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To cite this version:
Camille Danne, José M Entenza, Adeline Mallet, Romain Briandet, Michel Débarbouillé, et al.. Molecular characterization of a Streptococcus gallolyticus genomic island encoding a pilus involved in endocarditis.. Journal of Infectious Diseases, Oxford University Press (OUP), 2011, 204 (12), pp.1960-70. .

HAL Id: hal-01000633
https://hal.archives-ouvertes.fr/hal-01000633
Submitted on 5 Jun 2014
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Molecular Characterization of a *Streptococcus gallolyticus* Genomic Island Encoding a Pilus Involved in Endocarditis

Camille Danne, José M. Entenza, Adeline Mallet, Romain Briandet, Michel Débarbouillé, Farida Nato, Philippe Glaser, Grégory Jouvin, Philippe Moreillon, Patrick Trieu-Cuot, and Shaynoor Dramsi

**Background.** *Streptococcus gallolyticus* is a causative agent of infective endocarditis associated with colon cancer. Genome sequence of strain UCN34 revealed the existence of 3 pilus loci (*pil1*, *pil2*, and *pil3*). Pili are filamentous structures playing a key role as adhesive organelles in many pathogens. The *pil1* locus encodes 2 LPXTG proteins (Gallo2178 and Gallo2179) and 1 sortase C (Gallo2177). Gallo2179 displaying a functional collagen-binding domain was referred to as the adhesin, whereas Gallo2178 was designated as the major pilin.

**Methods.** *S. gallolyticus* UCN34, Pil1+, and Pil1-, expressing various levels of *pil1*, and recombinant *Lactococcus lactis* strains, constitutively expressing *pil1*, were studied. Polyclonal antibodies raised against the putative pilin subunits Gallo2178 and Gallo2179 were used in immunoblotting and immunogold electron microscopy. The role of *pil1* was tested in a rat model of endocarditis.

**Results.** We showed that the *pil1* locus (gallo2179-78-77) forms an operon differentially expressed among *S. gallolyticus* strains. Short pilus appendages were identified both on the surface of *S. gallolyticus* UCN34 and recombinant *L. lactis*-expressing pil1. We demonstrated that Pil1 pilus is involved in binding to collagen, biofilm formation, and virulence in experimental endocarditis.

**Conclusions.** This study identifies Pil1 as the first virulence factor characterized in *S. gallolyticus*.

*Streptococcus gallolyticus* subsp *gallolyticus* (formerly known as *S. bovis* biotype I) is an increasing cause of infective endocarditis (IE). Asymptomatic carriage of *S. gallolyticus* is commonly observed in the gastrointestinal tract of birds, ruminants, and a small proportion of humans (2.5%–15%) [1, 2]. Several studies have shown that endocarditis due to *S. gallolyticus* are frequently associated with colorectal carcinoma [1,3–6]. Whether the development of tumors is a cause or a consequence of *S. gallolyticus* infections remains to be investigated. To address this question, a better understanding of the pathophysiology of these diseases is required with a focus on the bacterial virulence factors responsible for the initiation of infections, that is, adhesion to host tissues.

The gallate-degrading strains of *S. bovis* group have been reassigned to a new species named *S. gallolyticus* sp [7–9]. Customarily, human isolates of *S. bovis* were classified into 3 biotypes designated as I, II/1, and II/2 that corresponds to *S. gallolyticus* subsp *gallolyticus*, the closely related subspecies *pasteurianus*, and the more distant subspecies *infantarius*, respectively. Finally, *Streptococcus macedonicus* is considered as a nonpathogenic *S. gallolyticus* subspecies. However, this taxonomic recategorization is still a matter of debate, and for simplification and readability these subspecies will be considered as species throughout this work.
Although the proportion of IE due to *S. galolyticus* has increased among streptococci, particularly in Southern Europe [10–12], its virulence and colonization factors remain largely unknown. Five serotypes have been described based on capsular typing in strains isolated from pigeons [13]. Electron microscopic studies of *S. galolyticus* pigeon strains revealed the existence of filamentous structures known as fimbriae or pili [14]. It was hypothesized that these structures could play a role in virulence. Bacterial pathogens associated with IE possess surface adhesins belonging to MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which mediate attachment to cardiac vegetations and are involved in valve colonization and infection [15]. Previously characterized collagen-binding proteins include Cna of *Staphylococcus aureus* [16], Acm of *Enterococcus faecium* [17], Ace of *E. faecalis* [18, 19], and Acb from *S. galolyticus* strain TX20005 [20]. Development of endocarditis is initiated by injury of the endothelium, which disrupts the normal valve structure and exposes underlying tissues, including extracellular matrix (ECM) material. Deposition of host proteins, such as fibrin and platelets, then leads to the formation of a sterile thrombotic vegetation that may become colonized by circulating bacteria [21].

*S. galolyticus* isolates responsible for IE were shown to display heterogeneous profiles of adherence to ECM proteins [22–24]. We recently participated in the complete sequencing of *S. galolyticus* strain UCN34, isolated from a human IE case [25]. It was recently reported that *S. galolyticus* strain UCN34 was also found in an intravenous catheter [26]. In silico analyses enabled the identification of 19 putative cell wall-anchored proteins. We focused on 3 putative pilus loci, each encoding 1 sortase C and 2 LPXTG motif proteins, referred herein as pil1 (gallo2179-77), pil2 (gallo1570-68), and pil3 (gallo2040-38). It was recently reported that *S. galolyticus* strain TX20005 (draft genome) also encodes 3 pilus loci [20]. Interestingly, only 2 of these loci are identical in both *S. galolyticus* strains, namely pil1 (gallo-sbs7-srtC1 in TX20005) and pil3 (sbs15-sbs14-srtC2 in TX20005). Gram-positive pili were first observed in *Corynebacterium renale* by electron microscopy [26] and have now been characterized genetically and biochemically in many important pathogens, for example *Streptococcus agalactiae* [27–30]. These pili consist of covalently cross-linked subunit proteins and are anchored to the peptidoglycan (for reviews, see [31, 32]). Sortase-mediated pilus assembly was first demonstrated in *Corynebacterium diptheriae* [33, 34], and the current model for pilus biogenesis is as follows: the major subunit is assembled into the pilus by a cis-encoded class C sortase that catalyzes the covalent attachment between the conserved lysyl residue of the pilus motif (WxXVxVYPK) of one subunit and the conserved threonyl residue of the LPXTG motif of another subunit [35]. In addition, one or more accessory subunits could also be incorporated into the pilus backbone [36].

Here, we studied the *pil1* pilus locus of *S. galolyticus* UCN34, which is present in 90% of *S. galolyticus* IE clinical isolates but absent in the closely related nonpathogenic species *S. macedonicus* (P. Glaser, unpublished data). Moreover, *pil1* was predicted to encode a pilus made of 2 pilin subunits: the major pilin (Gallo2178) and the adhesin (Gallo2179), which displays a functional collagen-binding domain. We identified pilus appendages on the surface of *S. galolyticus* and tested the role of Pil1 pilus in adhesion to ECM proteins, biofilm formation, and virulence in a rat model of endocarditis.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

Bacterial strains, plasmids, and primers are listed in Tables 1 and 2. *S. galolyticus* were grown at 37°C in Todd–Hewitt broth, in standing filled flasks. *L. lactis* strain NZ9000 [46] was grown in M17 medium supplemented with 1% glucose. Heterologous expression of *pil1* in *L. lactis* strain was realized as follows: an ~5-kilobase DNA fragment containing the 3 genes gallo2179-2178-2177 was amplified from UCN34 genomic DNA with primers pilUCN34-fwd and pilUCN34-rev (Table 2), digested by *Bam*H1 and *Nsi*I (New England Biolabs) and cloned into the high-copy-number erythromycin resistance shuttle vector pOri23 [38] digested by *Bam*H1-Pst1. After ligation, the resulting plasmid pOri23Ω*pil1* was introduced into electrocompetent *L. lactis* NZ9000 cells. Unless otherwise specified, antibiotics were used at the following concentrations: for *Escherichia coli*, 150 μg/mL erythromycin; for *S. galolyticus*, 10 μg/mL tetracycline; and for *L. lactis*, 5 μg/mL erythromycin.

**Real-time Polymerase Chain Reaction**

Total RNA (15 μg) were extracted and treated as described elsewhere [27]. Quantitative real-time polymerase chain reaction (PCR) analysis was performed as described elsewhere [27] with gene-specific primers (Table 2).

**Cell Wall Protein Extracts**

Bacteria were grown in Todd–Hewitt medium at 37°C and harvested for protein analysis during late exponential phase of culture. Cell wall extracts were prepared as described elsewhere [27].

**Expression and Purification of Recombinant 6xHis-Gallo2178 and 6xHis-Gallo2179**

DNA fragments internal to gallo2179 and gallo2178 were produced by PCR using genomic DNA of UCN34 as the template and the primers gallo2179-Nhe1 and gallo2179-BamH1, and gallo2178-Nhe1 and gallo2178-BamH1, respectively (Supplementary Table 1). These DNA fragments were digested with the appropriate enzymes (Nhe1 and *Bam*H1) and cloned into pET28-a+(Novagen). The resulting plasmids were introduced into *E. coli* strain DH5α for sequence analysis or BL21(DE3)
for protein expression. Recombinant 6xHis-Gallo2178 and 6xHis-Gallo2179 were purified under native conditions by affinity chromatography on nickel–nitrilotriacetic acid columns according to the manufacturers’ recommendations (Novagen). Protein purity was checked on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were determined with the BCA kit (Thermo Scientific).

**Generation of Rabbit and Mice Polyclonal Antibodies**

Rabbit polyclonal antibodies (pAbs) against Gallo2178 were generated by Covalab. For double-labeling experiments, pAbs against Gallo2179 were generated in mice using a very similar procedure except for the quantity of the antigen injected: 10 μg per boost in each mouse, with a total of 4 boosts at 2-week intervals.

**Immunoblots**

For analysis of Gallo2178 and Gallo2179 expression, cell wall proteins were boiled in Laemmli sample buffer, resolved on Tris-Acetate Criterion XT gradient gels (4–12% SDS-PAGE gels), and transferred to nitrocellulose membrane (Hybond-C; Amersham). Gallo2178 and Gallo2179 were detected using specific pAbs and horseradish peroxidase–coupled anti-rabbit or anti-mouse secondary antibodies (Zymed) and the West Pico Chemiluminescence kit (Thermo Scientific). Image capture and analysis were performed with a GeneGnome imaging system (Syngene).

**Immunogold Electron Microscopy**

Bacteria were grown in appropriate medium and collected after overnight growth. Scanning electron micrographs were produced as described elsewhere [28].

**Adherence Assay and Biofilm Formation**

Bacterial attachment and surface growth on microtiter plates were determined during growth of *S. gallolyticus* or recombinant *L. lactis* in Luria broth medium supplemented with 1% glucose and erythromycin when necessary. The 96-well polystyrene plates were coated with 0.1 mg/mL collagen I (rat tail; BD Biosciences) diluted in 1/3 phosphate-buffered saline overnight at 4°C. Overnight cultures grown in Todd–Hewitt were used to inoculate Luria broth glucose medium at an optical density at 600 nm of 0.1, and, after a brief vortexing, 180 μL of cell suspension was dispensed into 96-well plates (Costar 3799; Corning) and incubated at 37°C for 2 or 24 hours. Adherent bacteria were stained with 0.1% crystal violet as described elsewhere [27]. The assay was performed in quadruplicate and repeated in ≥3 independent experiments.

For confocal laser-scanning microscopy (CLSM) analysis of biofilm architecture, a similar cultivation procedure was applied in 96-well polystyrene microtiter plate, which allowed for high-resolution imaging (Greiner Bio-one; microplates with a μClear base of 190 ± 5 μm thickness), as described elsewhere [47].
Rat Model of IE
Sterile aortic vegetations were produced in female Wistar rats by insertion of a catheter through the aortic valve, as described elsewhere [48]. The catheter was left in place throughout the experiment. Groups of animals were inoculated intravenously with $10^3$ or $10^4$ colony-forming units (CFU) for *S. gallolyticus* UCN34 or $10^6$ CFU for *L. lactis* NZ9000/pOri23 and *L. lactis* NZ9000/pOri23 X pil1 prepared from overnight cultures. These inoculum sizes allowed the determination of the 90% infectivity rate (ID$_{90}$) of *S. gallolyticus* and permit differentiation in *L. lactis* virulence, based on previous studies in the same model [43, 45]. Rats were euthanized 24 hours after inoculation, aortic vegetations were removed, homogenized in 1 mL of saline, serially diluted, and plated for colony counts. Statistic analyses were performed using the $\chi^2$ test, and differences were considered significant at $P < .05$.

RESULTS AND DISCUSSION

Genetic Organization of the pil1 Locus in Strain UCN34
The pil1 locus of *S. gallolyticus* UCN34 is composed of 3 genes encoding 2 LPXTG proteins (*gallo2179* and *gallo2178*) and 1 sortase C (*gallo2177*) (Figure 1A). Such a simple genetic organization has been reported only in *Actinomyces naeslundii* [37]. The majority of previously characterized pilus loci in other gram-positive bacteria, such as other streptococci and enterococci, consist of 3 structural pilus genes, 1 coding for the major pilus subunit and 2 for accessory subunits acting as tip adhesin and basal anchor, and 1–3 class C sortases. A transcriptional regulatory gene is often found upstream and divergently transcribed from the pilus operon. The structural proteins Gallo2178 and Gallo2179 possess the characteristic features of pilin subunits, which is a signal peptide at the N-terminus, an LPXTG motif at its C-terminus, and a pilin motif PK in the central part. A search for conserved domains revealed the presence of structural CnaB domains (Pfam 05738) in both proteins. The genes surrounding this pilus gene cluster, *gallo2178* and *gallo2179* possess the characteristic features of pilin subunits, which is a signal peptide at the N-terminus, an LPXTG motif at its C-terminus, and a pilin motif PK in the central part. The proteins Gallo2178 and Gallo2179 possess the characteristic features of pilin subunits, which is a signal peptide at the N-terminus, an LPXTG motif at its C-terminus, and a pilin motif PK in the central part. A search for conserved domains revealed the presence of structural CnaB domains (Pfam 05738) in both proteins. In addition, Gallo2179 contains a putative collagen-binding (COL) domain (Pfam 05737). Basic Local Alignment Search Tool (BLAST) analyses showed that Gallo2179 shares strong similarities with other collagen-binding proteins whose prototype is the Cna protein of *S. aureus* [16]. The genes surrounding this pilus gene cluster, *gallo2180* and *gallo2176*, were annotated as genes encoding

### Table 2. Primers Used in Current Study

<table>
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<tr>
<th>Primers</th>
<th>Sequences 5’ → 3’a</th>
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<td>ATTAGGGATCCAGGGAGTGATAAAGTGTTGCT</td>
</tr>
<tr>
<td>pilUCN34-rev</td>
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<td><strong>Recombinant protein production</strong></td>
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<td><strong>Polymerase chain reaction analyses</strong></td>
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<tr>
<td>gallo2177-CD26</td>
<td>CGTAAATCTGCCGTTAACATTTCCAC</td>
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</tbody>
</table>

* Restriction sites are in bold.
transcriptional regulators belonging to the TetR family and trehalose repressor, respectively (Figure 1A).

**Transcription of pil1 Locus in S. gallolyticus**

We first analyzed the transcription levels of pil1 genes in the reference strain UCN34 by quantitative reverse-transcription PCR (qRT-PCR). The 3 genes of pil1 were transcribed at similar levels, suggesting an organization in operon. Complementary RT-PCR experiments using oligonucleotides mapping the intergenic regions were realized (Figure 1B). Only 2 PCR products of 451 and 271 base pairs were obtained, showing that gallo2179, gallo2178, and gallo2177 form an operon.

In the absence of genetic tools allowing the construction of deletion mutants in S. gallolyticus, we searched in our collection for clinical isolates expressing the pil1 locus at levels different from those of our reference strain UCN34. Interestingly, we characterized 1 strain, NEM2470 (designated Pil1\(^1\)), that expresses the 3 genes of pil1 operon at a higher level (gallo2179, 5.8-fold, gallo2178, 7-fold, gallo2177, 8.5-fold) than that of UCN34. We found another strain, NEM2474 (designated Pil1\(^2\)), that does not express pil1.

**pil1-Encoded Proteins Gallo2178 and Gallo2179 Form Polymers of High Molecular Weight**

Pil1 pilus biogenesis was assessed by Western blotting of cell wall protein extracts from S. galloyticus strains UCN34, Pil1\(^1\), and Pil1\(^-\) using specific antibodies directed against the pilus structural components Gallo2178 and Gallo2179. To unambiguously characterize this operon in isogenic strains, heterologous expression of pil1 was performed in the non-pathogenic Lactococcus lactis NZ9000 by cloning the 3 genes (gallo2179-77) into the shuttle vector pOri23 [38]. Antisera raised against both proteins are highly specific, as demonstrated by the absence of reactive protein in the extracts from S. galloyticus Pil1\(^-\) and from the control strain L. lactis NZ9000/pOri23 (Figure 2A). The antisera raised against the major pilin Gallo2178 recognized high-molecular-weight species both in S. galloyticus UCN34 and Pil1\(^+\) and in the recombinant L. lactis NZ9000/pOri23/p pil1 (Figure 2A). A similar profile of high-molecular-weight species was observed with the antiserum specific for Gallo2179 (Figure 2A). It is noteworthy that protein levels of Gallo2178 and Gallo2179 demonstrated by Western blot analysis in strains UCN34 and Pil1\(^+\) correlate perfectly with findings of qRT-PCR analyses.

**Electron Microscopy Evidence for Pilus Structure**

Immunogold electron microscopy in S. galloyticus UCN34 and Pil1\(^+\) carried out with an antiserum specific for the major pilin Gallo2178 showed that each labeled bacterium possessed a few (1–4) short pilus structures (Figure 3A and 3B). A strong heterogeneity in the population of S. galloyticus expressing the pilin Gallo2178 was observed in both UCN34 and Pil1\(^+\) (Figure 3A).
whereas the Pil1− strain was not labeled (data not shown). *L. lactis* strain expressing pil1 was labeled with the anti-Gallo2178 anti-body, and, interestingly, similar pilus structures were visualized on the bacterial cell surface (arrows, Figure 3J). The control strain *L. lactis* NZ9000/pOri23 was not labeled in the same experimental conditions (Figure 3I). Specific antiserum raised against the putative adhesin Gallo2179 in *S. gallolyticus* Pil1 revealed short homopolymers of Gallo2179 (arrowheads, Figure 3C and 3D). Similar structures were visualized on the surface of the recombinant *L. lactis* strain expressing pil1 (Figure 3K).

To confirm that Gallo2178 and Gallo2179 are part of the same pilus structure, we carried out a double-labeling experiment. *S. gallolyticus* Pil1+ was stained with rabbit anti-Gallo2178 pAb, followed by 10 nm of gold-labeled immunoglobulin IgG, and then with mouse anti-Gallo2179 pAb followed by 20 nm of gold-labeled IgG. Typical heteropolymeric structures containing both pilin subunits, Gallo2178 constituting the core of the pilus and Gallo2179 at the tip, are shown in Figure 3E (inset). However, fibers composed of Gallo2178 only (arrows, Figure 3B, 3F, and 3J), and more surprisingly of Gallo2179 only (arrowheads, Figure 3E and 3K), were also found. Interestingly, the collagen-binding protein Gallo2179 displays a typical pilin motif (PK) in its central part, raising the possibility of 3 types of fibers, that is, homopolymers of Gallo2179 or Gallo2178 and heteropolymers of Gallo2179-2178. To our knowledge, this structural peculiarity has never been described in other pilus-associated adhesins until now. The homologous staphylococcal and enterococcal genes, *cna, acm,* and *ace*, all occur as individual genes [20]. Therefore, Gallo2179 may represent a remarkable example of evolution of an individual adhesin incorporated into a pilus fiber, thus increasing its avidity and affinity for collagen.

**Role of Pil1 Pilus in Primary Attachment to Collagen**

Because Gallo2179 is highly similar to other collagen-binding proteins of gram-positive bacteria, we first tested the adhesion capacity of *S. gallolyticus* UCN34 to 4 ECM proteins (collagen I and IV, fibronectin, and fibrinogen) over a 2-hour period. The reference strain UCN34 adhered preferentially to collagen I, and to a lesser extent to collagen IV (Figure 4A). Adherence to fibronectin and fibrinogen was not significant. In similar

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**Figure 2.** Pilus polymerization in *Streptococcus gallolyticus* wild-type and *Lactococcus lactis* recombinant strains. A, Western blot analysis of cell wall protein extracts isolated from *S. gallolyticus* strains UCN34, Pil1+ and Pil1− and *L. lactis* strains NZ9000/pOri23 and NZ9000/pOri23Ωpil1, separated with 4%–12% Criterion XT sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detected by means of immunoblotting using specific anti-Gallo2178 (left) and anti-Gallo2179 (right) polyclonal antibodies (pAbs). Equivalent amount (~10 μg) of total proteins was loaded in each well. Theoretical positions of Gallo2178 and Gallo2179 monomers, based on their molecular weights, are indicated by a black arrow (m), and high-molecular-weight species corresponding to pilus polymers are also labeled (p). B, Western blot analysis of cell wall protein extracts (5 μg/well) isolated from 10 additional *S. gallolyticus* clinical strains revealed using specific anti-Gallo2178 pAbs (top). Note adherence of *S. gallolyticus* strains to immobilized collagen I. Each well was coated with 1 μg of collagen I, and 10^7 bacterial colony-forming units for each strain were added. Bound bacteria were detected using crystal violet (CV) staining after extensive washing. Optical density at 595 nm (OD595nm) values are presented as means ± standard deviations of 3 experiments performed in triplicate (bottom).

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**Figure 3.** Where the Pil1+ strain was not labeled (data not shown), *L. lactis* strain expressing pil1 was labeled with the anti-Gallo2178 antibody, and, interestingly, similar pilus structures were visualized on the bacterial cell surface (arrows, Figure 3J). The control strain *L. lactis* NZ9000/pOri23 was not labeled in the same experimental conditions (Figure 3I). Specific antiserum raised against the putative adhesin Gallo2179 in *S. gallolyticus* Pil1+ revealed short homopolymers of Gallo2179 (arrowheads, Figure 3C and 3D). Similar structures were visualized on the surface of the recombinant *L. lactis* strain expressing pil1 (Figure 3K).
experiments, strain Pil1⁺ was found more adherent to collagen than strain UCN34, whereas Pil1⁻ did not adhere to any of these proteins. We also showed a dose-dependent binding of Pil1⁺ to collagen I (data not shown).

Analysis of 10 additional *S. gallolyticus* clinical isolates confirmed that Pil1 expression is necessary for adherence to collagen I (Figure 2B). The strains that did not express Pil1 were unable to bind collagen, whereas those expressing Pil1 were adherent. However, there is not a strict quantitative correlation between Pil1 expression levels and collagen adhesion capacities, suggesting that other elements linked to the strain genetic background modulate this property. In addition, constitutive

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**Figure 3.** Immunogold electron microscopy analysis of the pilus subunits Gallo2178 and Gallo2179. *Streptococcus gallolyticus* strains UCN34, Pil1⁺ and Pil1⁻ and *Lactococcus lactis* strains NZ9000/pOri23 and NZ9000/pOri23pili were incubated with rabbit and mouse polyclonal antibodies raised against Gallo2178 and Gallo2179, respectively. Anti-rabbit and anti-mouse secondary antibodies were conjugated to 10- and 20-nm gold particles, respectively. E–H, For double-labeling experiments on *S. gallolyticus* strain Pil1⁺, thin arrows indicate homopolymers of Gallo2178, whereas arrowheads in E indicate Gallo2179 trimers. Inset in E shows high-magnification view of 2 heteropolymers, Gallo2178–2179.
expression of pil1 in *L. lactis* (NZ9000/pOri23Xpil1) confers to this bacterium the ability to bind to collagen I and IV with a preference for collagen I, but not to fibronectin or fibrinogen. The control strain NZ9000/pOri23 did not adhere to any of the tested ECM proteins (Figure 4B).

Finally, when *S. gallolyticus* Pil1+ was incubated beforehand with anti-Gallo2179 pAb directed against the pil1 operon adhesin, bacterial adhesion to collagen was totally inhibited (Figure 4C), whereas polyclonal anti-Gallo2178 antibody showed only a slight adhesion reduction capability, potentially because of a steric hindrance. As a control, we used normal rabbit IgG (isotype control) that did not perturb bacterial adhesion. This result clearly shows that Gallo2179 is the major adhesin responsible for *S. galloolyticus* adhesion to collagen.

Our results are in agreement with those of a previous study showing that recombinant rAcb35 (ie, Gallo2179) produced in *E. coli* was able to bind collagen I, IV, and V with different affinities (I > IV > V) [20]. Collagen, the most abundant protein in human bodies and the main component of ECM, forms the only supportive fiber of cardiac valves. Of the dry weight of the human mitral valve, 67% is collagen, 74% type I, 24% type III, and 2% type V [39]. Collagen IV is a major component of the basal lamina layer underlying epithelial tissues. It is worth mentioning a study of animal isolates of *S. gallolyticus* that revealed no adherence to collagen I [23], which may indicate that human and animal strains possess a different repertoire of adhesins.

### Role of Pil1 in Biofilm Formation

We subsequently analyzed the role of pili in biofilm formation, using 2 frequently applied experimental procedures. The first consisted of a global quantification of biofilm achieved by measuring the optical density of adhered cells with crystal violet staining [40, 41]. *S. galloolyticus* and recombinant lactococcal strains were tested for biofilm formation on polystyrene plates coated with or without collagen I at 24 hours. For *S. galloolyticus* strains UCN34 and Pil1+ and for the *L. lactis* recombinant strain
NZ9000/pOri23Δpil1, biofilm formation capacities perfectly correlated to primary attachment capacities (Figure 5A).

The second experimental procedure was carried out with a CLSM enabling direct in situ and nondestructive investigation of native multicellular structures formed by S. gallopyticus. Representative 28-hour biofilm structures of strains UCN34, Pil1+, and Pil1−, grown on polystyrene microplates coated with collagen I, were observed using CLSM (Figure 5B). The images corresponded to 3-dimensional reconstructions obtained from confocal stack images with IMARIS 7.0 software, including virtual shadow projection on the right (Figure 5B, top). Analysis of variance performed on the maximal biofilm thickness showed that Pil1+ formed thicker biofilm than UCN34 and Pil1− (P < .01) at both 6 and 28 hours (Figure 5B, bottom). No significant difference in biofilm thickness was found between UCN34 and Pil1−, probably owing to the absence of a washing step in this procedure.

Collectively, these results suggest that S. gallopyticus pil1 operon plays a critical role in adhesion and colonization of damaged tissues exposing collagen I. Our results are in agreement with those of a very recent study showing that S. gallopyticus can form biofilm on collagen-rich surfaces, which in vivo are found at damaged heart valves and (pre)cancerous sites with displaced epithelium [42].

Role of Pil1 in a Rat Model of Endocarditis

We first tested the ability of S. gallopyticus reference strain UCN34 to infect aortic vegetations in the rat model of experimental endocarditis. The ID90 was found to be 10^4 CFU. In rats challenged with this inoculum, 11 of 12 vegetations (92%) were infected, whereas in those challenged with a lower inoculum of 10^3 CFU, only 1 of 13 (7%) developed infected vegetations. This ID90 value is very close to those (10^6–10^7 CFU) of the major bacterial pathogens responsible for IE [43, 44].

Because the S. gallopyticus strains used in this study have different genetic backgrounds, we used the recombinant lactococcal strains, a nonpathogenic bacterium, as a surrogate organism to test the role of Pil1 in vivo. Rats inoculated with 10^6 CFU of L. lactis NZ9000/pOri23Δpil1 produced infection in 9 of 11 vegetations (82%) compared with 4 of 11 (36%) with the control strain NZ9000/pOri23 (P = .03) (Figure 6A). These results with recombinant lactococci expressing the S. gallopyticus pil1 locus, encoding a collagen-binding protein, suggest that pil1 plays a critical role during the initial attachment and colonization stage of IE development. Histological analyses confirmed these results. Most animals infected with S. gallopyticus UCN34 or L. lactis NZ9000/pOri23Δpil1 indeed displayed fibrinous valvular endocarditis associated with intralesional bacteria (Figure 6B). Furthermore, it has been shown elsewhere that recombinant lactococci expressing staphylococcal adhesins were found to increase their infectivity in experimental endocarditis [45].

CONCLUSION

This study represents the first functional characterization of a pilus locus in S. gallopyticus. The Pil1 pilus is made of 2 subunits (Gallo2178, the major pilin, and Gallo2179, the pilus-associated adhesin) covalently assembled by a sortase C (Gallo2177). Its involvement in the development of endocarditis identifies Pil1 as the first virulence factor in this intriguing pathogen. Future
studies aim to develop genetic toolbox in *S. gallolyticus*, define the pilus regulation, and investigate other potential virulence factors involved in colorectal carcinoma.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

**Acknowledgments.** We thank Samuel Bellais for help with purification of recombinant proteins 6xHis-Gallo2179 and 6xHis-Gallo2178 from *E. coli*, and Olivier Poupel for qRT-PCR experiments. We are also grateful to Marlyse Giddey and Jacques Vouillamoz for excellent technical assistance with animal experiments. We thank Claire Poyart for helpful discussion.

**Financial support.** This work was supported in part by the Network of Excellence EuroPathoGenomics (grant LSHB-CT-2005-512061 to P. T. C.) and the Swiss National Science Foundation (grant 310030-125325).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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**Figure 6.** A, Infectivity rate for *Lactococcus lactis* NZ9000/pOri23 and recombinant *L. lactis* NZ9000/pOri23Ωpil1 in rats with experimental endocarditis. Groups of rats were challenged with 10⁶ colony-forming units of *L. lactis* carrying either an empty vector (pOri23) or pil1-encoding (pOri23Ωpil1) plasmid. The percentage of infected vegetations was assessed after 24 hours. *P* = 0.03 (χ² test). B, Fibrinous endocarditis was observed in cardiac valves of rats infected with *Streptococcus gallolyticus* UCN34 and *L. lactis* NZ9000/pOri23Ωpil1. Both strains induced similar lesions, characterized by (1) endothelium ulceration, (2) accumulation of a fibrillar acidophilic material (fibrin) containing bacterial colonies (arrowheads and insets), and (3) peripheral infiltration of neutrophils and macrophages (hematoxylin-eosin staining with Gram staining in insets).