MAJOR ARTICLE

Molecular Characterization of a *Streptococcus gallolyticus* Genomic Island Encoding a Pilus Involved in Endocarditis

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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 long 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 *Streptococcus 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 reclassification is still a matter of debate, and for simplification and readability these subspecies will be considered as species throughout this work.

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Although the proportion of IE due to *S. gallolyticus* 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. gallolyticus* 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. gallolyticus* 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. gallolyticus 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. gallolyticus* strain UCN34, isolated from a human IE case associated with colorectal cancer [25]. 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. gallolyticus* strain TX20005 (draft genome) also encodes 3 pilus loci [20]. Interestingly, only 2 of these loci are identical in both *S. gallolyticus* strains, namely *pil1* (*acb-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 diphteriae* [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 pilin motif (WxxxVxVYP**K**) of one subunit and the conserved threonyl residue of the LPX**T**G 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. gallolyticus* UCN34, which is present in 90% of *S. gallolyticus* 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. gallolyticus* 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. gallolyticus* 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*1 (New England Biolabs) and cloned into the high-copy-number erythromycin resistance shuttle vector pOri23 [38] digested by *Bam*H1-*Pst*1. 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. gallolyticus*, 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-*Nhe*1 and gallo2179-*Bam*H1, and gallo2178-*Nhe*1 and gallo2178-*BamH*1, respectively (Supplementary Table 1). These DNA fragments were digested with the appropriate enzymes (*Nhe*1 and *Bam*H1) and cloned into pET28-a(+) (Novagen). The resulting plasmids were introduced into *E. coli* strain DH5α for sequence analysis or BL21(λDE3)

Table 1. Bacterial Strains and Plasmids Used in Current Study

Bacterial Strains and Plasmids	Characterization	Antibiotic Resistance	References
Escherichia coli			
DH5α	F$^-$ φ80*lacZ*△M15 △(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17($r_k^-m_k^+$) *pho*A *sup*E44 *thi*−1 *gyr*A96 *rel*A1 λ^-	Em	Invitrogen
BL21(λDE3)	B F$^-$ *dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(DE3)		Novagen, R&D Systems
Streptococcus gallolyticus			
UCN34	MLST 1, infectious endocarditis and colon cancer	Tc	R. Leclercq, [1]
Pil1$^+$ (NEM2470)	MLST 1, infectious endocarditis	Tc	R. Leclercq, Caen Hospital
Pil1$^-$ (NEM2474)	MLST 25, infectious endocarditis	Tc	R. Leclercq, Caen Hospital
Lactococcus lactis			
NZ9000/pOri23	*L. lactis* subsp *cremoris* MG1363, nisRK, pOri23	Em	Current work
NZ9000/pOri23Ω*pil1*	*L. lactis* subsp *cremoris* MG1363, nisRK, pOri23Ω*pil1*	Em	Current work
Plasmids			
pET-28a(+)	Km, *oriR* pBR322, origin *f1*, promoter T7, coding sequence His-Tag, terminator T7, *lacZa*	Km	Novagen, R&D Systems
pOri23	Em, *oriR*s pUC18 et pIP501 (Gram$^-$/Gram$^+$ shuttle vector)	Em	[32]

Abbreviations: Em, Erythromycin; Km, Kanamycin; MLST, MultiLocus Sequence Typing; Tc, Tetracyclin.

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× 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 quadriplate 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].

Table 2. Primers Used in Current Study

Primers	Sequences 5′→ 3′[a]
pil1 amplification	
pilUCN34-fwd	ATTAG**GGATCC**AGGGAGTGATAAAGTGGTTGCT
pilUCN34-rev	GCAAT**ATGCAT**TACCGTCGCCCAAACAGTTTGA
Recombinant protein production	
gallo2178-*Nhe*1	AGCT**GCTAGC**TATGATATTACTGTTGAGAACGGT
gallo2178-*Bam*H1	GTTCC**GGATCC**TCAAGTTGAAGGAAGTGTTGC
gallo2179-*Nhe*1	GAAGTA**GCTAGC**GCTGATGTTAGTAATCGTGTA
gallo2179-*Bam*H1	TAAT**GGATCC**TCAACCTGTATTAGGGAATAG
Transcriptional analyses	
CD5	GTGCTTTAGATAAGCGATGTTTGG
CD6	GCAGCCCTTACTTCTGATACTGTG
CD42	GATGGTGAAAAGATTGCATATACAGTC
CD43	ATATGTTCCACTACCACCGTTCTCAACAG
CD44	GACTGTTGTTATAGGCGTTAGGTTGCAAG
CD45	CGCTAACTGTCGGATACAATAACAAGGAC
CD31	GGATCCAAATGTTTACCGCAGATTACG
CD32	CCTGCAGTATGAGCCGTGACACTATCCGC
gallo2177-fwd	TGACTGGTACGGGGATTATGGCTT
gallo2177-rev	CGCTAAACCACGGTGACCTGAAAT
gallo2178-fwd	ACTGTTGAGAACGGTGGTAGTGGA
gallo2178-rev	GTTTGACCAGCTGTAGTGATGCCA
gallo2179-fwd	CACTATTGAGGTCACCTGGTCGAT
gallo2179-rev	CCCACCCTGATACATTTTCCATTG
gallo16SRNA-fwd	CAGGTCTTGACATCCCGATGCTAT
gallo16SRNA-rev	CGCTAGAGTGCCCAACTGAATGAT
Polymerase chain reaction analyses	
gallo2177-CD25	TTTGACGATTAGCCTCGTCGGCATA
gallo2177-CD26	CGTAATCTGCGGTAAACATTTACCAC

[a] Restriction sites are in bold.

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/pOri23Ω*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 χ^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 1*A*). 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. 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

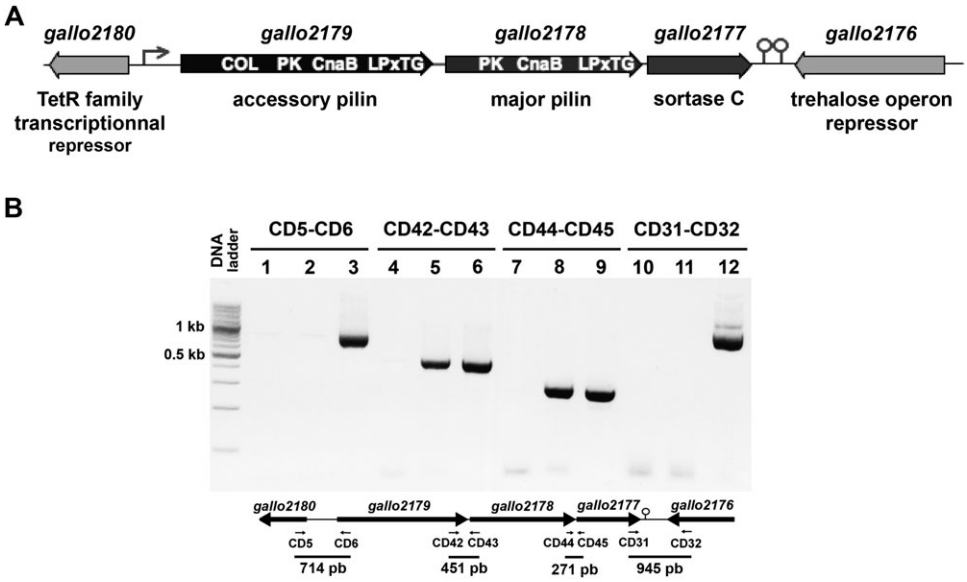

Figure 1. Physical map of *pil1* operon in *Streptococcus gallolyticus* strain UCN34. *A,* Schematic representation of the *pil1* locus. COL, functional collagen-binding domain (Pfam 05737); PK, pilin motif; CnaB, structural collagen-binding domain, immunoglobulinlike module (Pfam 05738); LPXTG, cell wall-anchoring domain; ↱, putative promoter; ♀, putative terminator. *B,* Determination of *pil1* operon. DNA polymerase chain reaction products were amplified using the indicated oligonucleotides pairs. Lanes 1, 4, 7, and 10 represent negative controls without reverse transcription; lanes 3, 6, 9, and 12, positive controls using UCN34 chromosomal DNA; lanes 2, 5, 8, and 11, cotranscriptional analyses of *gallo2179*, *gallo2178*, and *gallo2177*. Thin arrows below genes indicate oligonucleotides used, and black lines represent successfully amplified fragments.

transcriptional regulators belonging to the TetR family and trehalose repressor, respectively (Figure 1*A*).

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 1*B*). 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$^+$), 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$^-$), 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. gallolyticus* strains UCN34, Pil1$^+$, 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 nonpathogenic *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. gallolyticus* Pil1$^-$ and from the control strain *L. lactis* NZ9000/pOri23 (Figure 2*A*). The antiserum raised against the major pilin Gallo2178 recognized high-molecular-weight species both in *S. gallolyticus* UCN34 and Pil1$^+$ and in the recombinant *L. lactis* NZ9000/pOri23Ω*pil1*, with the typical laddering profile of covalently linked polymers as seen in many gram-positive bacteria [27, 33]. A similar profile of high-molecular-weight species was observed with the antiserum specific for Gallo2179 (Figure 2*A*). 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. gallolyticus* 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 3*A* and 3*B*). A strong heterogeneity in the population of *S. gallolyticus* expressing the pilin Gallo2178 was observed in both UCN34 and Pil1$^+$ (Figure 3*A*),

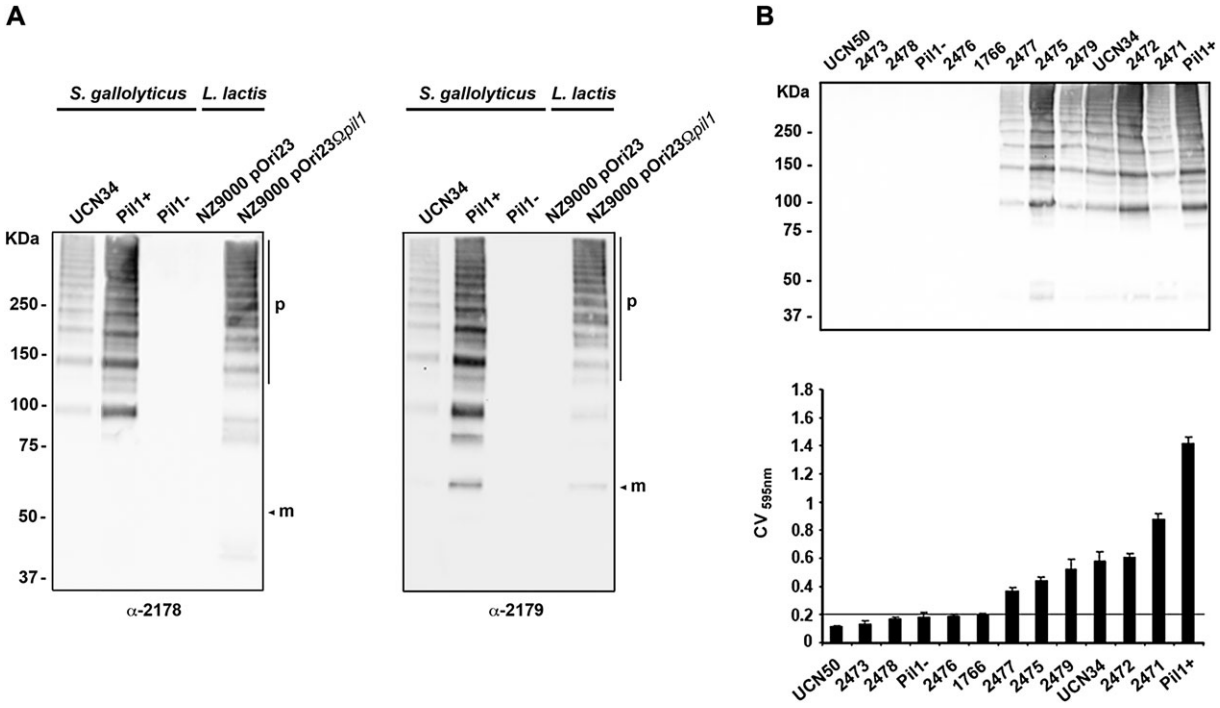

Figure 2. Pilus polymerization in *Streptococcus gallolyticus* wild-type and *Lactococcus lactis* recombinant strains. *A,* Western blot analysis of cell wall protein extracts isolated from *S. gallolyticus* strains UCN34, Pil1$^+$ and Pil1$^-$ and *L. lactis* strains NZ9000/pOri23 and NZ9000/pOri23Ω*pil1*, 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 pili 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 (OD_{595nm}) values are presented as means ± standard deviations of 3 experiments performed in triplicate (*bottom*).

whereas 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 3*J*). The control strain *L. lactis* NZ9000/pOri23 was not labeled in the same experimental conditions (Figure 3*I*). Specific antiserum raised against the putative adhesin Gallo2179 in *S. gallolyticus* Pil1$^+$ revealed short homopolymers of Gallo2179 (arrowheads, Figure 3*C* and 3*D*). Similar structures were visualized on the surface of the recombinant *L. lactis* strain expressing *pil1* (Figure 3*K*).

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 3*E* (inset). However, fibers composed of Gallo2178 only (arrows, Figure 3*B*, 3*F*, and 3*J*), and more surprisingly of Gallo2179 only (arrowheads, Figure 3*E* and 3*K*), 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 4*A*). Adherence to fibronectin and fibrinogen was not significant. In similar

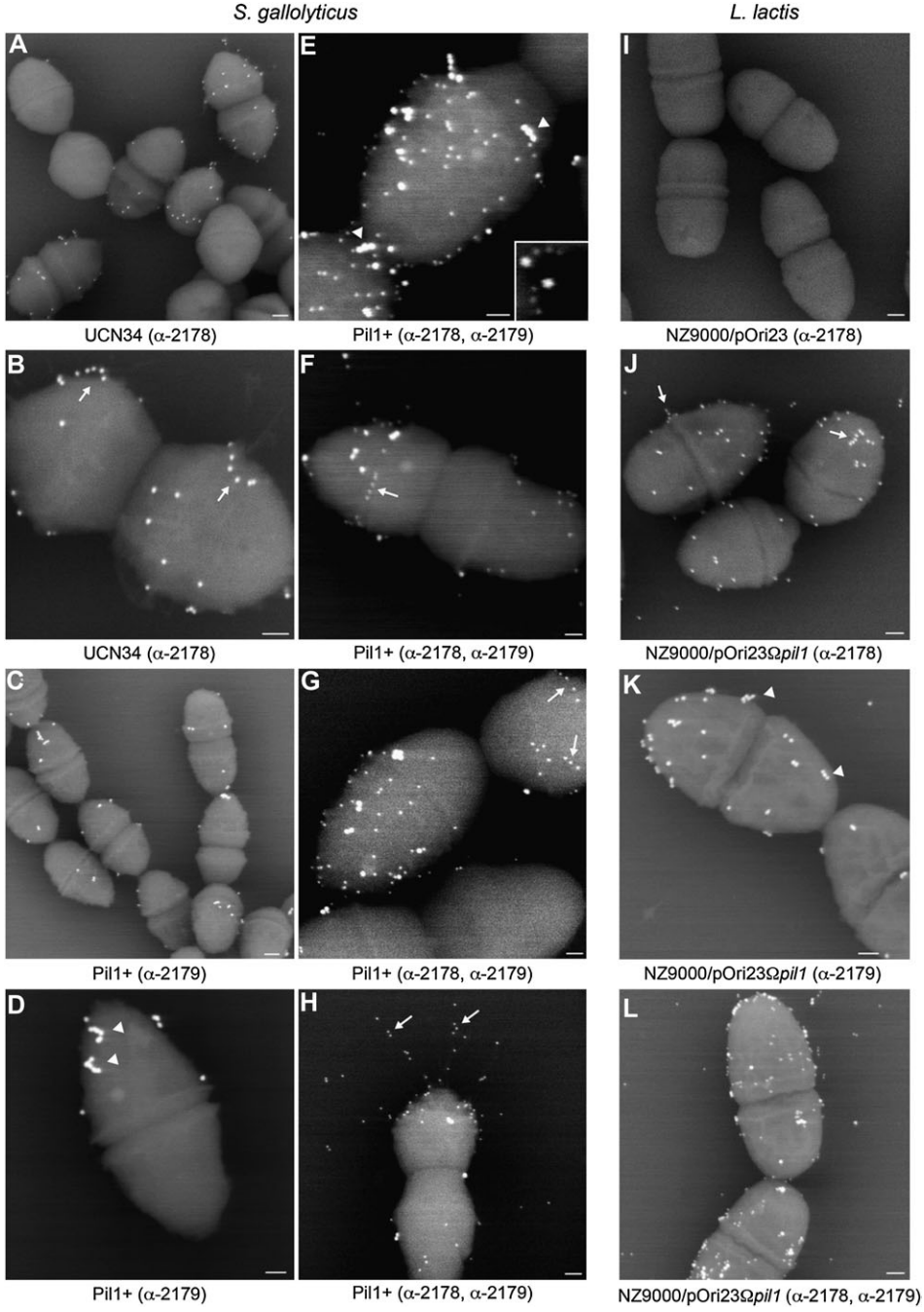

Figure 3. Immunogold electron microscopy analysis of the pilus subunits Gallo2178 and Gallo2179. *Streptococcus gallolyticus* strains UCN34, Pil1$^+$ and Pil1$^-$ and *Lactococcus lactis* strains NZ9000/pOri23 and NZ9000/pOri23Ω*pil1* were incubated with rabbit and mouse polyclonal antibodies raised 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.

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 2*B*). 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

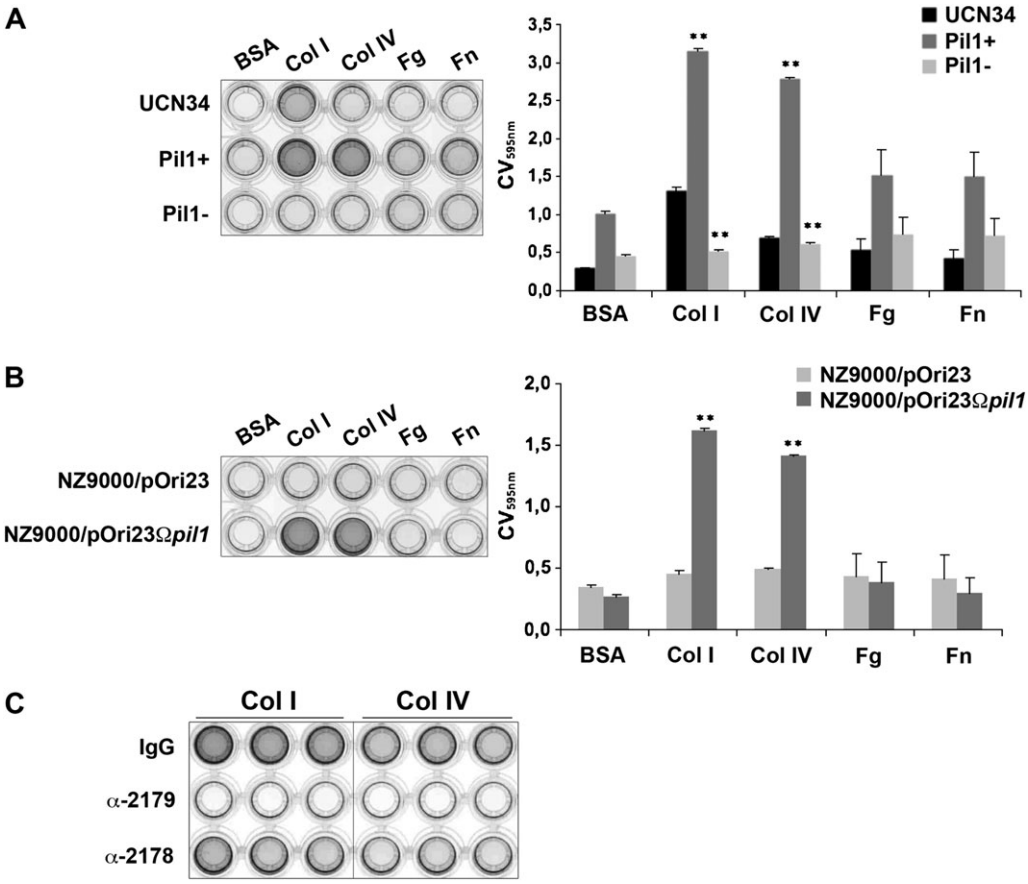

Figure 4. Adherence of *Streptococcus gallolyticus* wild-type strains and *Lactococcus lactis* recombinant strains to immobilized extracellular matrix (ECM) proteins. Microtiter wells were coated with 1 μg of ECM proteins, and 10^7 bacterial colony-forming units were added. The wells were washed, and bound bacteria were detected using crystal violet (CV) staining. Optical density at 595 nm (OD_{595nm}) values are presented as means ± standard deviations for 3 experiments performed in triplicate. *A,* Adherence of *S. gallolyticus* UCN34, Pil1$^+$, and Pil1$^-$ strains. *B,* Adherence of *L. lactis* NZ9000/pOri23 and NZ9000/pOri23Ω*pil1* strains. *C,* Inhibition of *S. gallolyticus* Pil1$^+$ adherence to collagen with antibody against Gallo2179. Abbreviations: BSA, bovine serum albumin; Col I, collagen I; Col IV, collagen IV; Fg, fibrinogen; Fn, fibronectin.

expression of *pil1* in *L. lactis* (NZ9000/pOri23Ω*pil1*) 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 4*B*).

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 4*C*), 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. gallolyticus* adhesion to collagen.

Our results are in agreement with those of a previous study showing that recombinant $rAcb_{35}$ (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. gallolyticus* and recombinant lactococcal strains were tested for biofilm formation on polystyrene plates coated with or without collagen I at 24 hours. For *S. gallolyticus* strains UCN34 and Pil1$^+$ and for the *L. lactis* recombinant strain

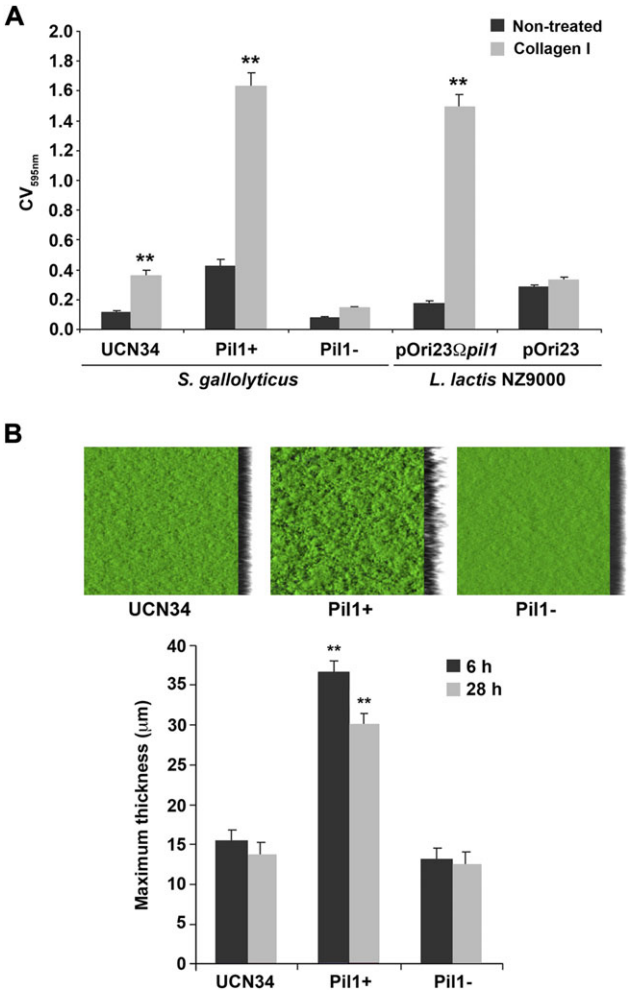

Figure 5. Role of the Pil1 pilus in biofilm formation. *A, Streptococcus gallolyticus* and *Lactococcus lactis* strains were grown in Todd–Hewitt broth at 30°C for 24 hours in 96-well polystyrene plates coated with 10 μg of collagen I or not coated. Adherent bacteria were stained with crystal violet (CV), and quantification was performed by measuring absorbance at 595 nm. Results are representative of 3 experiments; error bars show standard deviations. Plain bars, collagen I coating; hatched bars, not coated. *B,* top, Three-dimensional projections of biofilm architecture obtained from confocal z-stacks using IMARIS software. Images present aerial view of 28-hour biofilm structures obtained with the 3 strains of *S. gallolyticus.* Bacteria were stained in green, and shadow projection on right represents height of biofilm. Bottom, Biofilm maximal thickness (in micrometers) after 6 or 28 hours of growth (average values of 12 measurements in 4 wells). $*P < .01$ (1-way analysis of variance test).

NZ9000/pOri23Ω*pil1*, biofilm formation capacities perfectly correlated to primary attachment capacities (Figure 5*A*).

The second experimental procedure was carried out with a CLSM enabling direct in situ and nondestructive investigation of native multicellular structures formed by *S. gallolyticus*. Representative 28-hour biofilm structures of strains UCN34, $Pil1^+$, and $Pil1^-$, grown on polystyrene microplates coated with collagen I, were observed using CLSM (Figure 5*B*). 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 5*B*, 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 5*B*, 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. gallolyticus 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. gallolyticus* 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. gallolyticus* reference strain UCN34 to infect aortic vegetations in the rat model of experimental endocarditis. The ID_{90} 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 ID_{90} value is very close to those (10^4–10^5 CFU) of the major bacterial pathogens responsible for IE [43, 44].

Because the *S. gallolyticus* 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/pOri23Ω*pil1* produced infection in 9 of 11 vegetations (82%) compared with 4 of 11 (36%) with the control strain NZ9000/pOri23 ($P = .03$) (Figure 6*A*). These results with recombinant lactococci expressing the *S. gallolyticus 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. gallolyticus* UCN34 or *L. lactis* NZ9000/pOri23Ω*pil1* indeed displayed fibrinous valvular endocarditis associated with intralesional bacteria (Figure 6*B*). 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. gallolyticus*. 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

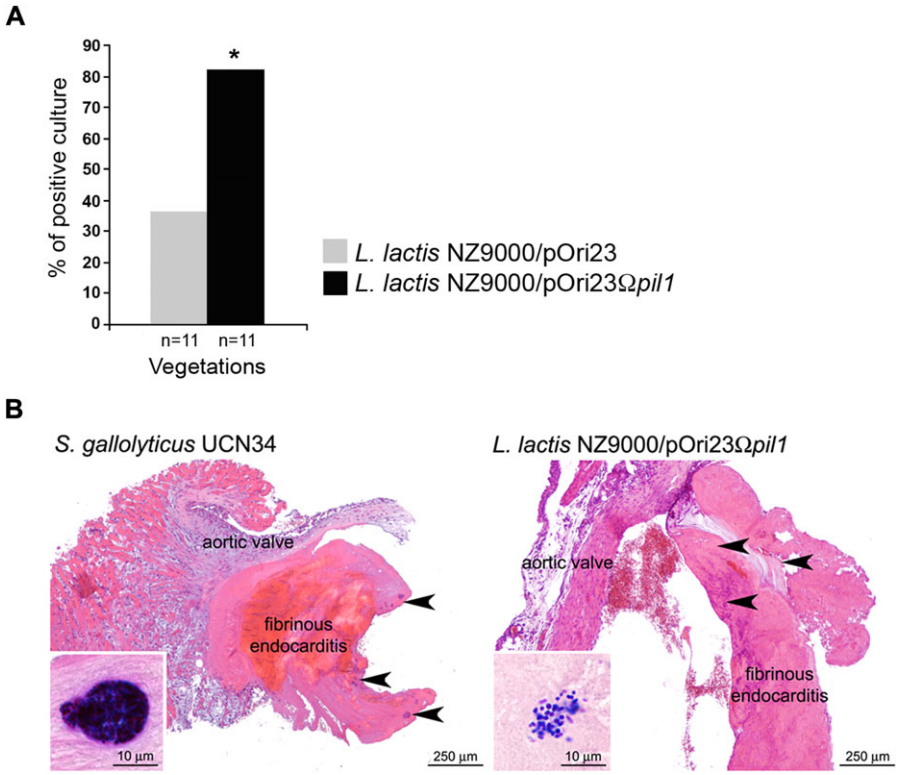

Figure 6. *A,* Infectivity rate for *Lactococcus lactis* NZ9000/pOri23 and recombinant *L. lactis* NZ9000/pOri23Ω*pil1* in rats with experimental endocarditis. Groups of rats were challenged with 10^6 colony-forming units of *L. lactis* carrying either an empty vector (pOri23) or *pil1*-encoding (pOri23 Ω*pil1*) plasmid. The percentage of infected vegetations was assessed after 24 hours. $*P = .03$ (χ^2 test). *B,* Fibrinous endocarditis was observed in cardiac valves of rats infected with *Streptococcus gallolyticus* UCN34 and *L. lactis* NZ9000/pOri23Ω*pil1*. 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).

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

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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.

References

1. Klein RS, Recco RA, Catalano MT, Edberg SC, Casey JI, Steigbigel NH. Association of *Streptococcus bovis* with carcinoma of the colon. N Engl J Med **1977**; 297:800–2.
2. Klein RS, Warman SW, Knackmuhs GG, Edberg SC, Steigbigel NH. Lack of association of *Streptococcus bovis* with noncolonic gastrointestinal carcinoma. Am J Gastroenterol **1987**; 82:540–3.
3. Abdulamir AS, Hafidh RR, Mahdi LK, Al-jeboori T, Abubaker F. Investigation into the controversial association of *Streptococcus gallolyticus* with colorectal cancer and adenoma. BMC Cancer **2009**; 9:403.
4. Boleij A, Schaeps RM, Tjalsma H. Association between *Streptococcus bovis* and colon cancer. J Clin Microbiol **2009**; 47:516.
5. Gupta A, Madani R, Mukhtar H. *Streptococcus bovis* endocarditis, a silent sign for colonic tumour. Colorectal Dis **2010**; 12:164–71.
6. zur Hausen H. *Streptococcus bovis*: causal or incidental involvement in cancer of the colon? Int J Cancer **2006**; 119:xi–xii.
7. Beck M, Frodl R, Funke G. Comprehensive study of strains previously designated *Streptococcus bovis* consecutively isolated from human blood cultures and emended description of *Streptococcus gallolyticus* and *Streptococcus infantarius* subsp. *coli*. J Clin Microbiol **2008**; 46:2966–72.
8. Poyart C, Quesne G, Trieu-Cuot P. Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (sodA) sequences: reclassification of '*Streptococcus infantarius* subsp. *coli*' as *Streptococcus lutetiensis* sp. nov. and of *Streptococcus bovis* biotype 11.2 as *Streptococcus pasteurianus* sp. nov. Int J Syst Evol Microbiol **2002**; 52:1247–55.
9. Schlegel L, Grimont F, Ageron E, Grimont PA, Bouvet A. Reappraisal of the taxonomy of the *Streptococcus bovis*/*Streptococcus equinus* com-

plex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and *S. gallolyticus* subsp. *pasteurianus* subsp. nov. Int J Syst Evol Microbiol **2003**; 53:631–45.
10. Corredoira J, Alonso MP, Coira A, et al. Characteristics of *Streptococcus bovis* endocarditis and its differences with *Streptococcus viridans* endocarditis. Eur J Clin Microbiol Infect Dis **2008**; 27:285–91.
11. Hoen B, Chirouze C, Cabell CH, et al. Emergence of endocarditis due to group D streptococci: findings derived from the merged database of the International Collaboration on Endocarditis. Eur J Clin Microbiol Infect Dis **2005**; 24:12–6.
12. Tripodi MF, Fortunato R, Utili R, Triassi M, Zarrilli R. Molecular epidemiology of *Streptococcus bovis* causing endocarditis and bacteraemia in Italian patients. Clin Microbiol Infect **2005**; 11:814–9.
13. De Herdt P, Haesebrouck F, Devriese LA, Ducatelle R. Biochemical and antigenic properties of *Streptococcus bovis* isolated from pigeons. J Clin Microbiol **1992**; 30:2432–4.
14. Vanrobaeys M, De Herdt P, Charlier G, Ducatelle R, Haesebrouck F. Ultrastructure of surface components of *Streptococcus gallolytics* (*S. bovis*) strains of differing virulence isolated from pigeons. Microbiology **1999**; 145:335–42.
15. Moreillon P, Que YA, Bayer AS. Pathogenesis of streptococcal and staphylococcal endocarditis. Infect Dis Clin North Am **2002**; 16:297–318.
16. Zong Y, Xu Y, Liang X, et al. A 'collagen hug' model for *Staphylococcus aureus* CNA binding to collagen. EMBO J **2005**; 24:4224–36.
17. Nallapareddy SR, Weinstock GM, Murray BE. Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol Microbiol **2003**; 47:1733–47.
18. Liu Q, Ponnuraj K, Xu Y, et al. The *Enterococcus faecalis* MSCRAMM ACE binds its ligand by the Collagen Hug model. J Biol Chem **2007**; 282:19629–37.
19. Singh KV, Nallapareddy SR, Sillanpaa J, Murray BE. Importance of the collagen adhesin ace in pathogenesis and protection against *Enterococcus faecalis* experimental endocarditis. PLoS Pathog **2010**; 6:e1000716.
20. Sillanpaa J, Nallapareddy SR, Qin X, et al. A collagen-binding adhesin, Acb, and ten other putative MSCRAMM and pilus family proteins of *Streptococcus gallolyticus* subsp. *gallolyticus* (*Streptococcus bovis* Group, biotype I). J Bacteriol **2009**; 191:6643–53.
21. Angrist AA, Oka M. Pathogenesis of bacterial endocarditis. JAMA **1963**; 183:249–52.
22. Sillanpaa J, Nallapareddy SR, Singh KV, Ferraro MJ, Murray BE. Adherence characteristics of endocarditis-derived *Streptococcus gallolyticus* ssp. *gallolyticus* (*Streptococcus bovis* biotype I) isolates to host extracellular matrix proteins. FEMS Microbiol Lett **2008**; 289:104–9.
23. Vanrobaeys M, Haesebrouck F, Ducatelle R, De Herdt P. Adhesion of *Streptococcus gallolyticus* strains to extracellular matrix proteins. Vet Microbiol **2000**; 74:273–80.
24. Vollmer T, Hinse D, Kleesiek K, Dreier J. Interactions between endocarditis-derived *Streptococcus gallolyticus* subsp. *gallolyticus* isolates and human endothelial cells. BMC Microbiol **2010**; 10:78.
25. Rusniok C, Couve E, Da Cunha V, et al. Genome sequence of *Streptococcus gallolyticus*: insights into its adaptation to the bovine rumen and its ability to cause endocarditis. J Bacteriol **2010**; 192: 2266–76.
26. Yanagawa R, Otsuki K. Some properties of the pili of *Corynebacterium renale*. J Bacteriol **1970**; 101:1063–9.
27. Dramsi S, Caliot E, Bonne I, et al. Assembly and role of pili in group B streptococci. Mol Microbiol **2006**; 60:1401–13.
28. Konto-Ghiorghi Y, Mairey E, Mallet A, et al. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. PLoS Pathog **2009**; 5:e1000422.
29. Lauer P, Rinaudo CD, Soriani M, et al. Genome analysis reveals pili in group B *Streptococcus*. Science **2005**; 309:105.
30. Rosini R, Rinaudo CD, Soriani M, et al. Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. Mol Microbiol **2006**; 61:126–41.
31. Mandlik A, Swierczynski A, Das A, Ton-That H. Pili in gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol **2008**; 16:33–40.
32. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol **2006**; 4:509–19.
33. Ton-That H, Schneewind O. Assembly of pili on the surface of *Corynebacterium diphtheriae*. Mol Microbiol **2003**; 50:1429–38.
34. Ton-That H, Schneewind O. Assembly of pili in gram-positive bacteria. Trends Microbiol **2004**; 12:228–34.
35. Dramsi S, Trieu-Cuot P, Bierne H. Sorting sortases: a nomenclature proposal for the various sortases of gram-positive bacteria. Res Microbiol **2005**; 156:289–97.
36. Hilleringmann M, Ringler P, Muller SA, et al. Molecular architecture of *Streptococcus pneumoniae* TIGR4 pili. EMBO J **2009**; 28:3921–30.
37. Mishra A, Das A, Cisar JO, Ton-That H. Sortase-catalyzed assembly of distinct heteromeric fimbriae in *Actinomyces naeslundii*. J Bacteriol **2007**; 189:3156–65.
38. Que YA, Haefliger JA, Francioli P, Moreillon P. Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis* subsp. *cremoris* using a new shuttle vector. Infect Immun **2000**; 68: 3516–22.
39. Cole WG, Chan D, Hickey AJ, Wilcken DE. Collagen composition of normal and myxomatous human mitral heart valves. Biochem J **1984**; 219:451–60.
40. Christensen GD, Simpson WA, Younger JJ, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol **1985**; 22:996–1006.
41. O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol **1998**; 28:449–61.
42. Boleij A, Muytjens CM, Bukhari SI, et al. Novel clues on the specific association of *Streptococcus gallolyticus* subsp *gallolyticus* with colorectal cancer. J Infect Dis **2011**; 203:1101–9.
43. Francioli P, Moreillon P, Glauser MP. Comparison of single doses of amoxicillin or of amoxicillin-gentamicin for the prevention of endocarditis caused by *Streptococcus faecalis* and by viridans streptococci. J Infect Dis **1985**; 152:83–9.
44. Moreillon P, Entenza JM, Francioli P, et al. Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. Infect Immun **1995**; 63:4738–43.
45. Que YA, Francois P, Haefliger JA, Entenza JM, Vaudaux P, Moreillon P. Reassessing the role of *Staphylococcus aureus* clumping factor and fibronectin-binding protein by expression in *Lactococcus lactis*. Infect Immun **2001**; 69:6296–302.
46. Kuipers OP, de Ruyter PGGA, Kleerebezem M, de Vos WM. Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol **1998**; 64:15–21.
47. Bridier A, Dubois-Brissonnet F, Boubetra A, Thomas V, Briandet R. The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. J Microbiol Methods **2010**; 82:64–70.
48. Heraief E, Glauser MP, Freedman LR. Natural history of aortic valve endocarditis in rats. Infect Immun **1982**; 37:127–31.