis* and the heterologous host *Lactococcus lactis*. *E. faecalis* OG1SSp and *L. lactis* NZ9800 were transformed with plasmid pMSP7517 that encodes Asc10 (9). We have used these transformants to clarify the effect of AS on bacterium-enterocyte interactions. Because pMSP7517 contains a gene for erythromycin resistance, bacteriological media were supplemented with 10 µg of erythromycin (Sigma Chemical Co., St. Louis, Mo.) per ml. For experiments, *E. faecalis* was cultivated overnight in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) in the absence of nisin or in broth supplemented with 25 ng of nisin (Sigma) per ml; nisin was present either throughout the incubation period or only during the final 2 h. Following incubation at 35°C with nisin, *E. faecalis* cells clumped, confirming Asc10 expression (6, 9). To obtain single-cell suspensions (verified by light microscopy), these inocula were sonicated, typically with 20 W for 10 s using a 40-W high-intensity ultrasonic processor (Sonics and Mate-

rials, Danbury, Conn.). *L. lactis* was cultivated in a similar manner except the incubation temperature was 30°C and the medium was M17 broth (Difco) supplemented with 0.5% glucose. As expected, AS expression on *L. lactis* was not associated with bacterial clumping. Scanning electron microscopy and Western blot analysis have been used to visualize nisin-induced expression of Asc10 on the surfaces of the *E. faecalis* and *L. lactis* strains used in this study (9).

Caco-2 and HT-29 cells were obtained from the American Type Culture Collection (Rockville, Md.) and were cultivated in 24-well plastic dishes as described previously (22–24). Briefly, Caco-2 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 4 mmol of L-glutamine per liter. HT-29 cells were cultivated in glucose-free Dulbecco's modified Eagle's medium supplemented with 15% dialyzed fetal bovine serum, 4 mmol of L-glutamine per liter, and 5 mM galactose. All tissue culture reagents were obtained from Sigma. Enterocytes were seeded at 2×10^4 cells per well (2 cm²) and incubated at 37°C in 9.5% CO₂. Caco-2 and HT-29 enterocytes were used after 15 to 18 and 21 to 24 days, respectively, when these enterocytes are polarized and differentiated (10, 17, 23). Caco-2 and HT-29 cells were used between passages 30 and 37 and passages 30 and 35, respectively.

Enterocyte internalization of viable bacteria was assayed as described previously (22–24), with minor modifications. Broth cultures of *E. faecalis* or *L. lactis* grown overnight were washed and diluted in the appropriate enterocyte tissue culture medium. One milliliter containing 10⁸ viable bacteria was added to each tissue culture well containing 10^{5.7} to 10⁶ confluent enterocytes. After 1 h at 37°C, enterocytes were washed five times with Hanks balanced salt solution, and antibiotic-supplemented tissue culture medium was added to kill residual viable extracellular bacteria. Residual *L. lactis* bacteria were eliminated with 50 µg of gentamicin sulfate (Sigma) per ml, and residual *E. faecalis* bacteria were eliminated with 50 µg of penicillin (Sigma) per ml plus 10 µg of gentamicin per ml. After 2.5 h, epithelial cells were washed five times with Hanks

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FIG. 1. Effect of nisin-induced expression of Asc10 on internalization of *E. faecalis* (A and B) and *L. lactis* (C and D) by HT-29 and Caco-2 enterocytes that had been incubated under standard conditions or preincubated for 1 h in calcium-free medium prior to addition of bacteria. Using both HT-29 and Caco-2 enterocytes, bacterial internalization was consistently higher with enterocytes pretreated with calcium-free medium than with the corresponding enterocytes incubated under standard conditions (§ and ‡, $P \leq 0.01$ and $P < 0.05$, respectively). (A) Using HT-29 enterocytes incubated under standard conditions, internalization of *E. faecalis* incubated overnight with nisin was increased compared to internalization of *E. faecalis* incubated in the absence of nisin for 2 h (†, $P < 0.05$). Incubation of *E. faecalis* with nisin had an additive effect on the increased internalization associated with enterocytes exposed to calcium-free medium compared to internalization of *E. faecalis* incubated in the absence of nisin (* and #, $P < 0.01$ and $P < 0.05$, respectively). (B) Incubation of *E. faecalis* with nisin had no noticeable effect on the numbers of bacteria internalized by Caco-2 cells that had been incubated under standard conditions or preincubated in calcium-free medium. (C and D) Using either HT-29 or Caco-2 enterocytes preincubated in calcium-free medium, overnight incubation of *L. lactis* with nisin was associated with greater numbers of internalized lactococci than incubation of *L. lactis* without nisin (*, $P < 0.01$). The horizontal lines represent the lower limits of assay detection. Error bars are not apparent if less than 0.1. Data represent the averages of at least four assays. SE, standard error.

balanced salt solution and lysed with 1% Triton X-100, and viable intracellular bacteria were quantified following serial dilution and incubation on the appropriate agar medium. Each bacterial strain was tested in at least four separate assays, performed on different days, with each assay result representing the average of triplicate tissue culture wells. Bacterial numbers were converted to log₁₀ units prior to statistical analysis. The lower limit of detection was 50 bacteria or 1.7 log₁₀ units, and values below this limit were assigned a value of 1.7. Data involving two treatment groups were analyzed by unpaired Student's *t* test. Data involving more than two treatment

groups were analyzed by one-way analysis of variance followed by Fisher's test for significant differences. Statistical analyses were performed with StatView 5.0 (Abacus Concepts, Berkeley, Calif.), and a P of ≤ 0.05 was considered statistically significant.

Immediately prior to bacterium-enterocyte incubation, some enterocyte cultures were preincubated for 1 h in calcium-free Krebs Ringer's solution (Sigma) as described previously (22). A low extracellular concentration of calcium has no noticeable effect on enterocyte viability but causes reversible disruption in the calcium-dependent junctional complex, causing confluent

FIG. 2. Scanning electron micrographs of *E. faecalis* cultivated overnight in nisin and then incubated for 1 h with Caco-2 enterocytes that had been preincubated for 1 h in calcium-free medium. (A) Relatively low-magnification view showing numerous *E. faecalis* bacteria (some highlighted with arrows) localized on the lateral surface of rounded enterocytes, with the apical enterocyte surfaces relatively devoid of adherent bacteria. (B) Higher-magnification view showing numerous *E. faecalis* bacteria adherent to the enterocyte lateral surface. Bars, 6 μ m (A) and 2 μ m (B).

enterocytes to pull apart from each other and exposing the enterocyte lateral surface (1, 3, 22). This phenomenon was verified by light microscopy of Wright-Giemsa-stained cultures.

To study the effect of hypoxia, some enterocyte cultures were cultivated in reduced oxygen as described previously (23). Briefly, after initial seeding, enterocytes were incubated at 37°C in 9.5% CO₂ for 2 days; enterocytes in this atmosphere were considered exposed to 20% oxygen. After 2 days, some 24-well plastic dishes were transferred to polycarbonate modular incubator chambers (Vanguard International, Neptune, N.J.). The chambers were flushed with one of two gas mixtures that were certified as accurate at 0.02% (GenEx, St. Louis, Mo.): (i) 10% oxygen plus 10% CO₂ plus 80% nitrogen or (ii) 5% oxygen plus 10% CO₂ plus 85% nitrogen. Preliminary experiments showed that enterocytes would not grow in 0% oxygen. Tissue culture medium was replaced twice weekly with fresh medium that had equilibrated in the appropriate gas mixture for at least 24 h, and the pH of the tissue culture media remained 7.22 to 7.26 after exposure to 20, 10, or 5% oxygen. This model may have clinical relevance because many clinical conditions associated with extraintestinal spread of normal enteric flora (such as *E. faecalis*) are also associated with mesenteric ischemia; these conditions include endotoxemia, burn wounds and other trauma, intestinal obstruction, and hemorrhagic shock (reviewed in reference 21). As previously noted (23), enterocytes cultivated in normoxia were confluent, while enterocyte cultures cultivated in 5% oxygen contained approximately 10-fold-less cells and appeared as islands of cells, with larger clusters following cultivation in 10% oxygen. Numbers of internalized bacteria were therefore normalized to 10⁵ enterocytes, and the lower limit of assay detection was 0.7 log₁₀ unit per 10⁵ enterocytes. In all experiments, mature Caco-2

and HT-29 cultures were $\geq 95\%$ viable as determined by staining with the vital dyes trypan blue (0.4%) and propidium iodide (20 mg/liter).

Using the nisin-induced expression system, we have now confirmed and extended our previous work wherein we reported that Asc10 facilitates *E. faecalis* internalization by HT-29 enterocytes and that these intracellular bacteria could be visualized using transmission electron microscopy (16). In the present study, overnight incubation of *E. faecalis* with nisin resulted in increased internalization by HT-29 but not Caco-2 enterocytes (Fig. 1A and B). Using *E. faecalis* cultivated in either the presence or absence of nisin, enterococcal internalization was consistently increased for enterocytes preincubated in calcium-free medium than for enterocytes cultivated under standard conditions (Fig. 1A and B). Nisin induction of Asc10 augmented the increased enterococcal internalization associated with HT-29 (but not Caco-2) enterocytes cultivated in calcium-free medium (Fig. 1A). It might be noted that similar internalization of *E. faecalis* (with and without nisin induction of Asc10) by Caco-2 enterocytes indirectly confirmed that the data were not affected by possible reaggregation of AS-expressing *E. faecalis* during the 1 h of bacterium-enterocyte incubation. Surface interactions of *E. faecalis* incubated with enterocytes pretreated with calcium-free medium were visualized by scanning electron microscopy as described previously (22, 25), and *E. faecalis* (with and without AS expression) appeared preferentially adherent on the exposed enterocyte lateral surface (Fig. 2).

Surprisingly, nisin induction of Asc10 had no effect on the numbers of *L. lactis* internalized by HT-29 or Caco-2 enterocytes cultivated under standard conditions (Fig. 1C and D). However, nisin-induced alterations in lactococcal internalization could have been obscured because the numbers of intra-

FIG. 3. Effect of cultivation of HT-29 and Caco-2 enterocytes in either 20, 10, or 5% oxygen on internalization of *E. faecalis* with and without nisin-induced expression of Asc10. Data show a consistent increase in *E. faecalis* internalization with enterocytes cultivated in hypoxia compared to the corresponding bacterium-enterocyte mixture containing enterocytes cultivated in 20% oxygen (* and †, $P < 0.01$ and $P < 0.05$, respectively). Data also show that nisin-induced expression of Asc10 had an additive effect on the increased bacterial internalization associated with HT-29 enterocytes cultivated in hypoxia compared to the corresponding bacterium-enterocyte mixture with *E. faecalis* incubated in the absence of nisin (§ and ‡, $P < 0.01$ and $P < 0.05$, respectively). Preincubation of *E. faecalis* with nisin had no noticeable effect on *E. faecalis* internalization by Caco-2 cells cultivated in 20, 10, or 5% oxygen. The horizontal line represents the lower limit of assay detection. Data represent the averages of at least seven assays. SE, standard error.

cellular lactococci were consistently at or near the lower limit of assay detection. Internalization of *L. lactis* was consistently higher with enterocytes incubated in calcium-free medium than with enterocytes incubated under standard conditions, and Asc10 expression appeared to have an additive effect (Fig. 1C and D). This latter observation was consistent with the speculation that, using enterocytes cultivated under standard conditions, nisin-induced augmentation of *L. lactis* internalization was below the limit of assay detection.

Thus, Asc10 expression appeared to augment internalization of *L. lactis*, but not *E. faecalis*, by Caco-2 enterocytes. Perhaps Caco-2 enterocytes have an additional receptor that interacted with a chromosome-encoded *E. faecalis* surface component. This could obscure the contribution of AS in the interaction of *E. faecalis* with Caco-2 cells. Although clarification of this mechanism was beyond the scope of the present study, data from experiments with *L. lactis* indicated that Asc10 expression (independent of other plasmid-encoded surface proteins) facilitated bacterial internalization by both HT-29 and Caco-2 enterocytes.

Internalization of *E. faecalis* was increased with enterocytes cultivated in hypoxia (Fig. 3). Statistical analysis to determine the effect of oxygen revealed that the numbers of intracellular *E. faecalis* were consistently greater with enterocytes cultivated in 5% oxygen than for those cultivated in 20% oxygen (Fig. 3). Enterocytes cultivated in hypoxia were not confluent, and there is evidence (albeit circumstantial) that bacterial internalization is higher with enterocytes that have had their lateral surfaces exposed by a variety of experimental means (22, 24, 25). Surface interactions of *E. faecalis* incubated with enterocytes cultivated in 5% oxygen were also visualized by scanning electron microscopy, and *E. faecalis* (with and without AS expression) appeared preferentially adherent on the exposed enterocyte lateral surface (not shown). Statistical analysis to determine the effect of Asc10 revealed that Asc10 expression augmented the increased enterococcal internalization associated with HT-29 enterocytes cultivated in hypoxia (Fig. 3).

The role of AS in *E. faecalis* pathogenesis remains contro-

versial. For example, the gene for AS was not strongly associated with isolates from human blood cultures (11) or with isolates from patients with endocarditis (2, 5). AS did not seem to play an important role in experimental endophthalmitis (14), rodent sepsis (7), and rat endocarditis (2). However, there is evidence that AS promotes rabbit endocarditis (4, 19) and that AS facilitates opsonin-independent binding of *E. faecalis* to human neutrophils (20) and may promote intracellular survival of *E. faecalis* within neutrophils (18). Kreft et al. (15) noted that AS mediated *E. faecalis* binding to cultured porcine renal tubular cells and speculated that AS might function as an adhesin mediating enterococcal binding to eucaryotic cells. Thus, the pathogenic role of AS may depend upon the type of infection and/or model system studied.

Data from the present study indicated that AS might facilitate internalization of *E. faecalis* by intestinal epithelial cells. Consistent with this observation, Isenmann et al. (13) recently reported that AS facilitated *E. faecalis* invasion of ex vivo rat colonic mucosa. It therefore seems reasonable to speculate that AS might facilitate internalization of *E. faecalis* by intestinal epithelial cells and that this phenomenon might be augmented in a subset of patients with mesenteric ischemia and/or with alterations in intestinal epithelial junctional integrity. This hypothesis should be tested in a relevant in vivo model.

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