ADDENDUM

The Pil3 pilus of Streptococcus gallolyticus binds to intestinal mucins and to fibrinogen

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\section*{ABSTRACT}

\textit{Streptococcus gallolyticus} is a commensal bacterium responsible for infectious endocarditis in the elderly, which has frequently been associated with colonic carcinoma. Whether this species is a cause or a consequence of colorectal cancer remains unknown. We recently demonstrated that \textit{S. gallolyticus} Pil3 pilus is required for adhesion to colonic mucus and for colonization of mouse distal colon. We show here that Pil3 pilus binds equally well to human colonic mucins derived from HT29-MTX cells and to human stomach mucins from healthy donors. In addition, we have found that Pil3 also binds to human fibrinogen, which expands the repertoire of Pil3 host ligands.

\section*{KEYWORDS}

colon; colonization; fibrinogen; MUC2; MUC5AC; mucins; Pil3 pilus; \textit{S. bovis}; \textit{S. gallolyticus}

\section*{Addendum}

\textit{Streptococcus gallolyticus} subsp. \textit{gallolyticus}, formerly known as \textit{S. bovis} biotype I\textsuperscript{,1,2} is one of the few opportunistic pathogens which has been unambiguously linked to colorectal cancer (CRC)\textsuperscript{,3-10} This bacterium is responsible for a growing number of infective endocarditis (IE) and septicemia cases in elderly and immunocompromised individuals\textsuperscript{,11-16} Firstly isolated from Koala feces and named for its capacity to degrade host tannins\textsuperscript{,17} \textit{S. gallolyticus} is a common inhabitant of the rumen flora of herbivores. This species has also been found in the gastrointestinal (GI) tract of a wide spectrum of animals,\textsuperscript{17-21} including marsupials, mammals and birds. In humans, \textit{S. gallolyticus} is detected at a low carriage rate (2.5 to 15\%) from stool samples\textsuperscript{,3-5} In 1977, Klein et al reported that patients displaying colonic neoplasia had an increased level (up to 5-fold) of \textit{S. gallolyticus} in fecal samples as compared to similar samples from healthy individuals.\textsuperscript{,3} In 2010, Abdulamir et al. provided the first molecular evidence for a specific enrichment of \textit{S. gallolyticus} in CRC-mucosal tissues as compared to healthy tissue.\textsuperscript{,5} Nevertheless, it remains unclear whether \textit{S. gallolyticus} is taking advantage of the tumor environment to outcompete other microbiota species or if it can promote colonic transformation through other unknown mechanisms.\textsuperscript{22,23}

Bacterial colonization of host tissues is often considered to be a crucial step for the establishment of a successful infection. Adhesion to host cells is mediated by surface-exposed adhesins, which can be found in Gram-positive bacteria at the tip of long covalent polymers of covalently bound proteins called pili.\textsuperscript{24} \textit{S. gallolyticus} reference strain UCN34,\textsuperscript{25} recovered from the blood of a 70 year-old patient with IE and subsequently diagnosed for CRC, possesses 3 pilus loci: pil1, pil2 and pil3. Among these, pil1 and pil3 are the most conserved loci in clinical isolates.\textsuperscript{26} We previously showed that Pil1 pilus allows \textit{S. gallolyticus} to bind to collagen type I, promoting IE in a rat model of experimental endocarditis.\textsuperscript{27} These surface-exposed structures are highly immunogenic and have been proposed as vaccine candidates in pathogenic streptococci.\textsuperscript{28} Interestingly, Pil1 is expressed heterogeneously at the single bacterium level by a novel regulatory mechanism in the promoter region,
combining phase variation in the leader peptide and transcriptional attenuation. Some evidence suggests that heterogeneity in Pil1 expression confers a fitness advantage to the bacterial population as it alleviates selective pressure from the host immune system.

How does \textit{S. gallolyticus} interact with the human colon was unexplored until our recent work. We previously developed a mouse model of gut colonization by HT29-MTX cells. Pil3 is composed of 2 subunits, the putative adhesin Pil3A and the backbone pilin Pil3B, forming a surface-exposed appendage. As for Pil1, Pil3 was found to be expressed heterogeneously at the single cell level in UCN34 as well as in other clinical isolates.

Pil3A was shown to mediate \textit{S. gallolyticus} binding to HT29-MTX cells derived mucus. Interestingly, no significant binding was observed when using bovine maxillary mucins (data not shown), which suggests specific traits in host mucins serving as Pil3 ligand. We previously developed a mouse model of gut colonization by \textit{S. gallolyticus} requiring pre-treatment of C57BL/6 mice with a cocktail of antibiotics to reduce endogenous microbiota and repeated oral inoculation with the strain UCN34. This protocol resulted in significant colonization of mice intestinal tissues (small intestine, cecum, colon). The UCN34Δpil3 mutant was significantly impaired (2-log reduction) in distal colon colonization as compared to a Pil3-overexpressing variant (Pil3+). Examination by confocal microscopy of mouse intestinal tissues showed that \textit{S. gallolyticus} localized within the colonic mucus layer. These in vivo experiments revealed the importance of Pil3 pilus for the colonization of mouse distal colon.

**Role of Pil3 pilus in the interaction with human mucins and extracellular matrix (ECM) components**

We previously showed that the adhesin Pil3A binds to MUC5AC mucin isolated from HT29-MTX cells. HT29-MTX is a human colonic cell line of particular relevance, because in contrast to other colonic cell lines such as HT29, Caco-2 or T84, it secretes a thick mucus layer upon differentiation for 15 to 20 days. Among all glycoproteins, mucins, which are major components of the mucus gel covering and protecting the intestinal epithelial layer, are frequently altered in CRC. Indeed, specific mucins (e.g. MUC5AC) can be overexpressed and differentially glycosylated in CRC, and HT29-MTX is the cell line that better mimics this tumor characteristic. In healthy conditions, MUC5AC mucin is only expressed in the stomach and not in the colon, whereas MUC2 is the predominant mucin in the colon. Interestingly, aberrant and mislocalized expression of MUC5AC mucin in colonic adenomas and carcinomas has been reported as well as modification of mucins glycosylation patterns during colonic carcinogenesis.

To investigate \textit{S. gallolyticus} ligand preferences, we tested the binding capacities of UCN34 WT, Pil3+ and Δpil3 strains to human mucins isolated from colon (MUC2) and stomach (MUC5AC) of healthy donors. As shown in Figure 1A, Pil3 pilus was equally able to interact with MUC2 and MUC5AC. Binding of Pil3+ strain to purified mucins was significantly increased when compared to WT and Δpil3 strains. We hypothesized that binding of Pil3 pilus to MUC2 mucin may be important for \textit{S. gallolyticus} commensal colonization, whereas binding to MUC5AC mucin could provide a colonization advantage over other microbiota species in the context of colonic tumor environment. This might contribute to the higher prevalence of \textit{S. gallolyticus} in patient colorectal tumor tissues.

Next, we tested the capacity of UCN34, Pil3+ and Δpil3 strains to interact with other ECM components, such as human fibrinogen and fibronectin. As shown in Figure 1B, Pil3+ strain adhered more efficiently to fibrinogen compared to UCN34 and Δpil3. No significant difference was found between the strains in fibronectin binding. Hence, Pil3 pilus contributes to the binding of \textit{S. gallolyticus} to fibrinogen, but not to fibronectin. Binding to fibrinogen contributes to the development of endovascular infections through multiple events: increased biofilm formation, adherence to endothelial cells and most importantly binding to the surface of human platelets. Since cardiac valve endothelial lesion activates platelet recruitment, this allows the bacteria attached to the platelets via fibrinogen cross-bridges, to colonize these injured sites then contributing to IE development. Thus, Pil3 pilus of \textit{S. gallolyticus} might also be important in a later stage of infection, such as survival in the blood or in adhesion to platelets through fibrinogen-mediated cross-bridging, thereby contributing in concert with Pil1 pilus to the development of IE.
Pil3A binds to mucins and to fibrinogen through its amino-terminal part

We previously showed that heterologous expression of Pil3A in Lactococcus lactis strain NZ9000 conferred to the recombinant bacteria an enhanced capacity for binding to colonic mucins, as compared to the control L. lactis strain harboring the empty vector. Pil3A is a large LPXTG protein of 1664 amino acids containing a putative mucus-binding domain (position 1154 to 1238). In order to identify the binding domains of Pil3A, the amino-terminal region (from AA 43 to 847) and the C-terminal region (from AA 848 to 1627) were cloned in the shuttle vector pAT28-covSP containing a promoter, a signal peptide, a spacer and an LPXTG motif allowing expression, secretion and cell wall anchoring of each domain separately (Pil3A-N or Pil3-C). Recombinant plasmids were introduced by electroporation into L. lactis strain NZ9000. We first checked the expression of the Pil3A-N and Pil3A-C at the surface of L. lactis by flow cytometry using specific antibodies raised against the N- or the C-terminal part of Pil3A. As shown in Figure 2A, each fragment of Pil3A is expressed properly at the surface of L. lactis and recognized by the cognate antibody. We then tested the capacity of these recombinant bacteria to bind to HT29-MTX mucus and to fibrinogen. As shown in Figure 2B, only the N-terminal domain of Pil3A confers enhanced binding to mucus and to fibrinogen as compared to the control L. lactis strain harboring the empty vector. These results question the significance of the mucin-binding domain identified in silico located in the C-terminal part of Pil3A.

How does S. gallolyticus cause disease?

S. gallolyticus is an opportunistic pathogen responsible for increased number of septicemia and/or IE in the elderly and immunocompromised individuals. Strong evidences indicate a link between the presence of S. gallolyticus and the occurrence of CRC in humans. Whether this bacterium is the cause or the consequence of CRC is not known. Although highly
prevalent in the rumen of herbivores, S. gallolyticus is considered as a weak colonizer of the human GI characterized by a low fecal carriage ranging around 5–10%. Recent experimental data indicate that metabolic alterations associated to CRC may favor S. gallolyticus outgrowth in this environment. In addition, physiological alterations occurring during CRC, such as the expression of mislocalized MUC5AC mucin and displaced epithelium with exposed collagen IV, could favor adhesion of S. gallolyticus through Pil3 and Pil1 pili respectively, and thereby explain the increased numbers of S. gallolyticus bacteria at these particular sites. CRC conditions may also increase the ability of S. gallolyticus to translocate through the epithelial barrier and reach the lamina propria. Mimicking this route of infection, Boleij et al have shown experimentally that S. gallolyticus is able to cross the intestinal barrier using a paracellular route. Moreover, the same study showed that S. gallolyticus remains quite invisible or silent to the host immune system (no IL8 and IL1-β-dependent pro-inflammatory responses), which in turn increases the chances of the bacteria to reach the bloodstream – Then, heterogeneity in Pil1 and Pil3 expression probably attenuates exposure and pressure from the immune system. Once in the bloodstream, S. gallolyticus expressing Pil3 probably binds to fibrinogen highly enriched in human plasma, which in turn will contribute to host-bacteria interactions in unknown manner. Pil1-expressing S. gallolyticus cells are then able to

Figure 2. Adhesion of recombinant L. lactis expressing Pil3A to mucus and to fibrinogen. (A) Surface display of Pil3A-N (black line) or Pil3A-C (gray line) regions in recombinant L. lactis was determined by flow-cytometry using specific polyclonal antibodies directed against the amino- (Pil3A_Nter) or carboxyl- (Pil3A_Cter) part of Pil3A. The negative control (L. lactis carrying the empty vector) is shown in dotted line. In this representative experiment, 10,000 bacteria were analyzed for each strain. (B) Recombinant L. lactis strains expressing only the first 800 aa of Pil3A (N-Pil3A) displays higher binding to human HT29-MTX mucus and to fibrinogen as compared to the respective controls harboring the empty vector. Values indicate the mean of 3 independent experiments assayed in triplicate. Statistical analysis was performed using a 2-way ANOVA test (**p < 0.01; ***p < 0.001).
colonize collagen I rich surfaces, such as damaged heart valves and cause IE. This physiopathological scenario is summarized in Figure 3.

The contribution of *S. gallolyticus* to CRC certainly needs further experimental exploration using relevant animal models. Identification and characterization of *S. gallolyticus* specific factors contributing to pathogenesis will definitely help in the development of new strategies for CRC diagnosis, treatment and prevention.

**Abbreviations**

AA Amino acids  
CRC Colorectal cancer  
ECM Extracellular matrix  
FG Fibrinogen  
GI Gastrointestinal  
IE Infective endocarditis

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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