Novel Clues on the Specific Association of *Streptococcus gallolyticus* subsp *gallolyticus* With Colorectal Cancer

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(See the editorial commentary by Hensler, on pages 1040–2.)

Background. The prevalence of *Streptococcus gallolyticus* subsp *gallolyticus* (*Streptococcus bovis* biotype I) endocarditis is in general low but very often linked to colorectal cancer. Therefore, this study aimed to reveal the virulence characteristics that distinguish this opportunistic pathogen from a panel of (closely related) intestinal bacteria.

Methods. The route of infection was reconstructed in vitro with adhesion, invasion, and translocation assays on differentiated Caco-2 cells. Furthermore, cellular immune responses upon infection and bacterial biofilm formation were analyzed in a comparative manner.

Results. *S. gallolyticus* subsp *gallolyticus* strains were demonstrated to have a relative low adhesiveness and could not internalize epithelial cells. However, these bacteria were uniquely able to paracellularly cross a differentiated epithelium without inducing epithelial interleukin 8 or 1β responses. Importantly, they had an outstanding ability to form biofilms on collagen-rich surfaces, which in vivo are found at damaged heart valves and (pre)cancerous sites with a displaced epithelium.

Conclusions. Together, these data show that *S. gallolyticus* subsp *gallolyticus* has a unique repertoire of virulence factors that facilitate infection through (pre)malignant colonic lesions and subsequently can provide this bacterium with a competitive advantage in (1) evading the innate immune system and (2) forming resistant vegetations at collagen-rich sites in susceptible patients with colorectal cancer.

The human intestinal tract is the habitat for several hundred different bacterial species with an increasing bacterial concentration and variability toward the distal colon. The commensal bacterial population aids human health by making dietary nutrients availableto the host, but it also prevents attachment and subsequent invasion of pathogenic bacteria [1]. Strikingly, however, the part of the intestine with the highest bacterial colonization, the colon, is also most affected by cancer, with 146,970 cases annually in the United States [2]. This, together with the fact that germ-free mice have lower rates of colon carcinogenesis [3], implies that intestinal bacteria play an important role in the development of colorectal cancer (CRC).

The gram-positive, opportunistic pathogen *Streptococcus bovis* is one of the few intestinal bacteria that have been consistently linked to CRC [4–6]. The first case report suggesting an association between *S. bovis* endocarditis and carcinoma of the sigmoid was already published in 1951 [7]. Since then, multiple studies have shown that a colon tumor or polyp was detected upon full-bowel examination in up to 90% [8] of patients with a *S. bovis* infection [5, 9]. Furthermore, fecal carriage of *S. bovis* in the healthy population is low but increases ~5-

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fold in patients with CRC [10]. After Schlegel et al introduced the new nomenclature of *S. bovis* strains [11] it became clear that *Streptococcus gallolyticus* subsp *gallolyticus* (*S. bovis* biotype I) (Table 1), a major cause of infective endocarditis, has the highest association with CRC [6, 8].

Although some studies have shown that *S. bovis* strains can directly promote carcinogenesis in a rat model for CRC [12, 13], an incidental relationship provides an alternative explanation for the association of *S. gallolyticus* subsp *gallolyticus* with CRC. In a normal healthy colonic environment the host has several defense mechanisms to shield itself from bacterial infection. Goblet cells within the polarized epithelium secrete a continuous layer of mucus that protects the epithelium and promotes transit of bowel contents [14], whereas enterocytes secrete antimicrobial peptides, cytokines and immunoglobulin A as preventive agents. However, CRC is characterized by several changes in this physical barrier including increased tight junction permeability [15] and altered mucus production and composition [16]. This distorted physical protection could make patients with CRC prone to rare opportunistic bacterial infections.

However, it is still unclear why *S. gallolyticus* subsp *gallolyticus* infections have such a high association with colon malignancies, whereas this is not the case for other (related) opportunistic pathogens that inhabit the human gastrointestinal tract. Therefore, the main aim of this study was to reveal the virulence characteristics that distinguish *S. gallolyticus* subsp *gallolyticus* from other bacteria to gain insight in how these features could specifically cause infections in patients with CRC. To this purpose, several host-pathogen interactions that are involved in this infective process were mimicked in vitro. These studies indicated that *S. gallolyticus* subsp *gallolyticus* avails of a unique repertoire of virulence characteristics that give it an advantage over related *S. bovis* strains and other intestinal bacterial species, to cross an epithelial layer, evade the immune system and form biofilms on collagen-rich surfaces.

MATERIALS AND METHODS

Cell Culture and Bacterial Strains

Colorectal adenocarcinoma cell lines HT-29 and Caco-2 (www.atcc.org) were cultured in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% fetal calf serum (FCS), 20 mmol/L HEPES, 2 mmol/L L-glutamine and 1× nonessential amino acids (Gibco) at 37°C/5% CO_2. The human monocytic cell line THP-1 was cultured in Roswell Park Memorial Institute 1640 medium (RPMI1640) supplemented with 10% FCS, 2 mmol/L L-glutamine, 1 μmol/L pyruvate, and 5 μg/mL gentamicin (Gibco). These media and culture conditions were used in experiments unless stated otherwise.

The following bacterial *S. bovis* strains were used, *S. gallolyticus* subsp *gallolyticus* UCN34 [17], *S. gallolyticus* subsp *gallolyticus* NTB1 (Radboud collection), *S. gallolyticus* subsp *gallolyticus* 1293 provided by Dr R. Zarrilli [18], *Streptococcus infantarius* subsp *infantarius* NCTC8133 [13] and *S. gallolyticus* subsp *macedonicus* CIP105685T (Pasteur collection). The new and old designations for *S. bovis* strains are depicted in Table 1.

Reference strains included *Enterococcus faecalis* 19433 (www.atcc.org), *Escherichia coli* NTB5, and *Salmonella typhimurium* NTB6 from the Radboud collection, *Lactobacillus plantarum* WCFS1 [19], and *Bacillus subtilis* 168 [20]. All strains were grown on Columbia blood agar or in brain-heart infusion broth (Difco) supplemented with 1% glucose at 37°C and 5% CO_2. *L. plantarum* was grown in de Man–Rogosa–Sharpe (MRS) broth at 37°C and 5% CO_2 and *E. coli* was grown at 200 rpm.

Adherence and Internalization Assay

Caco-2 and HT-29 cells were cultured in 24-well plates (Corning) to ~1 ×x 10^6 cells/well and infected with a multiplicity of infection (MOI) of 20. After 2 h of incubation, monolayers were washed 3 times with PBS to remove nonadherent bacteria and subsequently lysed in trypsin-PBS containing .025% triton-X100. Alternatively, extracellular adherent bacteria were killed with 200 μg/mL gentamicin and 50 μg/mL ampicillin for another hour to measure the amount of internalized bacteria. The amount of adherent or internalized bacteria was determined by counting colony-forming units. Adherence was expressed as a percentage of the inoculum, and internalization as the percentage of adherence.

Translocation Assay

Caco-2 cells were cultured on Transwell permeable supports with a polycarbonate membrane (3-μm pore size) (Corning).

Table 1. Nomenclature of *Streptococcus bovis* Strains

New Name	Old Name	Strains Used in Current Study
Streptococcus gallolyticus subsp *gallolyticus*	*S. bovis* biotype I	UCN34 (SG1), 1293 (SG2), NTB1 (SG3)
Streptococcus infantarius subsp *infantarius*	*S. bovis* biotype II.1	NCTC8133 (SI)
S. infantarius subsp *coli*	*S. bovis* biotype II.1	None
S. gallolyticus subsp *pasteurianus*	*S. bovis* biotype II.2	None
S. gallolyticus subsp *macedonicus*	*Streptococcus macedonicus*	CIP105865T (SM)

NOTE. Historically *S. bovis* strains were delineated into 2 biotypes according to their ability (biotype I) or inability (biotype II) to ferment mannitol [11]. The former *S. bovis* biotype I, *S. bovis* biotype II.2, and S. *macedonicus* are now designated in a single DNA cluster including 3 subspecies: *S. gallolyticus* subsp *gallolyticus*, *S. gallolyticus* subsp *pasteurianus*, and *S. gallolyticus* subsp *macedonicus*. *S. gallolyticus* subsp *gallolyticus* is most often linked with endocarditis-associated colonic cancer.

Transepithelial electrical resistance (TEER) measurements confirmed the formation of a polarized monolayer by a flattening of the TEER value (250–350 $\Omega^{*}cm^{2}$) at 21 days (Millipore ERS) [21]. Bacteria were added to the apical compartment (MOI, 50), and after incubation the numbers of viable bacteria in the apical and basolateral compartments were determined by counting colony-forming units. At every time point, medium in the lower compartment was replaced to prevent growth of translocated bacteria. Translocation was expressed as a percentage of the inoculum.

Confocal Microscopy

Bacteria (1×10^{9}) were washed in PBS and labeled for 30 min at room temperature (RT) in PBS containing .5 mg/mL fluorescein isothiocyanate (FITC) (Sigma). Next, bacteria were extensively washed to remove nonbound FITC before infection (MOI, 50) of polarized Caco-2 monolayers on Transwell permeable supports. After 4 h of incubation in the dark monolayers were stained for confocal microscopy, as described in the Supplementary Information.

Phagocytosis Assay

Human monocytic THP-1 cells were seeded in 24-well plates at 50,000 cells/well in RPMI 1640 containing 1% FCS and were differentiated to macrophages by 50 ng/mL phorbol 12–myristate 13–acetate 24 h before phagocytosis assay. Next, bacteria were added (MOI, 50), spun at 400 *g* for 5 min, and incubated for 30 min to allow phagocytosis. Extracellular bacteria were killed with 200 μg/mL gentamicin and 50 μg/mL ampicillin, and after incubation viable intracellular bacteria were quantified by macrophage lysis with 1% saponin. Killing was expressed as the percentage of phagocytosed bacteria at $t = 0$.

Real-Time Polymerase Chain Reaction

Caco-2, HT-29, and THP-1 cells were washed and lysed in RLT lysis-buffer (RNeasy Mini Kit; Qiagen), and RNA extraction was performed according to Qiagen protocol. The RNA concentration and purity were evaluated with a NanoDrop Spectrophotometer (NanoDrop Technologies). Next, reverse-transcription polymerase chain reaction (PCR) (Iscript; Bio-Rad) was performed to synthesize 1 μg of complementary DNA under the following conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Expression of interleukin (IL) 8 and *IL-1β* (gene expression assays Hs00174103_m1 and Hs00174097_m1; Applied Biosystems) was compared with expression of the gene for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; gene expression assay4310884E), using the following real-time PCR protocol: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C (7900 HT; Applied Biosystems). Data were analyzed via the **ΔΔ**Ct method using SDS software (version 2.2.1).

Biofilm Formation

Biofilm formation assays were essentially performed as described elsewhere, with some minor modifications [22]. Bacteria were cultured overnight in tryptone-soya broth containing .25% glucose, diluted to 10^{7} bacteria/mL and dispensed in polystyrene 96-well plates that were either coated with collagen type I or type IV or uncoated. Plates were incubated for 24 h at 37°C on a 3-dimensional plate rotator (30 rpm). The cell suspension was removed, and biofilms were washed 3 times with PBS. Then plates were dried for 1 h at room temperature, and biofilms were stained with crystal violet solution. After 15 min, excess crystal violet was removed, plates were washed 3 times with PBS, and crystal violet was dissolved in ethanol-acetone (80:20 vol/vol). The absorbance, which is representative of the amount of biofilm formed, was measured at 595 nm (A_{595}).

Electron Microscopy

Bacteria were grown in Todd-Hewitt medium and collected after overnight growth (stationary phase). Electron microscopic images were produced as described elsewhere [23]. For details, see Supplementary Information.

RESULTS

Adherence of *S. gallolyticus* Strains to Colon Epithelial Cells

The first important step to establish a gut-borne infection is adherence of bacterial cells to colonic tissue. Therefore, the binding capacity of *S. gallolyticus* subsp *gallolyticus* clinical isolates to colonocytes was compared with that of the pathogen *S. typhimurium*, the opportunistic pathogen *E. faecalis,* and nonpathogenic *E. coli* and *L. plantarum* strains (Figure 1A and B). These experiments showed that *E. faecalis* is by far the most efficient adhering bacterium to both HT-29 and Caco-2 cells, reaching adherence of about 80% and 98% of the inocula, respectively. The nonpathogenic strains *E. coli*, *S. gallolyticus* subsp *macedonicus,* and *L. plantarum* adhered moderately well (20%–50%) to the monolayers. Adherence of the *S. gallolyticus* subsp *gallolyticus* strains was similar to that of the pathogen *S. typhimurium*, all with adherence <15%. Thus, the adhesive properties of *S. gallolyticus* subsp *gallolyticus* strains resemble those of the pathogen *S. typhimurium* more than nonpathogenic bacteria. This may reflect one of the reasons that *S. gallolyticus* subsp *gallolyticus* cannot efficiently colonize a healthy human intestinal tract.

Paracellular Translocation of Colonic Differentiated Epithelial Cells by *S. gallolyticus* subsp *gallolyticus*

After adhesion to colonic tissue, several invading mechanisms can be used by (opportunistic) pathogens. For example, *Salmonellae* are efficient in transcellular crossing of intestinal epithelium [24], whereas paracellular crossing is described for group B streptococci [25]. To obtain insight into the translocation capacity of *S. bovis* strains, their internalization and translocation efficiencies were analyzed in Caco-2 Transwell cultures. As shown in Figure 2C, none of the *S. bovis* strains were

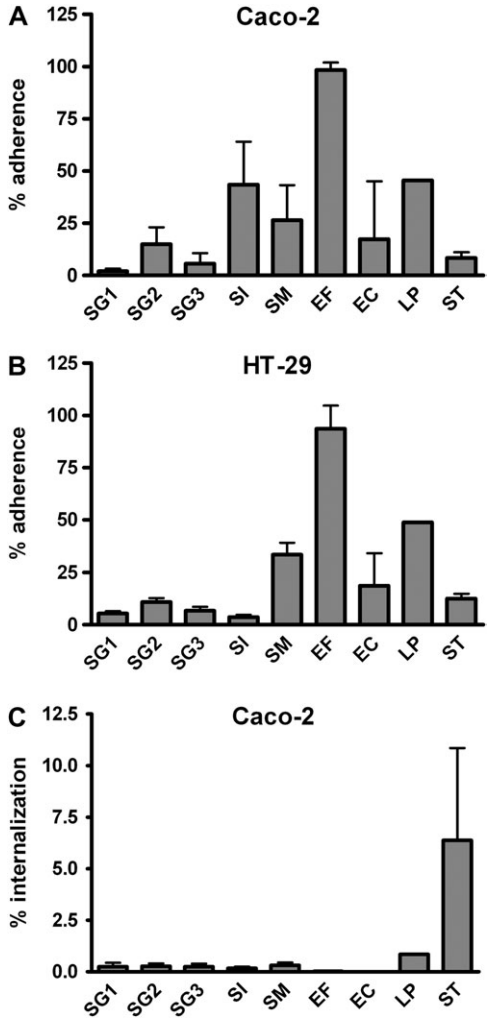

Figure 1. *A, B,* Bacterial adherence and invasion of epithelial cells. Adherence of indicated intestinal bacteria to Caco-2 (*A*) and HT-29 (*B*) colorectal cancer cells was analyzed after 2 h of bacterial exposure. *C,* Bacterial internalization after 2 h in Caco-2 cells. Adherence is presented as percentage of the bacterial inocula, and subsequent bacterial internalization as percentage of adherent bacteria. *EC, Escherichia coli*; *EF, Enterococcus faecalis*; *LP, Lactobacillus plantarum*; *SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius*; *SM, S. gallolyticus* subsp *macedonicus*; *ST, Salmonella typhimurium*.

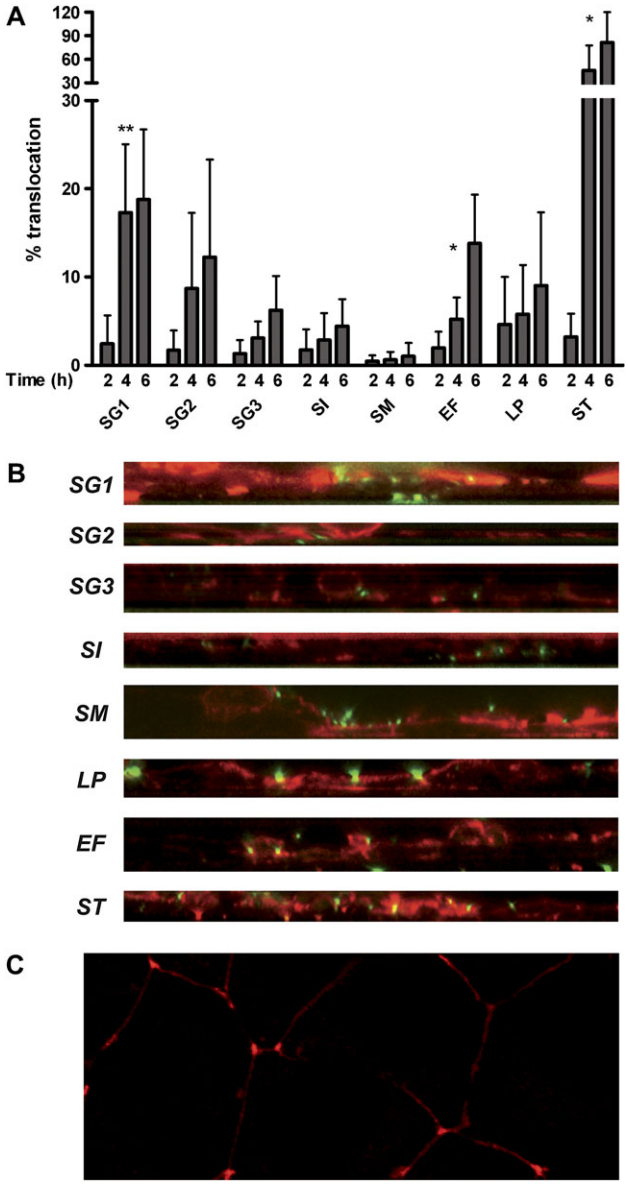

Figure 2. Bacterial translocation across an epithelial monolayer. *A,* Translocation of indicated bacteria across differentiated and polarized Caco-2 cells was measured after 2, 4, and 6 h. $*P < .05$; $**P < .01$ (significant increase in time; 1-way analysis of variance). *EF, Enterococcus faecalis*; *LP, Lactobacillus plantarum*; *SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius*; *SM, S. gallolyticus* subsp *macedonicus*; *ST, Salmonella typhimurium*. *B,* Confocal microscopy of fluorescein isothiocyanate–labeled bacteria (*green*) after translocation ($t = 6$ h). Cytoskeleton was stained with anti-actin antibodies (*red*). Top, lateral side; bottom, basolateral side of the epithelial monolayer. *C,* Zonula occludens 1 staining (*red*) of epithelial monolayer showing tight junction complexes in differentiated monolayer. Although *L. plantarum* displayed some discrepant results between translocation and confocal microscopy, it may be assumed that this bacterium cannot efficiently cross epithelial cells [26].

invasive (maximum, .2% of adherent bacteria), but 6%–19% of adhered *S. gallolyticus* subsp *gallolyticus* and *S. infantarius* subsp *infantarius* cells could translocate across the polarized and differentiated Caco-2 monolayer at efficiencies similar to those of the opportunistic pathogen *E. faecalis* (Figure 2A). In contrast, *S. gallolyticus* subsp *macedonicus* was unable to cross the differentiated monolayer (<2% of adhered bacteria), which clearly differentiates this strain from *S. gallolyticus* subsp *gallolyticus* and *S. infantarius* subsp *infantarius*. However, the data also showed that the only internalizing strain *S. typhimurium* displayed by far the highest translocation percentage (81%) of adhered bacteria.

Z-stack images made with confocal microscopy from the apical to the basolateral side confirmed that *S. gallolyticus* subsp *gallolyticus* (strains UCN34, 1293, and NTB1), *S. infantarius* subsp *infantarius, E. faecalis,* and *S. typhimurium* cells were indeed present at the basolateral side of the monolayer, whereas *S. gallolyticus* subsp *macedonicus* could be detected only at the apical side (Figure 2B). Zonula occludens 1 (ZO-1) visualization (Figure 2C) [26] and TEER measurements confirmed polarization and integrity of the monolayer during experiments, except for *S. typhimurium*, which induced a dramatic reduction in TEER after 6 h (data not shown) [27]. Therefore these data indicate that translocation of *S. gallolyticus* subsp *gallolyticus, S. infantarius* subsp *infantarius,* and *E. faecalis* cannot be attributed to passive leakage through a nonpolarized monolayer but instead constitutes an active process. Together, these data imply that *S. gallolyticus* subsp *gallolyticus* and *S. infantarius* subsp *infantarius,* but not *S. gallolyticus* subsp *macedonicus,* can translocate across a polarized epithelial monolayer via a paracellular mechanism.

Relative Invisibility of *S. gallolyticus* subsp *gallolyticus* to Epithelial Innate Immune System

When pathogens cross the intestinal barrier, the intestinal epithelium attracts macrophages by the production of alarm signals. To evaluate to which extent *S. bovis* strains induce an epithelial innate immune response, the expression of *IL-8* and *IL-1β* in Caco-2 cells was measured with real-time PCR on bacterial infection. As shown in Figure 3A and B, both interleukin *IL-8* and *IL-1β* were strongly increased 2 and 4 h after infection with *S. typhimurium* (maximum *IL-8*, 52-fold; *IL-1β,* 4-fold) and *E. coli* (maximum *IL-8*, 79-fold; *IL-1β*, 7-fold). The gram-positive strains *S. infantarius* subsp *infantarius, S. gallolyticus* subsp *macedonicus,* and *E. faecalis* also significantly increased *IL-8* messenger RNA levels after 4 h of infection but to a lesser extent than gram-negative strains (maximum induction, 6-fold). Surprisingly, however, all 3 *S. gallolyticus* subsp *gallolyticus* strains did not elicit a significant *IL-8* or *IL-1β* response (Figure 3A and B), similar to the probiotic bacterium *L. plantarum.*

To investigate to what extent *S. gallolyticus* subsp *gallolyticus* can withstand phagocytosis, *S. gallolyticus* subsp *gallolyticus* UCN34 cells were exposed to THP-1 derived macrophages. This experiment showed that ~14% of the *S. gallolyticus* subsp *gallolyticus* cells were still viable after 24 h (Figure 3C) in contrast to 0% of *L. plantarum* and *B. subtilis* cells, which were used as positive controls for bacterial killing [28, 29], whereas the pathogen *S. typhimurium* killed and escaped from macrophages within 5 h after infection (data not shown) [30]. However, no macrophage killing was observed by *S. gallolyticus* subsp *gallolyticus,* and bacterial cells remained confined within the macrophage. Accordingly, macrophages responded adequately to *S. gallolyticus* subsp *gallolyticus* infection by a 4-fold up-regulation of *IL-8* and a 3-fold up-regulation of *IL-1β* on the

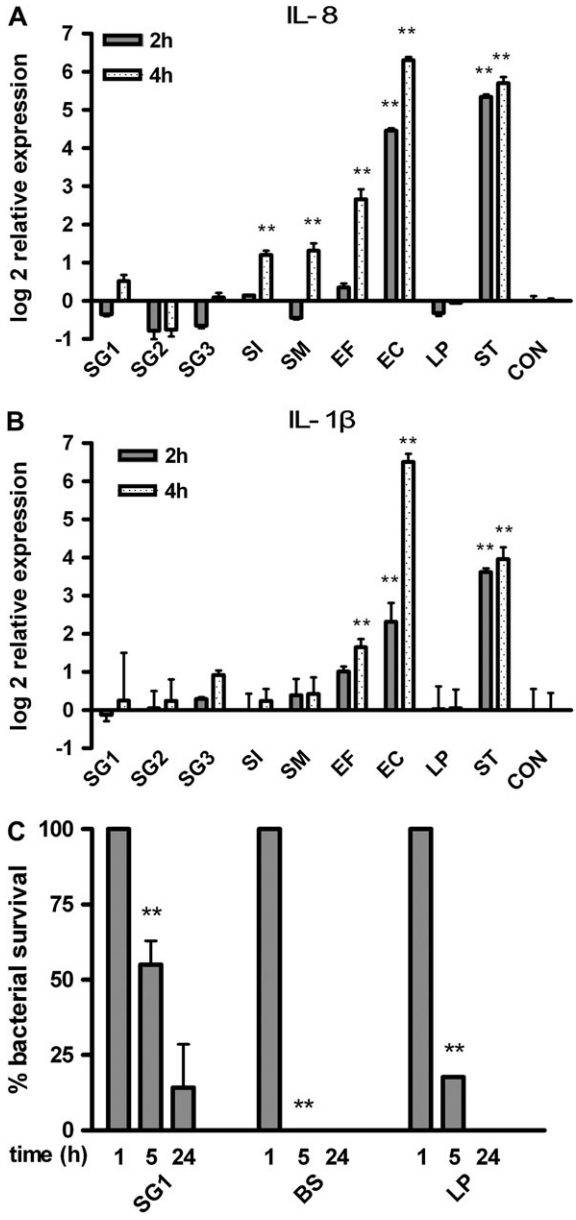

Figure 3. Bacterial interactions with innate immune system. *A, B,* Epithelial interleukin (IL) 8 (*A*) and *IL-1β* (*B*) immune response in Caco-2 cells elicited by indicated bacterial strains after 2 and 4 h of incubation. Data are presented as $\log_2$ values of fold changes. $^{**}P < .01$ (significant inductions; 2-way analysis of variance with Bonferroni posttests). CON, control; *EC, Escherichia coli*; *EF, Enterococcus faecalis*; *LP, Lactobacillus plantarum*; *SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius*; *SM, S. gallolyticus* subsp *macedonicus*; *ST, Salmonella typhimurium. C,* Bacterial killing by phorbol 12–myristate 13–acetate–stimulated THP-1 macrophages was analyzed after 5 and 24 h by counting colony-forming units of viable bacteria. $^{**}P < .01$ (significant reductions in time; 2-way analysis of variance with Bonferroni posttests). *BS, Bacillus subtilis.*

messenger RNA level after 4 h, similar to the response to *S. typhimurium*. Together, these findings indicate that *S. gallolyticus* subsp *gallolyticus* strains are relatively invisible to epithelial innate immunity upon infection, which could prolong their survival by the delayed recruitment of macrophages in the lamina propria.

Biofilm formation by *S. gallolyticus* subsp *gallolyticus* on Collagen-Coated Surfaces

After entry into the human body and escape from the immune system, *S. gallolyticus* subsp *gallolyticus* has the opportunity to establish endocarditis in susceptible patients. For endocarditis, it is known that bacterial binding to extracellular matrix proteins and biofilm formation are important characteristics to facilitate survival of bacterial vegetations on damaged or prosthetic heart valves [31, 32]. As shown in Figure 4, all *S. gallolyticus* subsp *gallolyticus* strains were indeed efficient in forming biofilms on surfaces coated with collagen I or IV (A_{595}, .4–1.4), while this was clearly not the case for uncoated polystyrene surfaces (A_{595}, <.15). In contrast, *S. gallolyticus* subsp *macedonicus*, *S. infantarius* subsp *infantarius,* and *E. faecalis* could form a biofilm on polystyrene surfaces (A_{595}, .2–.4) irrespective of the presence of collagens. In contrast, the probiotic bacterium *L. plantarum* did not form a biofilm under any of the tested conditions. These data demonstrate that *S. gallolyticus* subsp *gallolyticus* strains have exclusive features that enable them to form biofilms on collagen-rich surfaces.

DISCUSSION

In the present study, we reconstructed the route of gut-borne bacterial infections in patients with CRC. Basically there are 4 key events in establishing endocarditis from the intestinal tract: (1) fixing a dependable connection with the enterocyte or its extracellular matrix, (2) translocation of the epithelial barrier, (3) evasion of immune cells in the lamina propria, and (4) survival in the bloodstream and ability to establish a secondary infection. By comparative bacterial virulence analysis, we provided new clues on the underlying mechanism that specifically causes the increased incidence of clinical *S. gallolyticus* subsp *gallolyticus* infections in patients with CRC.

Focusing on the initial step of gut-borne infections, adhesion of *S. gallolyticus* subsp *gallolyticus* to epithelial cells can be categorized as low compared with related *S. bovis* strains and other intestinal bacteria. Genome exploration of *S. gallolyticus* subsp *gallolyticus* revealed that it contains a capsular operon that is highly similar in its organization to *S. pneumonia* serotype 23F [17], whereas *S. gallolyticus* subsp *macedonicus* contains a different capsule operon (P. Glaser, unpublished data). The diverse surface structures that are likely to determine the distinct adhesive properties of *S. gallolyticus* subsp *gallolyticus* and *S. gallolyticus* subsp *macedonicus* to epithelial cells are clearly visualized by electron microscopy (Figure 5). In general, capsular polysaccharides are known to negatively affect bacterial adhesion to host cells but may also shield the bacterial cell from the immune system and thereby be an important virulence factor [23, 33, 34]. In fact, encapsulation of *S. gallolyticus* subsp *gallolyticus* strains has already been shown to contribute to virulence in pigeons [23].

Our data clearly showed that *S. gallolyticus* subsp *gallolyticus* can translocate across an intestinal epithelial layer, whereas it was unable to invade epithelial cells as do pathogenic bacteria, such as *Salmonellae* [24]. Genome exploration of *S. gallolyticus* subsp *gallolyticus* revealed that this bacterium contains 3 pilus operons [17] with homology to the pilus backbone of group B streptococci that are known mediators of paracellular translocation [25, 35]. Strikingly, none of these operons are present in the genome of *S. gallolyticus* subsp *macedonicus* (P. Glaser,

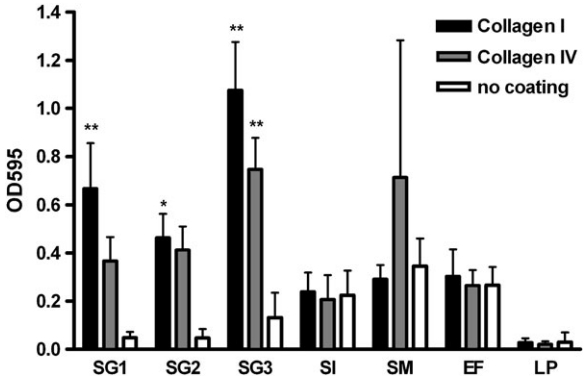

Figure 4. Bacterial biofilm formation. Biofilm formation of gram-positive bacteria on uncoated polystyrene and collagen type I– and type IV–coated surfaces. $^{*}P < .05$; $^{**}P < .01$ (2-way analysis of variance with Bonferroni posttests). *EF, Enterococcus faecalis*; *LP, Lactobacillus plantarum*; *SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius*; *SM, S. gallolyticus* subsp *macedonicus*.

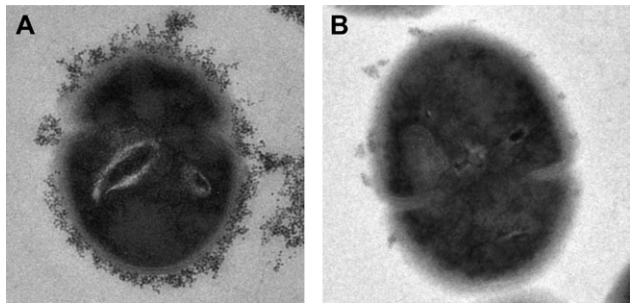

Figure 5. Distinct surface structure of *Streptococcus bovis* strains. Electron micrographic image of representative cells from the *Streptococcus gallolyticus* subsp *gallolyticus* UCN34 (*A*) and *S. gallolyticus* subsp *macedonicus* (*B*) strains, illustrating the different surface structures of these closely related strains.

unpublished data), for which no translocation was observed. These data suggest that pilluslike surface structures of *S. gallolyticus* subsp *gallolyticus* are important determinants for entry into the human body.

The third crucial step to establish an infection is the escape from the host immune system. On passage of the intestinal wall by a pathogen, immune cells in the lamina propria are normally alerted by the production of (for example) interleukin 8 and 1β originating from epithelial cells [36, 37]. In this study, we showed that epithelial cells were relatively unresponsive to *S. gallolyticus* subsp *gallolyticus* compared with other intestinal bacteria, which will delay *IL-8* and *IL-1β* gene expression on its infection. Notably, a functional Toll-like receptor 2 pathway is present in Caco-2 cells [38, 39], which is underscored by *IL-8* and *IL-1β* induction after exposure to *E. faecalis*. This implicates that the unresponsiveness to *S. gallolyticus* subsp *gallolyticus* is not due to lack of Toll-like receptor 2–mediated recognition of gram-positive bacteria. In contrast, macrophages infected by *S. gallolyticus* subsp *gallolyticus* yielded immune responses similar to other bacterial strains, and phagocytosed *S. gallolyticus* subsp *gallolyticus* were unable to escape from macrophages. Together, these findings suggests that the increased incidence of these infections in patients with CRC relates (in part) to a reduced epithelial immune response to these bacteria and subsequent delayed recruitment of tissue macrophages but not to resistance to macrophage-mediated killing itself, which increases the chance that *S. gallolyticus* subsp *gallolyticus* will reach the circulation after translocation of the bowel wall.

The final phase in the infective process toward bacterial endocarditis is survival in the bloodstream and infection of the heart endothelium. In general, gram-positive bacteria are relatively resistant to complement killing [40]. The fact that silent *S. gallolyticus* subsp *gallolyticus* infections can occur is nicely illustrated by the fact that this bacterium was found in the blood of a "healthy" blood donor who appeared to have a colon malignancy upon endoscopic examination [41]. Furthermore, we have shown elsewhere that patients with early-stage CRC can have increased antibody titers against *S. gallolyticus* subsp *gallolyticus* antigens without clinical signs of infection [42, 43]. Our observation that these bacteria have an advantage over other gram-positive intestinal bacteria in forming biofilms on collagen types I and IV may be crucial for explaining the pathology of *S. gallolyticus* subsp *gallolyticus* endocarditis. The aforementioned pilus structures may also, in addition to their role in paracellular transport, play an important role in the binding of these bacteria to extracellular matrix proteins [31, 44, 45], which is especially evident from the fact that one of the *S. gallolyticus* subsp *gallolyticus* pilus operons encodes a collagen-binding protein [17, 32]. Thus, a prerequisite for *S. gallolyticus* subsp *gallolyticus* to establish a clinical infection in patients with CRC seems to be the coincidental presence of collagens at the secondary infection site, including damaged heart valves, hepatic cirrhosis, and total knee replacements [46], which could explain the low co-occurrence of *S. gallolyticus* subsp *gallolyticus* infections in patients with CRC (estimated at <.1%). Although *S. infantarius* subsp *infantarius* has a similar translocation efficiency, it lacks the improved ability to form biofilms on collagen, in line with the fact that *S. infantarius* subsp *infantarius* is less often found in endocarditis but is more often the cause of bacteremia in patients with CRC [47].

Based on our current comparative virulence analysis, the most outstanding characteristic of *S. gallolyticus* subsp *gallolyticus* is its ability to form biofilms on collagen-rich surfaces. This finding inspired us to mine the scientific literature for additional links between *S. gallolyticus* subsp *gallolyticus*, collagens, and CRC. Intriguingly, this yielded previous histologic observations that polyps and early colorectal tumors are characterized by a continuous expression of collagen type IV in basement membranes that surround the crypts in the mucosa of hyperplastic polyps [48]. Accordingly, the collagen type IV containing basement membrane showed sawlike protrusions into the basal parts of the adenomatous epithelium [49]. In this situation, opportunistic pathogens such as *S. gallolyticus* subsp *gallolyticus,* which have low adhesion to epithelial cells, could gain a competitive advantage in colonizing these (pre)malignant sites. This may very well explain why these bacteria colonize only 10% of the normal population, compared with >55% of patients with CRC [14]. Furthermore, recent molecular analyses have indeed pointed toward increased colonization of CRC tissue by *S. gallolyticus* subsp *gallolyticus* strains [50] (unpublished observations).

Summarizing the above, we hypothesize that 2 surface features of *S. gallolyticus* subsp *gallolyticus* are the main determinants for its specific association with endocarditis and CRC. First, it contains a polysaccharide capsule that lowers its adhesive capabilities to epithelial cells, but this same capsule allows it to stay invisible for the host immune system for a prolonged period of time. Second, *S. gallolyticus* subsp *gallolyticus* contains piluslike structures that facilitate colonization of polyps and adenomatous epithelium, paracellular translocation and the formation of resistant vegetations on collagen-rich sites. A model that summarizes these virulence features is depicted in Figure 6. It goes without saying that our experiments did not fully mimic the complex ecosystem of the gut or the human immune system and that future studies should concentrate on the actual role of the virulence factors highlighted here (ie, capsular polysaccharides and pili) in invasion, immune evasion, or biofilm formation. Preferably this would be done by evaluating mutant *S. gallolyticus* subsp *gallolyticus* strains in live infection models. Nevertheless, our in vitro approach allowed us to gain new insights into the infective mechanisms used by *S. gallolyticus* subsp *gallolyticus* in patients with CRC, which provides clear leads for these future explorations. Finally, our study underscores the importance of proper microbiologic

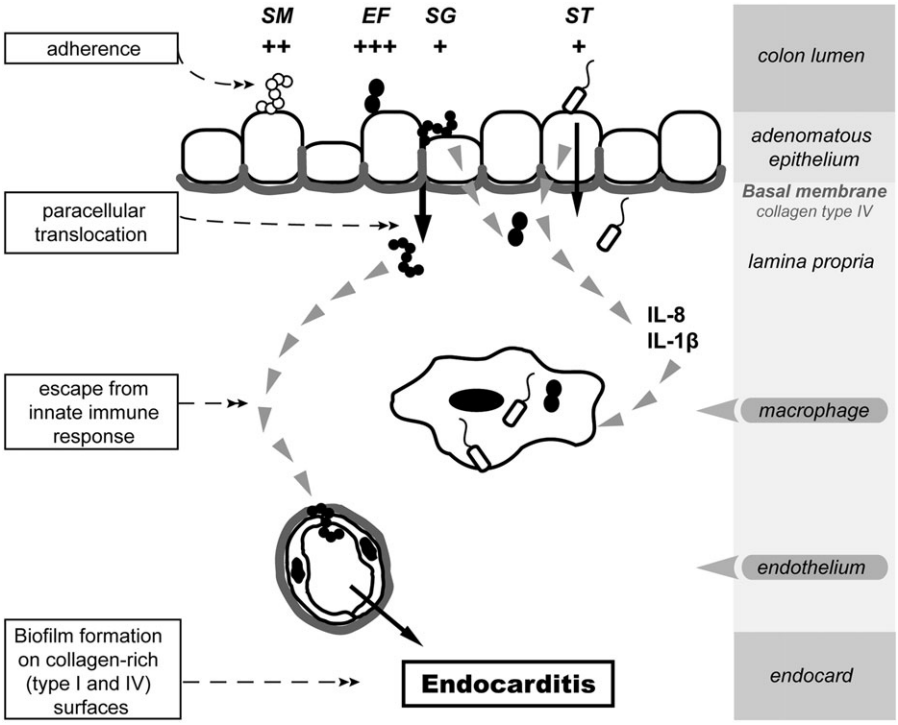

Figure 6. Model for specific association of *Streptococcus gallolyticus* subsp *gallolyticus* (*SG*) endocarditis with colorectal cancer (CRC). Based on our data and that of others, we postulate that *S. gallolyticus* subsp *gallolyticus* is an inefficient colonizer of a healthy intestinal tract and that it benefits from adenomatous epithelial tissue with displaced collagen type IV expression to translocate the epithelium via a paracellular mechanism. In comparison, the closely related strain *S. gallolyticus* subsp *macedonicus* (*SM*) is very effective in adhesion but unable to cross an epithelial layer. On infection, *S. gallolyticus* subsp *gallolyticus* is relatively invisible for the epithelial immune system causing a delayed recruitment of tissue macrophages, which increases its chances to reach the bloodstream. In contrast, other invading bacteria, such as *Salmonella typhimurium* (*ST*) and *Enterococcus faecalis* (*EF*), induce a (strong) epithelial immune response in which these infections are readily cleared by attracted macrophages. It is important to note that *S. gallolyticus* subsp *gallolyticus* infections remain subclinical in most individuals owing to low virulence in humans. However, in a very small fraction of patients with CRC and coincidental collagen depositions at, for example, damaged heart valves, these infections can become clinically manifest through the effective formation of resistant bacterial vegetations, which present as endocarditis. IL, interleukin.

classification of *S. bovis* subspecies. Because only *S. gallolyticus* subsp *gallolyticus* seems to have virulence characteristics that clearly associate endocarditis with underlying colon malignancies, the specific diagnosis of *S. gallolyticus* subsp *gallolyticus* infection might become a valuable tool for the early detection of CRC.

Supplementary Data

Supplementary data are available at http://www.oxfordjournals.org/our_journals/jid/online.

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