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FOOD MICROBIOLOGY

Biofilm Formation on Stainless Steel by *Streptococcus thermophilus* UC8547 in Milk Environments Is Mediated by the Proteinase PrtS

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ABSTRACT In *Streptococcus thermophilus*, gene transfer events and loss of ancestral traits over the years contribute to its high level of adaptation to milk environments. Biofilm formation capacity, a phenotype that is lost in the majority of strains, plays a role in persistence in dairy environments, such as milk pasteurization and cheese manufacturing plants. To investigate this property, we have studied *S. thermophilus* UC8547, a fast-acidifying dairy starter culture selected for its high capacity to form biofilm on stainless steel under environmental conditions resembling the dairy environment. Using a dynamic flow cell apparatus, it was shown that *S. thermophilus* UC8547 biofilm formation on stainless steel depends on the presence of milk proteins. From this strain, which harbors the *prtS* gene for the cell wall protease and shows an aggregative phenotype, spontaneous mutants with impaired biofilm capacity can be isolated at high frequency. These mutants lack the PrtS expendable island, as confirmed by comparison of the genome sequence of UC8547Δ3 with that of the parent strain. The *prtS* island excision occurs between two 26-bp direct repeats located in the two copies of the IS*Sth1* flanking this genomic island. The central role of PrtS was confirmed by analyzing the derivative strain UC8547Δ16, whose *prtS* gene was interrupted by an insertional mutation, thereby making it incapable of biofilm formation. PrtS, acting as a binding substance between the milk proteins adhered to stainless steel and *S. thermophilus* cell envelopes, mediates biofilm formation in dairy environments. This feature provides *S. thermophilus* with an ecological benefit for its survival and persistence in this environment.

IMPORTANCE The increased persistence of *S. thermophilus* biofilm has consequences in the dairy environment: if, on the one hand, the release of this microorganism from biofilm can promote the fermentation of artisanal cheeses, under industrial conditions it may lead to undesirable contamination of dairy products. The study of the molecular mechanism driving *S. thermophilus* biofilm formation provides increased knowledge on how an ancestral trait affects relevant phenotypes, such as persistence in the environment and efficiency of growth in milk. This study provides insight into the genetic factors affecting biofilm formation at dairy plants.

KEYWORDS biofilm, genome, milk, PrtS, stainless steel, *Streptococcus thermophilus*

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For many years, the dairy industry has benefited from *Streptococcus thermophilus* natural activity, as that species is the most used thermophilic starter culture for yogurt and cheese manufacture (1). Complete genome analysis revealed that this microorganism harbors genes that well describe its adaptation to milk environments, seen after experiencing a series of horizontal gene transfer (HGT) episodes and loss-of-function events that differentiated this bacterium from its original commensal nature

(2–4). Even if the exact source is not well known and its presence was originally correlated to *Lactococcus lactis* (5), the cell envelope proteinase PrtS in *S. thermophilus* could be indicated as an example of a gene acquired by HGT that recently appears to be highly conserved and therefore selected in the industrial strains (6). This is supported by the fact that in *S. thermophilus*, the *prtS* gene, coding for an LPXTG-containing serine proteinase of the subtilisin family (5), is part of a genomic island flanked by conserved insertion sequence (IS) elements (6–8). This mobile island has been shown to be involved in recombination events involving the flanking IS elements that lead to the loss of the *prtS* genomic island (6–8), therefore influencing gene homeostasis and species heterogeneity (7). The close proximity of these IS elements to genes involved in adaptation to milk environments reveals that their acquisition in this bacterium's genome could represent a relatively recent excision event (8). *prtS* genetic adoption generated beneficial properties in *S. thermophilus*, like fast growth in milk substrate and the ability to hydrolyze casein with a consequent high acidification rate (6); moreover, additional unknown functions could be conferred by these horizontal genomic rearrangements compared to *prtS*-deficient strains.

Another trait that *S. thermophilus* shows prior to adaptation to the milk environment and that is maintained in the domestication process in milk is the ability to form biofilm (9). High levels of contamination by biofilms formed by thermophilic bacteria have been detected in the heating sections of cheese-milk pasteurization equipment, where the temperature ranges from 30 to 73°C, and these contaminated sections are consequently released in the pasteurized milk (10, 11). This can cause defects in milk and cheese quality, such as acidic flavor and undesirable texture (11, 12). Even if further studies are needed, biofilm formation by streptococci seems to be influenced by the different milk components and by the surface material composition of the dairy equipment as well (13, 14). When cheeses such as Sicilian PDO Ragusano (15) and Caciocavallo Palermitano (16) are produced using Tina wooden vats, *S. thermophilus* appeared to be one of the dominant species in the biofilm isolate analysis. Due to the variabilities in their life environments, many streptococcal species develop the capacity to form a biofilm in order to survive unfavorable conditions, thereby obtaining an ecological advantage.

Recently, Couvigny et al. (9) provided new information about the biofilm-associated genes that *S. thermophilus* can activate and express for biofilm formation. When a collection of *S. thermophilus* strains obtained from different dairy products and with diverse geographical origins were studied for their capacity to produce biofilm, their varied behavior raised interest, as it was observed that a few strains acted as strong biofilm producers while the majority were poor biofilm producers. Three niche-specific genes involved in adhesion to and biofilm formation on polystyrene can justify this peculiarity (9). One of these, STH8232_017, is located in a genomic island which harbors open reading frames (ORFs) encoding transposases and appears to be acquired by HGT. This gene is heterogeneously found in *S. thermophilus* strains. Orthologs of the other two ORFs, STH8232_0714 and STH8232_1361, are detected in many host-associated bacteria, being involved in adhesion to mammalian cells, but they rarely are found in *S. thermophilus*. These observations led to the hypothesis that these genes code for ancestral functions that have been lost in the majority of *S. thermophilus* strains (9).

The aim of this study was therefore to investigate the mechanism behind biofilm formation by *S. thermophilus* strains in milk environments and to understand in more detail the molecular basis of and the gene exchange episodes driving adhesion and persistence on stainless steel surfaces.

RESULTS

Biofilm formation capacity of *S. thermophilus* UC8547. We investigated the capacity to form biofilm on abiotic surfaces in the presence of milk, under conditions resembling the natural environment of *S. thermophilus* species, by analyzing 25 strains (Fig. S1). Since a static biofilm assay showed that *S. thermophilus* UC8547 had the highest ability to produce biofilm (Fig. S1), this strain was selected for further studies.

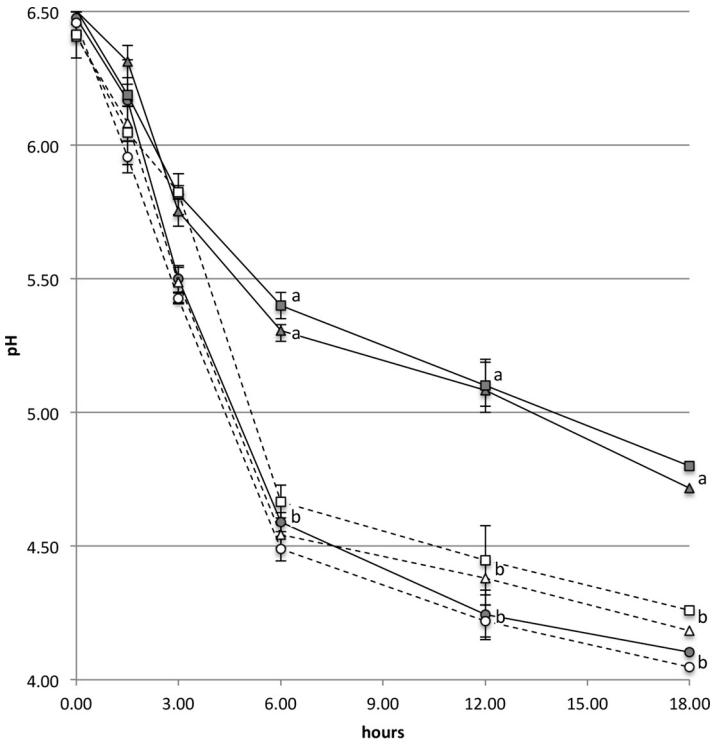

FIG 1 Acidification rate of *S. thermophilus* UC8547 (○), its spontaneous mutant UC8547Δ3 (△), and the *prtS*-null mutant UC8547Δ16 (□). Strains were grown in reconstituted skim milk (gray symbols and continuous line) and in reconstituted skim milk supplemented with 5 g/liter of hydrolyzed casein (white symbols and dotted line). The two derivative strains show a reduced acidification rate, which is restored by the addition of hydrolyzed casein. Data represent the means of the results from three independent experiments. Standard deviations are indicated by bars. A statistically significant difference ($P < 0.05$, one-way analysis of variance [ANOVA]) was observed at 6 h and thereafter between the two mutants (UC8547Δ3 and UC8547Δ16) grown in milk and the other analyzed samples, as indicated by different letters.

UC8547 is a fast acidifier, dropping the milk pH below 4.8 after 6 h (Fig. 1). PCR and reverse transcription-PCR (RT-PCR) experiments showed that it harbors the gene coding for the *S. thermophilus* serine protease PrtS and expresses it when grown in LM17 (data not shown) (5). Moreover, this strain showed an aggregation phenotype when grown in liquid medium and a hydrophobic phenotype when tested with the microbial adhesion to solvents (MATS) assay (Fig. 2).

When tested in the flow cell apparatus using dRSM as the medium, the counts of *S. thermophilus* UC8547 sessile and planktonic cells (Fig. 3) show that adhered cells increased over time. At 3 h, the count was 6.2×10^3 CFU/cm^2; at 6 h, the count was 6.3×10^6 CFU/cm^2, and the highest cell density was reached at 18 h, with a count of 5.0×10^7 CFU/cm^2. The density of planktonic cells increased during the first 6 h and then remained constant over the 36 h of the experiments. When the same samples were analyzed by scanning electron microscopy (SEM) (Fig. 4), it was observed that after 3 h, the stainless steel surface was covered by a matrix, presumably composed of adhered milk proteins, in which are enclosed *S. thermophilus* cells (Fig. S2). The thickness of the biofilm increased over time, and after 30 h, the wire was covered by a multilayer biofilm (up to 20 μm) of *S. thermophilus* cells. When LM17 was used as the medium, the cell density in the biofilm on stainless steel was significantly lower, reaching the highest value of 1.5×10^3 CFU/cm^2 after 24 h, while the counts of planktonic cells were comparable to those observed in the experiment with milk (Fig. 3).

***prtS* mutants have impaired biofilm formation ability.** To identify the cellular mechanisms at the basis of biofilm formation, isogenic spontaneous mutants of the

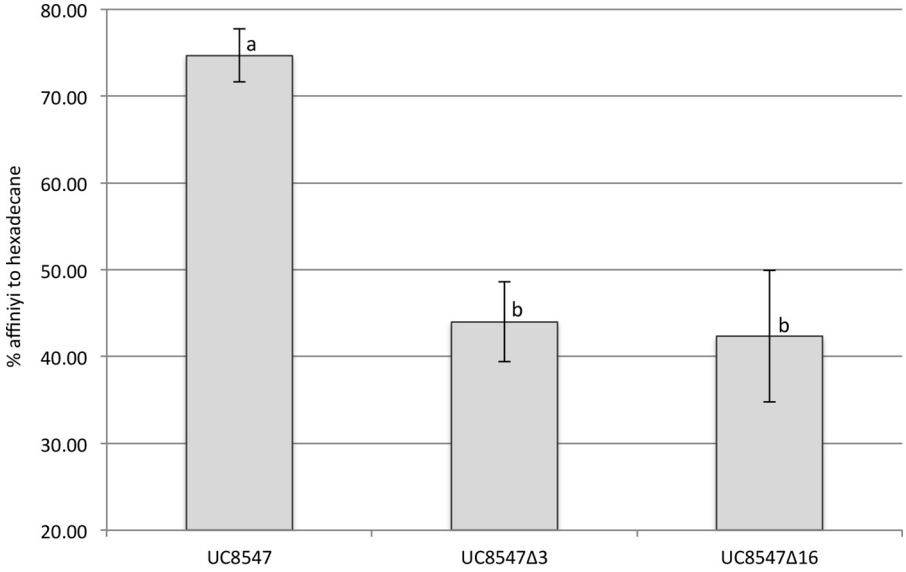

FIG 2 Cell surface hydrophobicity of *S. thermophilus* UC8547 and its *prtS*-null derivatives UC8547Δ3 and UC8547Δ16. The lack of the *prtS* gene reduced the cell affinity to hexadecane solvent. Data represent the means of the results from three independent experiments. Standard deviations are indicated by error bars. Values with different letters are significantly different ($P < 0.05$, one-way analysis of variance [ANOVA]).

parent strain *S. thermophilus* UC8547 were selected after subculturing for 30 days. After this period, 6% of the analyzed colonies lost the aggregative phenotypes. Five nonaggregative derivatives were isolated and when tested showed poor biofilm formation in static-mode experiments. When grown in milk, all five derivatives had a reduced

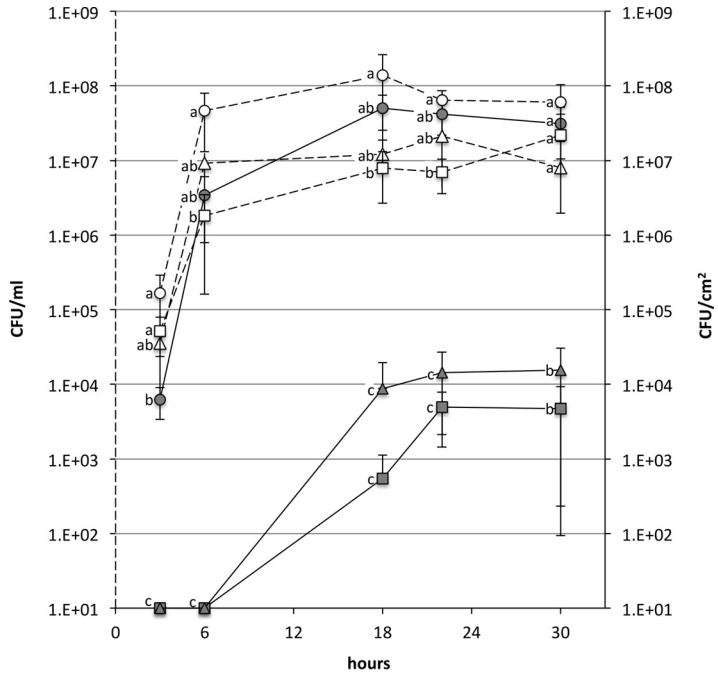

FIG 3 Biofilm formation in dynamic milk flow by *S. thermophilus* UC8547 (○), its spontaneous mutant UC8547Δ3 (△), and the *prtS*-null mutant UC8547Δ16 (□) over 36 h. Planktonic cells (white symbols and dotted line) are expressed as CFU per milliliter, while sessile cells (gray symbols and continuous line) are expressed as CFU per square centimeter. The reported data are the averages of the results from three independent experiments. Standard deviations are indicated by bars. No significant differences were observed in the planktonic cells counts between the three isogenic strains. The numbers of sessile cells adhered to stainless steel indicate that UC8547Δ3 and UC8547Δ16 have impaired biofilm formation. Values with different letters are significantly different ($P < 0.05$, one-way ANOVA).

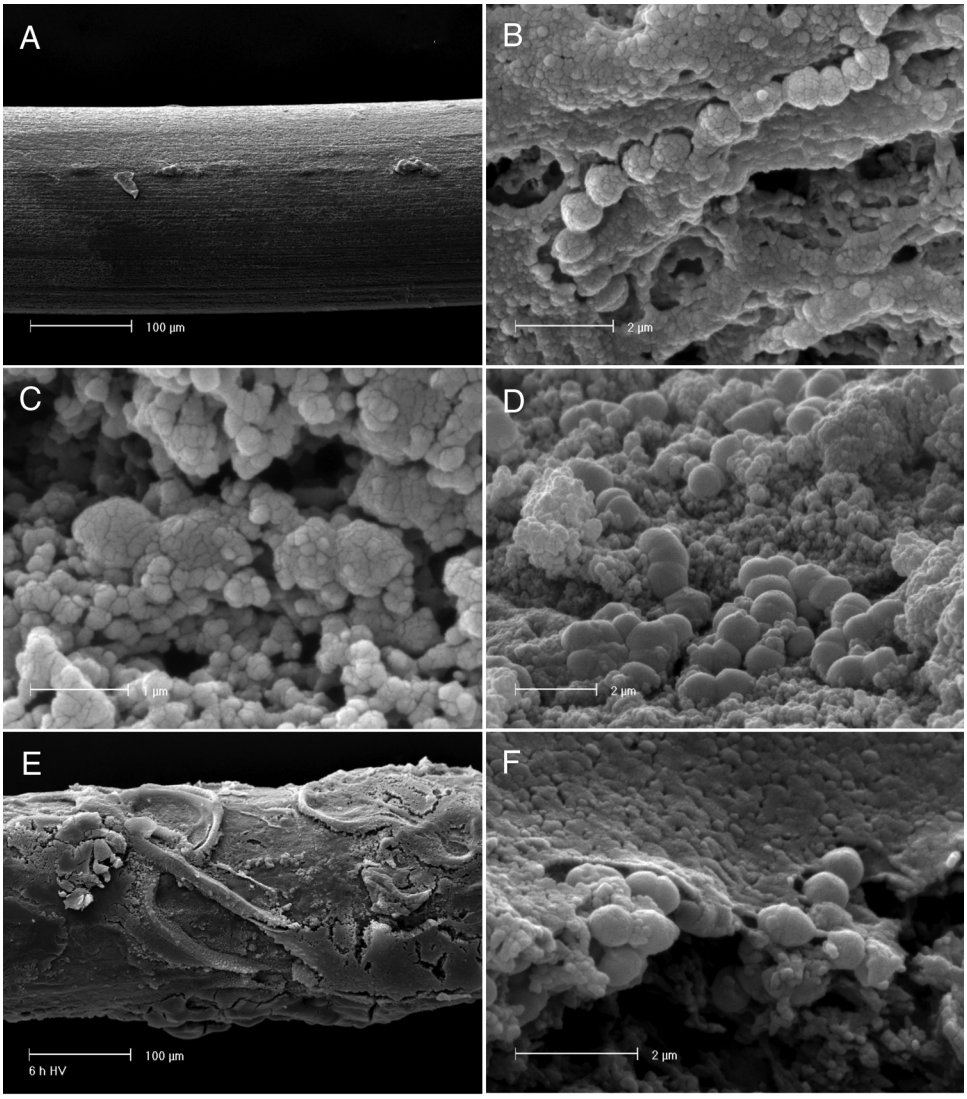

FIG 4 SEM observation of *S. thermophilus* UC8547 biofilm on stainless steel. (A) The stainless steel wire at the beginning of the experiment. (B) *S. thermophilus* cells adhered to the surface after 3 h. (C) Sessile cells at 6 h and 18 h. (D) After 30 h, an evident biofilm is present over the stainless steel wire (E and F) and cells are included in a multilayer structure.

acidification compared to the parent strains, as shown in Fig. 1 for one representative of them, named UC8547Δ3. All the nonaggregative mutants investigated by PCR lacked the gene coding for PrtS. Additionally, all the studied spontaneous mutants presented lower cell hydrophobicity than *S. thermophilus* UC8547 (Fig. 2). As shown in Fig. 3, a selected spontaneous mutant, named UC8547Δ3, showed a limited ability to form biofilm on stainless steel in milk, reaching the highest counts of sessile cells (1.5×10^4 CFU/cm^2) after 30 h. The counts of planktonic cells were substantially equal to those of the parent strain. The addition of hydrolyzed casein to RSM did not significantly affect the numbers of planktonic and sessile *S. thermophilus* UC8547Δ3 cells (data not shown). The SEM observation confirmed that a limited biofilm structure is formed over the stainless steel surface compared to that with the parent strain (Fig. 5).

To confirm the role of the cell wall-associated proteinase in biofilm formation, a mutant was constructed by a single-crossover insertional mutation. The resulting knockout mutant, named *S. thermophilus* UC8547Δ16, showed impaired biofilm formation in the presence of milk or casein, similar to that of the spontaneous mutant UC8547Δ3 (Fig. 3). While the counts of planktonic cells of the two mutants were

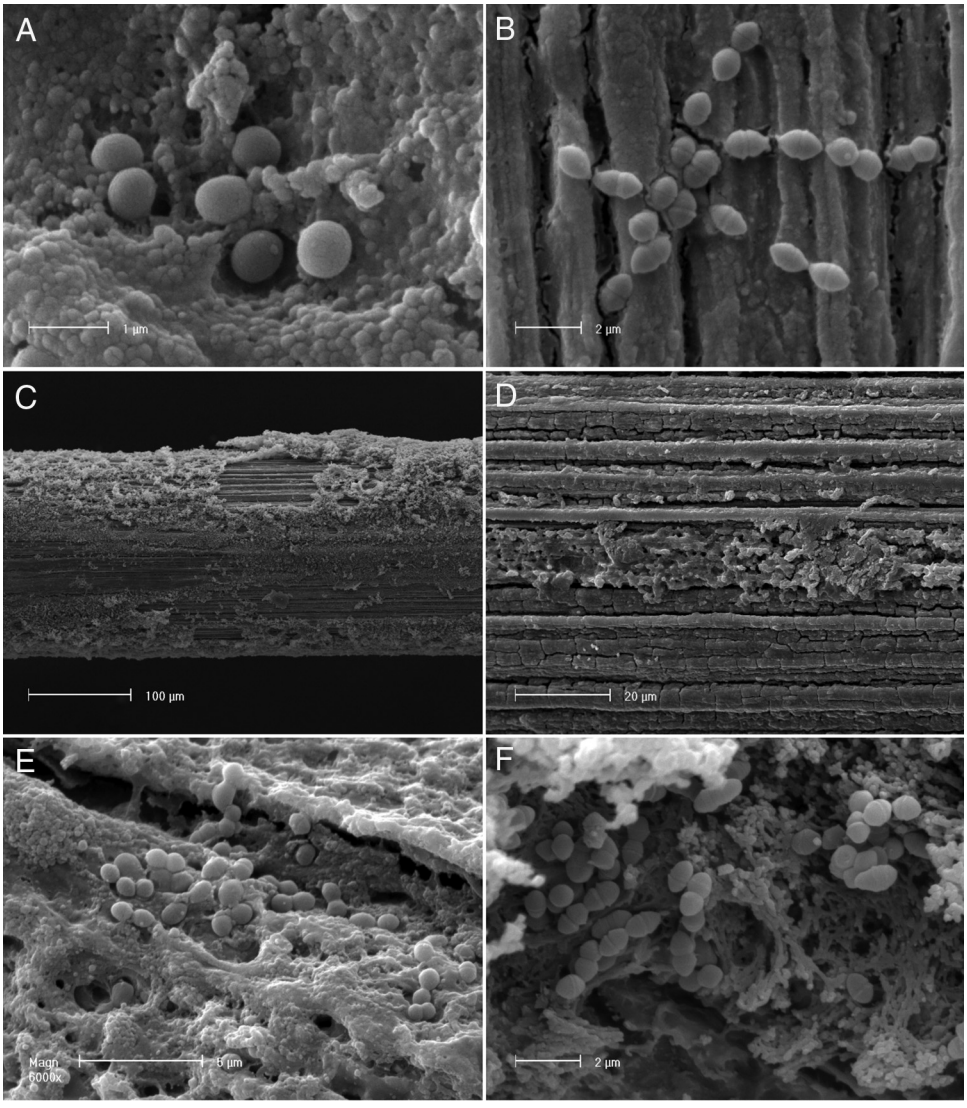

FIG 5 (A and B) SEM observations of UC8547Δ3 and UC8547Δ16 biofilm on stainless steel. *S. thermophilus* UC8547Δ3 (A) and UC8547Δ16 (B) cells adhered to the surface after 6 h. (C and D) Low-magnification observation of the stainless steel wire at 30 h for *S. thermophilus* UC8547Δ3 (C) and UC8547Δ16 (D). The biofilm matrix is substantially limited compared to that of the parent strain UC8547. (E and F) Higher magnification of the same sample shows that adhered cells of UC8547Δ3 (E) and UC8547Δ16 (F) are not included in a multilayer biofilm structure.

comparable to those of the parent strain UC8547, the cell density on the stainless steel was substantially lower. Electron microscopy observations confirmed the limited capacity to form biofilm under the tested conditions, as shown in Fig. 5. Differently from the parent strain UC8547, but as observed in the UC8547Δ3 experiments, few cells of UC8547Δ16, mainly in a monolayer, were detected over the abiotic surface. As expected, inactivation of the gene coding for the cell wall-associated protease resulted in a reduced acidification rate of *S. thermophilus* UC8547Δ16 in milk (Fig. 1). A reduction in cell hydrophobicity (Fig. 2) and the loss of the aggregative phenotype were also observed, indicating that, as observed by Couvigny et al. (9), cell hydrophobicity has a central role in the biofilm formation.

PrtS affects the conjugal transfer of pAMβ1 plasmid. The observation that both the spontaneous and the knockout mutants lost the aggregative phenotype when grown in LM17 led us to assess if the loss of PrtS affects horizontal gene transfer. For this purpose, we used as a donor *L. lactis* SH4174 harboring pAMβ1 (17), an erythro-

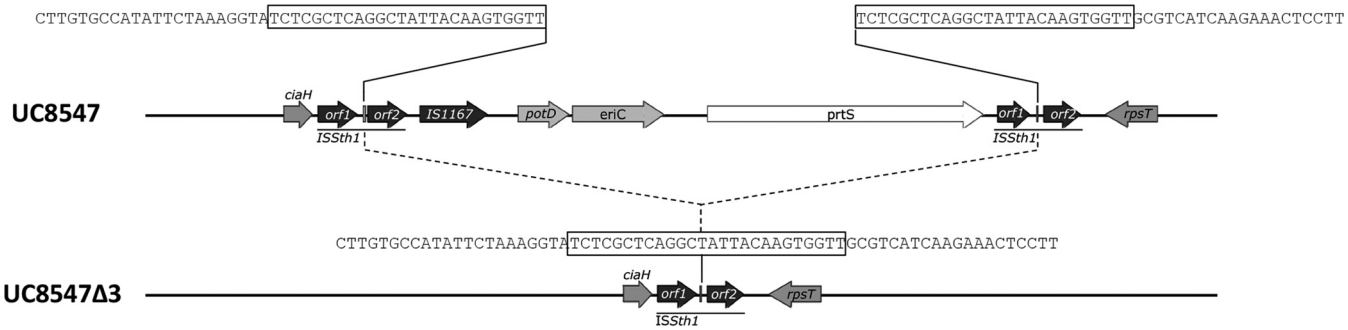

FIG 6 Schematic representation of the *prtS* genomic island of *S. thermophilus* UC8547. Genes are represented by arrows. In black are the IS elements IS*Sth1*, composed of two ORFs, and IS*1167*. In the spontaneous mutant UC8547Δ3, the excision event occurred via recombination between the two flanking IS*Sth1* elements. The nucleotide sequences of direct repeats of the deletion junctions are boxed.

mycin resistance plasmid with a broad host range in *Firmicutes* (18). The data from three independent plate mating experiments demonstrated that the transfer of pAMβ1 occurred at a higher frequency in *S. thermophilus* UC8547 (3.7×10^{-4}) than in the PrtS-negative mutants UC8547Δ3 (8.5×10^{-8}) and UC8547Δ16 (9.2×10^{-8}), indicating that in *S. thermophilus* UC8547, the aggregative phenotype associated with the cell wall proteinase PrtS positively affects horizontal gene transfer.

Genome analysis of *S. thermophilus* UC8547 and UC8547Δ3. *De novo* shotgun sequencing of *S. thermophilus* UC8547 and its spontaneous mutant UC8547Δ3 was performed. A 1,795,194-bp assembly was obtained from the genome analysis of the parent strain UC8547, consisting of a total of 77 contigs. These annotated contigs contain 1,960 putative coding sequences (CDSs) and 55 predicted RNAs. In the case of *S. thermophilus* UC8547Δ3, a slightly smaller assembly of 1,779,153 bp was obtained, consisting of a total of 66 contigs. The annotated contigs of the mutant contain 1,954 putative CDSs and 55 predicted RNAs. The whole-genome sequence analysis of *S. thermophilus* UC8547 and UC8547Δ3 made it possible to search for the genes involved in the biofilm formation on polystyrene plates and responsible for adhesion to epithelial cells (9). Both *S. thermophilus* UC8547 and its isogenic mutant harbor the STH8232_1361 gene, coding for a transmembrane protein of the polysaccharide transporter (PST) family, and lack the STH8232_017 gene, coding for a predicted cytoplasmic protein of unknown function. Both strains harbor an identical copy of a gene that shows 68% identity with STH8232_0714, a gene that codes for a putative surface protein that contains the mucin binding domain MucBP. The genome analysis showed that *S. thermophilus* UC8547 contains the *prtS* gene on the mobile genetic element previously identified in the fast-acidifying *S. thermophilus* strains. This genomic island is located in the *ciaH-rpsT* chromosomal region, as observed in other *S. thermophilus* strains (6, 8), downstream of the *ciaH* pseudogene and between two tandem IS*Sth1* elements (Fig. 6). The comparative genomic analysis of the parent and derivative strains revealed that UC8547Δ3 has encountered a major deletion event involving the 11,677-bp *prtS* island. Figure 6 shows that the deletion involved *orf2* of IS*Sth1*, IS*1167*, the *eriC* gene encoding a chloride channel protein, the *potD* gene coding for the ABC transporter spermidine putrescine-binding protein PotD, the *prtS* gene, and *orf1* of the second IS*Sth1* (Fig. 6). In *S. thermophilus* UC8547Δ3, the excision event involved two 26-bp direct repeat sequences (TCTCGCTCAGGCTATTACAAGTGGTT) located between the two open reading frames of IS*Sth1* (Fig. 6). The excision event occurred through recombination between the two copies of IS*Sth1*, resulting in the reconstruction of a complete copy of this insertion sequence. The excision site identified by genome comparison of *S. thermophilus* UC8547 and UC8547Δ3 differs from that identified in other *S. thermophilus* strains, with the left junction 769 bp downstream and the right junction 883 bp upstream of two direct repeat sequences homologous to those described in *S. thermophilus* LMD-9 (8). The comparison of the two genomes did not show other relevant mutations.

DISCUSSION

The scientific hypothesis we have investigated is that the cell wall-associated proteinase PrtS has a central role in adhesion to stainless steel in milk environments. The rationale behind this hypothesis is that biofilm formation is based on two major stages: biofilm starts with the adsorption of milk proteins to stainless steel, which is followed by *S. thermophilus* adhesion and colonization of this preconditioned surface. The adsorption of milk proteins to stainless steel is a multistep process that has been extensively studied. The first step is the electrostatic adsorption of β-lactoglobulin to negatively charged surfaces (19), such as that of stainless steel at neutral pH (20), and that is followed by the adsorption of κ-casein to the β-lactoglobulin-saturated layer. This adsorption process is temperature dependent, increasing when the temperature rises (21). The adsorbed layer of milk protein has been demonstrated to affect bacterial adherence and biofilm formation: Barnes et al. (22) observed that preconditioning of stainless steel surfaces with milk or individual milk proteins reduced the adhesion of *Staphylococcus aureus*, *Pseudomonas fragi*, *Escherichia coli*, *Listeria monocytogenes*, and *Serratia marcescens* to stainless steel after 2 h of incubation. In contrast, in flow cell experiments, Al-Makhlafi et al. (23) showed that β-lactoglobulin increases the initial adhesion of *Listeria monocytogenes* and the bacterial colonization over time. The hypothesis that PrtS protein is the binding force between milk-conditioned stainless steel and *S. thermophilus* cells is based on the presence in this proteinase of a substrate binding site, recognizing caseins, and of the sortase target site LPXTG motif, which allows a covalent bond to the cell wall (5). Moreover, PrtS lacks the B domain involved in the autoproteolytic process, which results in the release from the cell wall of other proteinases of lactic acid bacteria (5). To investigate if the ability to form biofilm under environmental conditions resembling the dairy environment is mediated by PrtS, we have selected a strain of *S. thermophilus* with a high biofilm formation capacity, isolated a spontaneous mutant, made the *prtS* gene-null mutant, assessed the biofilm formation in a flow cell apparatus, and compared the whole-genome sequences of the parent and derivative strains. The following observations support the hypothesis of a PrtS-mediated biofilm formation in dairy environment: (i) *S. thermophilus* UC8547 adheres to and forms a biofilm on stainless steel in milk; after 3 h of milk flow, cells attached to the conditioned surface are visible, and then a multilayer biofilm in which cells are embedded in an extracellular matrix is formed. Since the *S. thermophilus* exopolysaccharide gene cluster (24) is not present in the UC8547 genome and this strain does not produce a capsule, the exopolysaccharide (EPS) cannot be involved in the extracellular matrix formation and adhesion. In contrast, when the same experiments were performed in LM17, a very limited biofilm was observed, indicating the fundamental role of milk proteins in this process. (ii) PrtS is involved in the adhesion process. When this gene was interrupted by a single-crossover insertional mutation, the derivative strain UC8547Δ16 lost the proteolytic activity and failed to form biofilm. Moreover, when the whole-genome sequence of the spontaneous mutant UC8547Δ3 with impaired biofilm formation was compared with that of the wild-type strain, a deletion involving the expendable *prtS* island was detected. No differences in the genes involved in biofilm formation onto polystyrene genes, recently described by Couvigny et al. (9), were observed. The results of this study strongly indicate that the cell wall-associated proteinase PrtS, besides improving the growth and acidification rate in milk, mediates the adhesion of *S. thermophilus* cells to surfaces conditioned by milk. Two potential mechanisms at the basis of adhesion can be hypothesized: PrtS acts as a binding substance between the cell envelope and the milk proteins through its binding domain or, affecting cell hydrophobicity, it improves cell aggregation and biofilm formation, as reported by Habimana et al. (25) for the cell wall protease PrtP in *Lactococcus lactis*.

Biofilm formation ability provides an ecological advantage for the survival and persistence of this species, which is strongly adapted to dairy environments. Interestingly, proteolytic activity and biofilm formation are associated with the *prtS* gene, which is located on a mobile genetic element that has been demonstrated to be a horizontally

acquired trait (6–8). The mechanism of *prtS* island excision involves two copies of the IS*Sth1* insertion sequence flanking the *prtS* gene and is similar to that already described by Delorme et al. (8) in different *S. thermophilus* strains, and to that observed by Dandoy et al. (6), inserting by natural transformation the island in PrtS$^-$ strains. Unlike other *S. thermophilus prtS* islands (8), in UC8547, there is a single copy of IS*1167*.

The comparison of the whole-genome sequence of UC8547 and its PrtS$^-$ spontaneous mutant demonstrated the natural excision event in an isogenic model and confirmed the involvement of IS*Sth1* in the recombination, supporting the observation of IS-dependent recombination that leads to population heterogeneity in *S. thermophilus* (7). These authors, using the engineered endogenous clustered regularly interspaced short palindromic repeat 3 (CRISPR3) type II system of strain LMD-9 targeted to the *lacZ* genomic island, showed that IS elements flanking this expendable genomic island are responsible for large chromosomal deletions. Moreover, in the same study, using the engineered CRISPR3 repeat-spacer arrays targeted to a 30-bp internal region of the *prtS* gene, the excision of the *prtS* island was induced (7). We observed that the excision event involving the *prtS* island occurred at a relatively high frequency when the culture was grown in medium containing hydrolyzed proteins, such as in LM17, and the selective pressure of milk was absent. This phenomenon results in a heterogeneous population of fast and slow acidifiers in milk.

Additionally, we have noticed that PrtS influences the cell hydrophobicity and the aggregation phenotype when *S. thermophilus* is grown in broth. Since this phenotype has been associated with high-frequency plasmid conjugation in other lactic acid bacteria, such as *Lactococcus*, *Lactobacillus*, and *Enterococcus* (26–28), we assessed the ability to acquire the conjugative plasmid pAMβ1 of *S. thermophilus* UC8547 and its *prtS*-null mutants. The frequency of pAMβ1 transfer was substantially higher in the wild-type strain than in its mutants. A relationship between biofilm formation and high frequency of pAMβ1 conjugal transfer was observed in *Lactococcus lactis* expressing the CluA protein, a binding substance that induces cell aggregation, high conjugal transfer, and biofilm formation (29).

The recognition of the role of IS in the instability of mobile genetic elements coding for technologically important traits, such as the Prt$^+$ and biofilm phenotypes, provides clearer insight into the genome plasticity of *S. thermophilus* and its adaptation to environmental conditions: in the presence of milk this bacterium, which is highly adapted to the dairy environment, maintains the ability to persist in the habitat and to retrieve amino acids from milk proteins. Thus, it can be hypothesized that the biofilm forming on milk residues provides an ecological advantage for the persistence of *S. thermophilus* in the dairy environment, such as in pipelines and vats, and the proteolytic activity allows a rapid restoration of growth when milk is present. The identification of the molecular mechanisms behind biofilm formation in the milk environment represents an important finding in terms of both ecological advantage for persistence in this habitat and practical consequences in the dairy industry.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. thermophilus* strains were cultivated in M-17 medium (Merck KGaA, Darmstadt, Germany) containing 1% lactose (LM17) or 1% glucose (GM17) at 42°C. A total of 25 strains were tested in the study; the strains were used as industrial starter cultures or isolated from raw milk cheeses. The proteinase phenotype was determined on bacterial colonies grown on fast-slow differential agar (FSDA) medium after 48 h, as previously described (6, 30). Protease-positive strains form large yellow opaque colonies surrounded by a yellow area different from the small translucent colonies of the slow-acidifying phenotype. The acidification rate was assessed by growing *S. thermophilus* strains in 10% (wt/vol) reconstituted skim milk (RSM) (Oxoid, Thermo Fisher Scientific, Inc.) treated at 110°C for 10 min as follows: an overnight culture grown in LM17 was inoculated (1%) in RSM, incubated at 42°C, and the pH was measured over 16 h. In the studies with PrtS-negative mutant, 5g/liter hydrolyzed casein (tryptone; Sigma-Aldrich) was added to RSM before heat treatment. A high rate of acidification was considered when the pH was 4.8 or lower after 6 h of incubation.

Lactococcus lactis was cultivated in GM17 at 30°C. *E. coli* was grown in Luria-Bertani medium at 37°C. When needed, erythromycin (4 μg/ml for *S. thermophilus* and 200 μg/ml for *E. coli*) was added to broth and agar media.

Plasmid and DNA/RNA manipulations. The plasmid pRV300 harboring erythromycin resistance (31) was utilized as a vector to perform a Campbell-like integration. A 595-bp internal fragment of the *prtS* gene was amplified by PCR with oligonucleotides *prtS*f (5′-GTGAGGCTTTGGCAGCTAAC-3′) and *prtS*r (5′-TCGCGATATAGACCGGATTC-3′), digested with EcoRI-ClaI (Sigma-Aldrich, St. Louis, MO), and ligated with pRV300 digested with the same enzymes. The obtained construct was electroporated in *Escherichia coli* TB1 and named pPC7020. This plasmid was electrotransformed in *S. thermophilus* UC8547 as previously described (32), and mutants were selected on LM17 with 4 μg/ml erythromycin. The Campbell-like integration was confirmed by PCR, and a mutant, named UC8547Δ16, was selected for the study.

Total RNA was extracted from log-phase *S. thermophilus* cultures grown in LM17 using the RNeasy minikit (Qiagen, Inc., Hilden, Germany), with an additional step of treatment with RNase-free DNase (Qiagen). The concentration of RNA was determined by measuring the absorbance at 260 nm using an Ultrospec 2100 Pro UV-visible spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK). Reverse transcription reactions were performed on total RNA using the Reverse-iTTM one-step RT-PCR kit (ABgene, Epsom, UK), according to the supplier's recommendations, in a final volume of 25 μl containing 30 ng of total RNA. Reaction mixtures were incubated at 47°C for 30 min, followed by denaturation at 94°C for 2 min. cDNA products were amplified as described above (33). PCR products were analyzed on 0.8% agarose gels. To control for the residual presence of DNA, RT-PCR was also performed in the absence of reverse transcriptase. The experiments were performed in triplicate.

Biofilm formation. The biofilm formation capacity by *S. thermophilus* was assessed under static conditions over stainless steel (SS) by crystal violet staining. Five sterile spheres of SS (0.5 mm diameter) were introduced in a 5-ml tube containing 3.3% (wt/vol) diluted reconstituted skim milk (dRSM). The tube was inoculated with 50 μl of an overnight *S. thermophilus* culture and incubated at 42°C for 18 h. The spheres were removed from milk, washed three times with saline solution to remove unattached cells, and then resuspended in a 0.3% crystal violet solution for 15 min. The crystal violet solution was removed and the spheres rinsed three times with deionized water to remove the residual dye. Then, 2 ml of 95% (vol/vol) ethanol was added and the tube vortexed for 1 min. The ethanol solution was transferred to a 1-cm-path cuvette and the absorbance at 590 nm determined. Three independent replicates were performed.

Biofilm formation under dynamic conditions was studied using a flow chamber system composed of 5 glass tubes (10 cm long; internal diameter, 0.4 mm) in parallel, into which a stainless wire (0.2-mm diameter) was introduced. The tubes were connected by a Teflon pipeline to a solution dispenser, and the flow rate (1 ml/min) was maintained using a peristaltic pump. The apparatus was chemically disinfected with a succession of disinfection solutions (0.5 M NaOH and 50% ethanol) and washed with sterile saline solution. The apparatus was maintained at 42°C. An overnight culture of the *S. thermophilus* strain grown in RSM was introduced into the system at a 1 ml/min flow rate, and when the pipeline was full, the flow was stopped and the whole system was incubated at 42°C for 1 h. After this step, the nutrient solution, dRSM, dRSM–0.5% hydrolyzed casein (tryptone; Sigma-Aldrich), or LM17 was introduced inside the apparatus at a flow rate of 1 ml/min. At defined time points (0, 3, 6, 18, 24, and 30 h), the flow was stopped and aliquots of the wire (1 cm) and of the eluate were taken. Planktonic cell numbers were obtained by serially diluting the collected milk samples. The count of sessile cells was achieved by analyzing a fragment of the wire, collected under aseptic conditions and transferred to a sterile tube. To remove nonadhered cells, two washing steps with sterile saline solution were performed and then 1 ml of phosphate buffer (pH 7) was added to the tube. Detachment of sessile cells was performed by vortexing for 3 min. Counts were obtained by plating onto LM17 agar and incubating at 42°C. All the biofilm formation experiments were performed in triplicate.

MATS and aggregation assays. MATS was performed to determine the cell hydrophobicity, as previously described (34), by measuring the affinity of cells for hexadecane. Aggregation experiments were performed, as already described (26), in LM17 and GM17 media.

SEM analysis. Samples for SEM were prepared as follows. A 1-cm portion of the stainless steel wire was dehydrated stepwise in 75%, 85%, 95%, and 100% ethanol for 1 h each at room temperature. Critical point drying was performed in a Bal-Tec CPD030 critical point dryer. Samples were gold coated, as described by Palumbo et al. (35), and observed with a Philips XL30 environmental scanning electron microscope (ESEM).

Isolation of biofilm formation-negative mutants. The selection of nonaggregative mutants was achieved by subculturing *S. thermophilus* UC8547 in LM17 for 30 days. The inoculation of a new tube was performed with 10 μl from the top of an overnight culture. After 30 days, a culture lacking the aggregation phenotype was streaked on LM17 agar plates, and the resulting colonies were tested for aggregative properties.

Conjugation experiments. Mating experiments were performed as follows. *Lactococcus lactis* SH4174 carrying the erythromycin resistance broad-host-range conjugative plasmid pAMβ1 was used as a donor strain. The *S. thermophilus* strains UC8547, UC8547Δ3, and UC8547Δ16 were used as recipients. Donor and recipient log-phase cultures (0.2 ml) grown in GM17 were mixed in a 1:1 ratio, plated on a GM17 plate, and incubated for 24 h at 30°C. Transconjugants were selected on LM17 agar with 10 μg/ml erythromycin at 45°C, a limiting temperature for the donor strain.

Whole-genome sequencing and bioinformatic analyses. Shotgun sequencing of *S. thermophilus* strains UC8547 and UC8547Δ3 was performed. The genomes were sequenced using an Illumina HiSeq 1000 platform. Quality-filtered reads were assembled using the Velvet software (version 1.1.04) (36), and contig sequences were annotated in the RAST server (37) and the NCBI Prokaryotic Genome Annotation Pipeline. To fill the gap between contigs 20 and 61 of the *S. thermophilus* UC8547 genome, containing

a region flanking the *prtS* island, primers were designed immediately upstream of the gap and used for amplification and sequencing. Multiple alignments were performed using Geneious 9.0.5 software. Basic bioinformatics analysis was performed with the SnapGene Viewer 3.0.1 software. The OrthoMCL software was used for the identification of orthologous genes within the genomes of the two *S. thermophilus* strains. The protein sequences obtained from the annotation of the two *S. thermophilus* genomes were subjected to a BLAST all-versus-all search using a cutoff E value of 10^{-5}, and the results were submitted to OrthoMCL to generate groups of orthologous genes (OGs).

Accession number(s). The *S. thermophilus* genome sequences have been deposited in GenBank with accession numbers NZ_MCHW00000000.1 for UC8547 and NZ_MCHV00000000.1 for UC8547Δ3.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02840-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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