Lactobacillus reuteri, a Gram-positive bacterial species inhabiting the gastrointestinal tract of vertebrates, displays remarkable host adaptation. Previous mutational analyses of rodent strain L. reuteri 100-23C identified a gene encoding a predicted surface-exposed serine-rich repeat protein (SRRP_{100-23}) that was vital for L. reuteri biofilm formation in mice. SRRPs have emerged as an important group of surface proteins on many pathogens, but no structural information is available in commensal bacteria. Here we report the 2.00-Å and 1.92-Å crystal structures of the binding regions (BRs) of SRRP_{100-23} and SRRP_{53608} from L. reuteri ATCC 53608, revealing a unique β-solenoïd fold in this important adhesin family. SRRP_{53608}-BR bound to host epithelial cells and DNA at neutral pH and recognized polygalacturonic acid (PGA), rhhamnogalacturonan I, or chondroitin sulfate A at acidic pH. Mutagenesis confirmed the role of the BR putative binding site in the interaction of SRRP_{53608}-BR with PGA. Long molecular dynamics simulations showed that SRRP_{53608}-BR undergoes a pH-dependent conformational change. Together, these findings provide mechanistic insights into the role of SRRPs in host–microbe interactions and open avenues of research into the use of biofilm-forming probiotics against clinically important pathogens.

**Significance**

Gut bacteria play a key role in health and disease, but the molecular mechanisms underpinning their interaction with the host remain elusive. The serine-rich repeat proteins (SRRPs) are a family of adhesins identified in many Gram-positive pathogens. We previously showed that beneficial bacterial species found in the gut also express SRRPs and that SRRP was required for the ability of Lactobacillus reuteri strain to colonize mice. Here, our structural and biochemical data reveal that L. reuteri SRRP adopts a β-solenoïd fold not observed in other structurally characterized SRRPs and functions as an adhesin via a pH-dependent mechanism, providing structural insights into the role of these adhesins in biofilm formation of gut symbionts.

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The authors declare no conflict of interest.

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Data deposition: The structure factors and coordinates reported in this work have been deposited in the Protein Data Bank (PDB), www.wwpdb.org [accession ID codes 5NXK (SRRP_{100-23}-BR) and 5NY0 (SRRP_{53608}-BR)].

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an associated SRRP that shared the same domain organization as SRRP100-23 (8).

SRRPs belong to a growing family of adhesins in Gram-positive bacteria that mediate attachment to a variety of host and bacterial surfaces, and many of them are virulence factors that contribute to bacterial pathogenesis and biofilm formation (9). SRRPs are characterized by (i) two heavily glycosylated serine-rich regions (SRRs), (ii) one or two species-unique nonrepeat regions [NR domains, which include the binding region (BR) domain] toward the N terminus that facilitate specific interactions with a diverse array of host receptors and share little sequence homology to each other, and (iii) a C-terminal cell wall anchor domain (9, 10). Export of SRRPs onto the bacterial surface occurs through a dedicated noncanonical Sec translocation, Sec-Y2A2, following recognition of an extended atypical signal sequence peptide of around 90 aa at the N terminus (10, 11). The domain organization of SRRPs is highly conserved in pathogenic streptococci and staphylococci and includes Srr-1 and Srr-2 of Streptococcus agalactiae, Psr of Streptococcus pneumoniae, Fap1 of Streptococcus parasanguinis, GspB and Hsa of Streptococcus gordonii, SraP of Staphylococcus aureus, and SrpA homologs from Streptococcus sanguinis and Streptococcus crista tus (9, 12, 13). However, the structure and function of SRRPs in gut commensal bacteria have not yet been determined. Here we used a number of complementary approaches to provide a structural basis for the role of L. reuteri SRRPs (LrSRRPs) in bacterial adaptation to the host. We show that the LrSRRP-BR adopts a right-handed parallel β-helical or “β-solenoid” fold not observed in other structurally characterized SRRPs and functions as an adhesin via a pH-dependent mechanism. These findings provide insights into the role of LrSRRPs in biofilm formation and structural insights into intra- and interspecies adhesins across Gram-positive pathogenic and commensal bacteria.

Results

Bioinformatics Analysis of SRRPs from Lactobacilli. SRRPs and corresponding specialized secretion systems are being defined in a growing number of pathogens, but their occurrence and characterization in commensal bacteria have been reported only infrequently (14, 15). Our bioinformatics analysis of lactobacilli genomes revealed genes encoding fully functional SRRPs and SecY2-SecY2 secretion systems in a number of Lactobacillus species, including strains of L. reuteri, Lactobacillus oris, Lactobacillus salivarius, Lactobacillus johnsonii, and Lactobacillus fructivorans, with none found so far in other major lactobacilli species such as Lactobacillus plantarum (SI Appendix, Table S1). In other cases, strains possessed what appeared to be only an incomplete SecA2–SecY2 gene cluster, a SRRP that lacked a C-terminal cell wall anchor (possibly the result of a pseudogene capable of exporting a SRRP extracellularly which would not be covalently linked to the cell surface), or an obvious pseudo-SRRP whose domains were encoded by at least two adjacent ORFs. These include strains of Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus murinus, Lactobacillus nagelli, and Lactobacillus mucosae, a species closely related to L. reuteri (SI Appendix, Table S1) (16). When some strains of lactobacilli harbor two SRRPs, at least one of them is encoded by pseudogene fragment(s), highlighting their loss of function due to lack of selective pressure.

Analysis of 58 available genome-sequenced L. reuteri strains showed that homologs of functional SRRPs (and the corresponding linked SecA2–SecY2 gene cluster) were found exclusively in some rodent and pig isolates with the exception of one chicken strain and one sourdough strain [previously reported to be of intestinal origin with a genome content similar to that of the model rodent isolate 100-23 (17)] (SI Appendix, Table S2). The putative LrSRRPs from L. reuteri rodent strain 100-23C (SRRP100-23) and pig strain ATCC 53608 (SRRP53608) possess a LPXTG cell wall anchor and display characteristics of a protein secreted through the SecA2–SecY2 system, i.e., the presence of an unusually long N-terminal signal peptide and two extremely serine-rich regions, SRR-1 and SRR-2, the second of which contains many repeat motifs (Fig. 1).

Further comparative sequence analysis of SRRP-BR domains was carried out in a total of 76 lactobacilli SRRPs/pseudo-SRRPs and 18 pathogen/clinical-associated SRRPs to generate a set of SRRP homologs from 38 species-unique SRRPs (SI Appendix, Fig. S1). In the case of most L. reuteri strains, SRRP-BRs formed groups relating to the host or source from which the strain was isolated, such as the two main groups of pig-derived SRRP-BRs/pseudo-SRRP-BRs and the one main group of rodent/sourdough-derived BRs. Similar relationships were observed for the BRs from L. oris, L. salivarius, and the pathogenic streptococcal BRs. There were a few exceptions where SRRP-BRs/pseudo-SRRP-BRs of L. reuteri strains crossed this host-specific divide, such as the relatedness of (i) the pseudo-SRRP-BRs from rodent strains Lpup, LR0, and TD1 and a number of pig strain SRRP-BRs; (ii) the SRRP-BR from chicken isolate 1366 and the pseudo-SRRP-BRs from three pig strains KLR2001, KLR3005, and pg-3b; and (iii), most importantly, the BR domain from SRRP100-23 of rodent origin and one group of porcine SRRP-BRs that included SRRP53608-BR. The SRRP53608-BR and SRRP100-23-BR shared ~49% amino acid identity. This compares with the very low amino acid identity of <15% between SRRP53608-BR or SRRP100-23-BR and pathogenic bacterial BRs such as GspB-BR, Fap1-NR0, Hsa-BR, PsrP-BR, Srr-1-BR, and Srr-2-BR. Typically, SRRP-BRs neighbor-joining phylogram (SI Appendix, Fig. S1). In the case of between each other, but one exception was the pseudo-SRRP-BR from L. mucosae pig strain L1M and the predominantly pseudo-SRRP-BRs from 18 L. reuteri pig strains and one L. reuteri bovine strain, providing some insight into the evolutionary relationships between these two related species (16, 18). We confirmed the presence of the full-length srrp gene in five pig strains of L. reuteri by PCR and showed that the encoded SRRPs were also secreted extracellularly, as previously shown for SRRP100-23 during growth in vitro (SI Appendix, Fig. S2) (6).

Crystal Structures of L. reuteri SRRP-BR Reveal a Unique Fold Within the SRRP Family. SRRP3608-BR and SRRP100-23-BR crystals were obtained via in situ-limited proteolysis of the full-length LrSRRP-BR proteins with α-chymotrypsin and thermolysin, respectively. The structure of SRRP3608-BR was determined at 1.92 Å resolution between residues 262 and 571, excluding 39 and 97 residues from the N and C termini, respectively (Fig. 2A). The structure of SRRP100-23-BR was determined at a resolution of 2.00 Å. The
model contains residues 257–623, excluding 55 and 64 residues from the N and C termini, respectively. There are two gaps in the structure (indicated by black spheres in Fig. 2B) which could not be modeled. The first gap of nine residues is from amino acids 413–421, and the second gap of 16 residues is from amino acids 568–583. The data collection and refinement statistics for SRRP\(_{53608}\)-BR and SRRP\(_{100\text{-}23}\)-BR are provided in Table 1.

**Overall fold of LrSRRP-BRs.** The structures of SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) and SRRP\(_{100\text{-}23}\)-BR\(_{257\text{-}623}\) share 43% sequence identity with an rmsd of 0.912 over 293 aligned Ca atoms. Both structures adopt a solenoid-type fold comprising \(\beta\)-strands coiled in a repetitive pattern to form a right-handed helix with three parallel \(\beta\)-sheets (Fig. 2). A Dali search (19) revealed structural homology of LrSRRP-BRs with proteins predominantly belonging to CATH superfamilies 2.160.20.10 and 2.160.20.20 (32, 33), and a structure with that of pectate lyase TM-Pel in complex with Salmonella typhimurium \(\beta\) (583). The data collection and refinement statistics for SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) and SRRP\(_{100\text{-}23}\)-BR\(_{257\text{-}623}\) share 43% sequence identity with an rmsd of 3.18 Å over 220 aligned Ca atoms and 3.48 Å over 190 aligned Ca atoms to P.09 pertactin and phage P22 TSP, respectively. P.09 pertactin from the pathogen Bordetella pertussis facilitates adhesion to mammalian epithelial proteins via a conserved Arg-Gly-Asp motif and two proline-rich regions (37). Phage P22 TSP is an endorhamnosidase acting on the O-antigen of Salmonella typhimurium; sugar-binding features observed from the structure of the TSP-O-antigen complex are hydrophobic stacking of aromatic sidechains with sugar pyranose and \(\alpha\)-bonds to polar and ionic sidechains (38). Another \(\beta\)-solenoidal extracellular adhesive protein is the N-terminal TPS-antigen (39) from Bordetella spp., which also has an Arg-Gly-Asp motif that recognizes macrophage CR3, a heparin-binding domain, as well as a carbohydrate-recognition domain, for adhesion to lung epithelial cilia (rmsd of 3.18 Å to SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) over 197 aligned Ca atoms) (40). However, no such Arg-Gly-Asp motif was identified in any of the LrSRRP-BR proteins.

**Putative binding sites of LrSRRP-BRs.** Superposition of the SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) structure with that of pectate lyase TM-Pel in complex with chitooligosaccharide trisaccharide-CGA (TGA) (Fig. 3C) revealed a potential binding site in LrSRRP-BRs. In TM-Pel, predominantly basic residues maintain polar contacts and salt bridges with the acidic TGA molecule as shown in Fig. 3B. In SRRP\(_{53608}\)-BR, the area under the lower loop corresponds to TM-Pel’s binding site and has four basic solvent-exposed residues (K377 on T3 adjacent to \(\beta\)13 and a triad, K485, R512, and R543, on \(\beta\)26, \(\beta\)29, and \(\beta\)32, respectively), one acidic residue (D487 on \(\beta\)26), and four solvent-exposed aromatic residues (a triad of W375, Y425, and W450 on \(\beta\)13, \(\beta\)20, and \(\beta\)23, respectively, and Y482 on T2, next to \(\beta\)26) (Fig. 3D). Given the role of these charged and aromatic residues for sugar binding in PelC-like proteins, the aforementioned surface-exposed residues are postulated to form a putative binding site (PuBS) in SRRP\(_{53608}\)-BR. The positions of Y482 and R512 in the PuBS are conserved with TM-Pel’s binding site. Superposition of SRRP\(_{100\text{-}23}\)-BR\(_{257\text{-}623}\) upon SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) revealed a high degree of conservation of amino acid residues between their PuBSs (depicted in Fig. 3E). However, the most notable difference is that the lower loop in SRRP\(_{100\text{-}23}\)-BR\(_{257\text{-}623}\) is five amino acids longer and includes two aromatic residues, H414 and Y415, although it could not be modeled in the crystal structure. In addition, solvent-accessible surface electrostatic potential maps of SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) and SRRP\(_{100\text{-}23}\)-BR\(_{257\text{-}623}\) revealed that, like the TM-Pel binding site, the PuBS in SRRP\(_{53608}\)-BR\(_{262\text{-}571}\) is enveloped by positive electrostatic potential (SI Appendix, Fig. S3), whereas the corresponding region in SRRP\(_{100\text{-}23}\)-BR\(_{257\text{-}623}\) is acidic, although this may be due to
the absence of the (unmodeled) loop in SRRP<sub>100-23</sub>-BR<sub>257-623</sub>. Indeed, removing the lower loop in the SRRP<sub>53608</sub>-BR<sub>262-571</sub> model from F411–T422 led to a reduced positive surface charge around the PuBS (<em>SI Appendix</em>, Fig. S3), implying that the residues in this loop may play a role in maintaining the basicity of the PuBS.

**Molecular Dynamics Simulations Suggest a pH-Dependent Conformational Change in the L<sub>5</sub>SRRP-BR PuBS.** Starting from the crystal coordinates of SRRP<sub>53608</sub>-BR<sub>262-571</sub>, hydrogen atoms were added to the structure according to known protein chemistry (<em>Methods</em>). This included prediction of the protonation state of acidic and basic residues at both pH 4.0 and 7.4. The resulting models differed in that 14 acidic residues (E263, E269, D334, E338, E400, E409, E434, D448, E475, E481, D487, E518, E527, and E566) were protonated at the carboxylate sidechain at pH 4.0 (<em>SI Appendix</em>, Fig. S4). Furthermore, four histidine residues (H311, H413, H493, and H535) were singly protonated at either the δ- or ε-nitrogen positions at pH 7.4 but were protonated at both positions at pH 4.0. Five of these residues (H413, D448, E481, D487, and E518) are in close proximity to the PuBS. As a result, the PuBS exhibits a more positive surface electrostatic potential at pH 4.0 (Fig. 4 and <em>SI Appendix</em>, Fig. S5), which is expected to facilitate binding to anionic polysaccharides at this pH. Furthermore, the majority of differentially protonated residues were found on the surface of SRRP<sub>53608</sub>-BR<sub>262-571</sub>, which predicts an interruption of key interactions between symmetry-related molecules, potentially explaining why no crystallization could be achieved at pH 4.0 (<em>SI Appendix</em>, Fig. S4).

Molecular dynamics (MD) simulations in the microsecond timescale revealed a pH-dependent conformational change in the loop connecting β30 and β31, close to the PuBS, resulting in greater solvent exposure of putative binding residues (Fig. 4). This change is caused by rotation about the Cα–C bond (ψ) of I514 so as to facilitate hydrogen bond formation between the backbone carboxyl of I514 and the hydroxyl of the D487 carboxylic sidechain (<em>SI Appendix</em>, Fig. S6). This is possible only at pH 4.0 due to the protonation of the D487 carboxylate group. Furthermore, a resulting reduction in steric interactions between I514 and R512 led to subtle sidechain rearrangements of R512 and Y482 (<em>SI Appendix</em>, Fig. S7), which may prearrange these residues for ligand recognition.

**SRRP<sub>53608</sub>-BR Displays Specific Binding to Polyanionic Ligands via a Low-pH Mode of Adhesion.** Glycan arrays were first used in an attempt to identify the potential ligands of L<sub>5</sub>SRRP-BR. Due to the reported binding specificity of several SRRP-BRs from Gram-positive pathogenic bacteria to sialylated structures, we first tested the binding of SRRP<sub>53608</sub>-BR against a sialoglycan microarray presenting over 70 synthetically recreated, naturally occurring oligosaccharide structures with diverse sialic acid forms, glycosidic linkages, and underlying glycans (41, 42) using sodium acetate buffer (pH 4.0) or sodium phosphate buffer (pH 7.4). However, no significant binding was observed under the tested conditions. No significant binding was detected at neutral pH using version 5.1 mammalian glycan arrays from the Consortium for Functional Glycomics (CFG) that contain 610 distinct potential glycan receptors.

### Table 1. Data collection and refinement statistics for SeMetSRRP<sub>53608</sub>-BR, SRRP<sub>53608</sub>-BR, and SRRP<sub>100-23</sub>-BR

<table>
<thead>
<tr>
<th>Protein</th>
<th>SeMetSRRP&lt;sub&gt;53608&lt;/sub&gt;-BR</th>
<th>SRRP&lt;sub&gt;53608&lt;/sub&gt;-BR</th>
<th>SRRP&lt;sub&gt;100-23&lt;/sub&gt;-BR</th>
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<tr>
<td>Beamline</td>
<td>i04</td>
<td>i03</td>
<td>i04</td>
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<td>Wavelength, Å</td>
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<td>P 3&lt;sub&gt;2&lt;/sub&gt; 2 1</td>
<td>H 3 2</td>
</tr>
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<td>Cell parameters: a, Å</td>
<td>144.80, 144.80, 110.73</td>
<td>146.70, 146.70, 110.42</td>
<td>162.36, 162.36, 146.78</td>
</tr>
<tr>
<td>α, β, γ, *</td>
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<td>90, 90, 120</td>
<td>90, 90, 120</td>
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<td>Resolution, Å</td>
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<td>73.35–1.92 (1.95–1.92)</td>
<td>63.40–2.00 (2.05–2.00)</td>
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<td>l/Å</td>
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<td>9.9 (2.2)</td>
<td>16.8 (2.2)</td>
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<td>Unique reflections</td>
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<td>104,897 (5,168)</td>
<td>50,080 (3,650)</td>
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<tr>
<td>Completeness, %</td>
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<td>100.0 (99.6)</td>
<td>100.0 (99.9)</td>
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<tr>
<td>Multiplicity</td>
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<td>18.0 (15.5)</td>
<td>9.2 (9.5)</td>
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<td>0.061 (0.945)</td>
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<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;</td>
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<td>0.286 (3.191)</td>
<td>0.069 (1.060)</td>
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<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;</td>
<td>0.022 (0.178)</td>
<td>0.067 (0.797)</td>
<td>0.031 (0.472)</td>
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<tr>
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<td>Molprobity score</td>
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<td>1.60 (94rd percentile)</td>
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<tr>
<td>Clashscore</td>
<td>2.98 (99th percentile)</td>
<td>3.98 (99th percentile)</td>
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</tr>
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</table>
Following the structural homology of SRRP<sub>35608</sub>-BR and SRRP<sub>100-23</sub>-BR with PelC-like proteins, binding of SRRP<sub>35608</sub>-BR was performed with polygalacturonic acid (PGA)-containing pectin fragments using a carbohydrate array of well-characterized plant polysaccharides and oligosaccharides produced by partial hydrolysis from polysaccharides (43, 44). Interestingly the binding was found to be pH-dependent, with SRRP<sub>35608</sub>-BR showing specific and reproducible binding to pectin structures at pH 4.0 but not at pH 7.4 (SI Appendix, Fig. S8 and Table S4). In most cases, the glycans showing binding were lime pectin fractions with a low degree of esterification (DE ≤ 31%) of the galacturonic acid moieties present or PGA isolated from citrus pectin, whereas pectin fractions with higher DE values gave little or no binding. Such pH-dependent interaction was confirmed by bio-layer interferometry using the Octet system where biotinylated-SRRP<sub>35608</sub>-BR was immobilized on streptavidin-coated optical biosensors and probed with rhamnogalacturonan I (RGI), pectin esterified from citrus fruit (PECF), or PGA as ligand. A sensorgram showing the rate of association (k<sub>a</sub>) and rate of dissociation (k<sub>d</sub>) of SRRP<sub>35608</sub>-BR binding to PGA or RGI at pH 4.0 is shown in SI Appendix, Fig. S9A. The kinetic parameters for the interaction of SRRP<sub>35608</sub>-BR and PGA were determined through global fitting of raw data using a 1:1 (Langmuir) binding model with a k<sub>a</sub> = 3.03 × 10<sup>6</sup> M<sup>−1</sup> s<sup>−1</sup> ± 0.54% and k<sub>d</sub> = 7.17 × 10<sup>−5</sup> s<sup>−1</sup> ± 0.69%, yielding an equilibrium dissociation constant, K<sub>d</sub> = 0.237 × 10<sup>−6</sup> M with an R<sup>2</sup> of 0.997077 and full χ<sup>2</sup> = 0.23 (Table 2), the latter two values confirming the fitting of the model (SI Appendix, Fig. S9A). While binding was observed with RGI at pH 4.0, it was not possible to fit an acceptable model to the data, although it is apparent that the interaction dissociates rapidly upon removal of the ligand (SI Appendix, Fig. S9B). Binding was not observed for two commercially available pectins, PECF and pectin P7536, both obtained from Sigma (SI Appendix, Fig. S9C). Additionally, no binding could be detected when the experiments were performed at pH 7.4. Preliminary screening assays using pH 7.4, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, and 4.0 demonstrated the pH-dependent increase in binding to PGA at lower pH (pH 5.0 and below). Initial assays omitting EDTA or Tween 20 in the run buffers resulted in nonspecific binding. As shown in Fig. 3, the superposition of the SRRP<sub>35608</sub>-BR<sub>262–571</sub> and TM-Pel-TGA

Fig. 3. Comparison of the TM-Pel-binding pocket with the PuBS of SRRP<sub>35608</sub>-BR and SRRP<sub>100-23</sub>-BR. (A) Crystal structure of TM-Pel (Protein Data Bank ID code 3ZSO) (orange) in complex with TGA (cyan). (B) A close-up view of the TM-Pel–binding pocket, with residues involved in TGA binding represented as sticks. (C) Superposition of SRRP<sub>35608</sub>-BR<sub>262–571</sub> (light pink) and TM-Pel (orange) structures, with an rmsd of 2.63 over 210 residues, showing that the PuBS of the former overlaps with that of TM-Pel. (D) Surface-exposed aromatic and charged residues on PB3 in SRRP<sub>35608</sub>-BR<sub>262–571</sub> PuBS (light pink). This includes the aromatic residue triad W375, Y425, and W450 and the basic residue triad K485, R512, and R543. (E) Solvent-exposed residues of PuBS in the overlaid structures of SRRP<sub>35608</sub>-BR (light pink) and SRRP<sub>100-23</sub>-BR (green), with an rmsd of 0.912 over 293 residues, showing that the latter two values confirming the fitting of the model (SI Appendix, Fig. S9A). While binding was observed with RGI at pH 4.0, it was not possible to fit an acceptable model to the data, although it is apparent that the interaction dissociates rapidly upon removal of the ligand (SI Appendix, Fig. S9B). Binding was not observed for two commercially available pectins, PECF and pectin P7536, both obtained from Sigma (SI Appendix, Fig. S9C). Additionally, no binding could be detected when the experiments were performed at pH 7.4. Preliminary screening assays using pH 7.4, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, and 4.0 demonstrated the pH-dependent increase in binding to PGA at lower pH (pH 5.0 and below). Initial assays omitting EDTA or Tween 20 in the run buffers resulted in nonspecific binding. As shown in Fig. 3, the superposition of the SRRP<sub>35608</sub>-BR<sub>262–571</sub> and TM-Pel-TGA

Fig. 4. The pH-dependent conformational change affects the PuBS, as predicted by MD simulations. (A) Combined surface and cartoon models of SRRP<sub>35608</sub>-BR<sub>262–571</sub> (pink) and SRRP<sub>100-23</sub>-BR<sub>257–623</sub> (green). (B) Surface representation of SRRP<sub>35608</sub>-BR<sub>262–571</sub> at pH 7.4 and pH 4.0 in the same orientation as in A, showing surface electrostatics (Upper) and surface-exposed putative binding residues (Lower, green). At pH 4.0, PuBS exhibits a more positive electrostatic potential as well as an open conformation that exposes a greater number of putative binding residues to the solvent. Coordinates were obtained from representative frames of each respective MD trajectory (Methods). Surface electrostatics were calculated in PyMOL and are color-coded as blue (positive), white (neutral), and red (negative).
structures allowed the identification of possible binding residues in SRRP_{53608}-BR (SI Appendix, Methods, Figs. 3F, and Table 2). To further investigate the specificity of the interaction, a series of SRRP_{53608}-BR mutants was generated by site-directed mutagenesis (Methods, Fig. 3F, and SI Appendix) and tested against PGA. These included two alanine-substituted single mutants, K377A and R512A, and one where the lower loop from F411 to T422 was deleted, designated “ΔF411–T422.” K377 and R512 were selected due to their localization at the extremities of the proposed PuBS. Furthermore, MD simulations also indicated a possible role of R512 in ligand binding. The ΔF411–T422 mutant was created to evaluate the importance of the flexible loop in ligand binding. All mutants showed similar circular dichroism spectra at pH 7.4, suggesting correct folding of the recombinant proteins (SI Appendix, Fig. S10). Additionally, no differences were observed between wild-type SRRP_{53608}-BR at pH 7.4 and pH 4.0 (SI Appendix, Fig. S10). The mutations led to reduced $k_p$ values, showing similar $k_b$ but increased $k_D$, in comparison with the wild-type protein (Table 2). Chondroitin sulfate A (from bovine trachea) was also tested against immobilized SRRP_{53608}-BR under the above conditions. Similarly, no binding was observed at pH 7.4. However, at pH 4.0, concentration-dependent binding could be observed with chondroitin sulfate A (SI Appendix, Fig. S9D). Binding analysis of chondroitin sulfate A (as above) determined a $k_b = 9.2 \times 10^{-6}$ M$^{-1}$ s$^{-1}$ and $k_D = 8.72 \times 10^{-6}$ s$^{-1}$ at pH 7.4 and $k_D = 3.04\%$, yielding a $K_D = 9.47 \times 10^{-7}$ M with an $R^2 = 0.9935$ and $\chi^2 = 0.21$.

**LrSRPP-BR Promotes L. reuteri Adhesion to the Intestinal Epithelium.** To determine the contribution of LrSRPP-BR adherence to the host tissue following the reported ability of *L. reuteri* 100-23 (but not the *L. reuteri* 100-23 srrp mutant) to form biofilm in vivo (6), we performed adhesion assays to tissue sections of the mouse epithelium using soluble recombinant SRRP_{53608}-BR at pH 7.4. Interestingly, we could detect binding of SRRP_{53608}-BR to both stomach and colonic epithelium. In both types of tissue, the staining patterns correlated with wheat germ agglutinin (WGA) binding. No staining was observed in negative controls (SRRP_{53608}-BR-free) (Fig. 5 A and B). Binding of SRRP_{53608}-BR to colonic tissue sections was significantly reduced following treatment with periodate at pH 4.0 (Fig. 5C), suggesting binding to glycoproteins. In the healthy stomach of mice and humans, the MUC5AC and MUC6 mucins dominate and are produced by the surface epithelium and glands, in line with the mucins produced by human HT-29-MTX cells. Direct binding of LrSRPP-BR to the HT-29-MTX cells was carried out at pH 7.4. The SRRP_{53608}-BR staining pattern correlated with WGA lectin and, to some degree, also with MUC5AC staining. No staining was observed in the negative control (SRRP_{53608}-BR-free) (Fig. 5D). Furthermore, binding was also observed to HT-29 cell lines (Fig. 5E), suggesting that at pH 7.4 SRRP_{53608}-BR can recognize mucins and/or other epithelial receptors.

To identify the nature of the ligands, we investigated the binding of SRRP_{53608}-BR to individual components of the epithelium and mucus layer including purified mucins and DNA by atomic force microscopy (AFM). Compared with other techniques used to measure the force magnitude of ligand–receptor interactions, AFM provides specific information on the distance of interactions between molecules, i.e., the distance to the functionalized tip as it moves along the immobilized ligand and retracts from the surface, as shown in the examples of force curves in Fig. 6. Insets. Fig. 6 shows quantification of the magnitude of adhesion captured in the retraction curves of the force spectroscopy measurements between SRRP_{53608}-BR and mucin. In neutral buffer (pH 7.4) the modal value of adhesion events was 105 pN, whereas bimodal values of adhesion events, at 72 pN and 120 pN, were obtained in acidic buffer (pH 4.0). The addition of PGA appeared to abolish SRRP_{53608}-BR–mucin interactions at pH 4.0, as PGA binds specifically to SRRP_{53608} only in acidic conditions. Furthermore, despite the adhesion magnitudes being similar in both buffers, the SRRP_{53608}-BR and mucin interactions in the acidic buffer produced a significantly larger range of the length of adhesion events compared with the data obtained in neutral conditions (Fig. 6B). This suggests that SRRP_{53608}-BR has a better ability to interact along the entire length of the mucin chains at acidic pH compared with neutral pH. Fig. 6 A and B shows a set of the typical force–distance curves from each of the experiments to reveal the distance variations between pH 7.4 and 4.0. In Fig. 6 C, direct binding of SRRP_{53608}-BR to PGA was further confirmed by AFM at pH 4.0. There was a significantly large range of adhesion magnitudes, from 33–428 pN. The modal value was 36 pN at pH 4.0 and 18 pN in the neutral buffer, which corresponds to the noise level in the force spectra. Adding free PGA to the AFM liquid cell led to a significant reduction in adhesion events due to competitive interactions between free PGA and the glass-attached PGA molecules, as shown in the force curve example, thereby confirming the specificity of the interaction.

![Fig. 5. Adhesion of SRRP_{53608}-BR to GI tissue.](image-url)
Species addition, the binding ligand of bacterial attachment to host surfaces is a pivotal event in the biological and infectious processes of both commensal and pathogenic bacteria. SRRPs and their associated secretion systems are being defined in a growing number of Gram-positive bacteria, indicating their crucial roles in mediating interaction with the host.

Atomic-resolution structures of seven SRRP binding regions have been reported for Gram-positive pathogens to date, highlighting a relationship between their structural folds and binding ligands. These include S. parasanguinis Fap1 [Protein Data Bank (PDB) ID codes 2KUB and 2X12] (45), S. gordonii GspB (PDB ID code 3Q5C5/6) (13), S. sanguinis SrpA (PDB ID code 5EQ2) (46), Srr-1 and Srr-2 paralogues of S. agalactiae (PDB ID codes 4MO(B/R) (47, 48), S. aureus SrrA [PDB ID codes 4M0(0–3) (49), and S. pneumoniae PsrP (PDB ID codes 3ZGH/I) (50)], Srr-1, Srr-2, and PsrP each adopt variations of the Dev-IgG fold (47, 50, 51) and bind to long β-strands in their target proteins, thereby forming a complementary strand along one of the Ig-like domains of the Dev-Ig protein, with Srr-1 binding to cytokeratin 4 (52–54), Srr-1 and Srr-2 binding to fibrinogen Aα (47, 55–58), and PsrP adhering to cytokeratin 10 (59) and to DNA (60). Other SRRP-BR regions are composed of two or more subdomains and include (from the N to the C terminal) the helical and CnaA folds for Fap1 (45), the CnaA, sialic acid-binding Ig-like lectin (siglec), and unique subdomains for GspB (13), the siglec and unique subdomains for SrpA (46), and a legume lectin-like fold, a β-sandwich, and two eukaryotic cadherin-like modules for SrrA (49). The GspB, Hsa, SrpA, and SrrA SRRP-BRs have all been shown to bind to different types of sialylated ligands (13, 49, 61–71), whereas the binding ligand of Fap1 remains to be identified (45). Lrrsrp-BR did not recognize sialylated glycans but was found to bind to host epithelium or pectin-like components in a pH-dependent manner. This difference in ligand specificity can be explained by the Lrrsrp-BR right-handed β-solenoid topology, which is typically adopted by extracellular, enzymatic PeC-like proteins. Such a fold has not previously been reported in SRRP-BRs and in proteins from L. reuteri species. Additionally, the Lrrsrp-BR structural data indicate a high representation of Trp, Tyr, and basic residues in the PuBS, suggesting an involvement in carbohydrate binding, which may correlate with Lrrsrp-BRs’ recognition of mucin glycoproteins and plant-derived anionic polysaccharides. In addition, mutagenesis confirmed the importance of the binding loop and residues R512 and K377 within the PuBS in the interaction of SRRP53608-BR with PGA.

**Discussion**

**Structural Differences Between SRRPs from Lactobacilli and Pathogenic Streptococci and Staphylococci Reflect Differences in Ligand Specificity.** Bacterial attachment to host surfaces is a pivotal event in the biological and infectious processes of both commensal and pathogenic bacteria. SRRPs and their associated secretion systems are being defined in a growing number of Gram-positive bacteria, indicating their crucial roles in mediating interaction with the host.

Force spectroscopy was also used to investigate the binding of SRRP53608-BR to DNA (Fig. 7). Increasing the NaCl molarity from 137 mM to 1 M in the PBS buffer caused a minor reduction of the adhesion frequency (from 15 to 12) of SRRP53608-BR to DNA but increased the modal values of the adhesion events (from 123 to 160 pN) (Fig. 7A), suggesting that binding was unlikely to be solely due to electrostatic interactions. To further assess the specificity of the interaction between SRRP53608-BR and DNA, free DNA sample was added into the AFM liquid cell, resulting in a minor reduction of adhesion event frequency (from 33 to 30) and of modal values (from 83 to 60 pN) (Fig. 7B). Adding single nucleotides separately (Fig. 7C–E) or in tandem (Fig. 7F, Insets) did not cause any major inhibition (only minor reductions in the frequency and modal values of the adhesions were observed). In contrast, the simultaneous addition of all four nucleotides led to a significant inhibition in terms of the adhesion event frequency (from 92 to 12) with similar modal values of adhesion (71 and 86 pN, respectively) (Fig. 7F), which is expected from single ligand–receptor interaction events as measured in the timescale of AFM experiments. These results suggest that all four nucleotides are recognized by SRRP53608-BR.

**Fig. 6.** AFM force spectroscopy histograms of SRRP53608-BR interacting with mucin or PGA. Insets show examples of force curves for each assay. (A) Quantification of adhesion values to mucin in buffers of neutral and acidic pH values or following PGA addition. (B) Quantification of variations in the mucin adhesion event distances. (C) Quantification of adhesion values to PGA in buffers of neutral and acidic pH values.

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**LrSRRP-BR Binds to Polyanionic Ligands via a pH-Dependent Binding Mode.** Three major carbohydrate structures are found in pectin, which include homogalacturonan, RGI, and rhamnogalacturonan II. Strong pH dependency was observed when SRRP\textsubscript{53608}BR binding was tested against chondroitin sulfate A and selected pectin ligands. SRRP\textsubscript{53608}BR bound PGA and RGI at pH 4.0, but no binding was observed at pH 7.4 or against the two other commercial pectins tested at either pH. A similar preference for acidic pH was observed for binding to chondroitin sulfate A. Long MD simulations (at a microsecond timescale) at pH 4.0 and pH 7.4 showed that SRRP\textsubscript{53608}BR undergoes a pH-dependent conformational change close to the PuBS, so that a greater region of the PuBS is solvent-exposed at pH 4.0. Coupled with a rearrangement of postulated key sidechains (D487, R512, I514, and Y482) at low pH, this suggests a mechanism for the observed differential binding to anionic PGA, RGI, and chondroitin sulfate A polysaccharides (SI Appendix, Fig. S11) at pH 4.0 and pH 7.4. Furthermore, the models suggest a notable difference in surface charge distribution in the PuBS at the two pH values, exhibiting a more positive potential at pH 4.0, which would certainly have a marked impact on the ability of SRRP\textsubscript{53608}BR to bind anionic substrates. These results also suggest that other linear polyanionic glycosaminoglycan polysaccharides may be relevant biological ligands for SRRP in the gut. Such binding pH-dependent conformational change has previously been reported for the pectin lyase PelA (32) and also for the S. parasanguinis SRRP adhesin, Fap1, where the low-pH-driven conformational change modulates adhesion and likely plays a role in survival in acidic environments (72).

**L. reuteri** and many other *Lactobacillus* spp. are primary colonizers of the proximal GI tract and therefore are exposed to acidic stress in the stomach. The pH values in the rodent forestomach region range between 3.8 and 5.1 depending on feeding (73). Similarly, in the porcine stomach, the pH is relatively low at the esophageal terminus of the stomach and is higher toward the pylorus (74). In addition to a longitudinal pH gradient along the GI tract, there is a pH gradient across mucus, as demonstrated in rodents in vivo, supporting a role for this barrier in gastric mucosal protection (75, 76). Our findings that *Lr* SRRP-BR binds to dietary components at low pH and to the mucosal epithelium at higher pH are in line with the observed pH gradient from the lumen to the epithelium surface. It is also worth noting that in the stomach, the *Helicobacter pylori* sialic acid-binding adhesin (SabA) shows a charge-low-pH-dependent mechanism likely to play different roles during colonization of the oral to gastric niches and during long-term infection (77).

**Lessons on Niche Specificity and Biofilm Formation.** SRRPs comprise a large family of adhesins in Gram-positive bacteria (45) which are exported by an accessory Sec system (the SecA2–SecY2 system) (11) and are important for biofilm formation (64, 79). Here we showed that in *L. reuteri* strains genes encoding homologs of SRRPs are generally colocalized within the SecA2–SecY2 gene cluster with only a few exceptions of unlinked SRRP genes or pseudogenes. While the overall domain organization of SRRPs is conserved between Gram-positive bacteria species, the individual SRRP-BR domains are highly diverse with limited to no sequence homology (80). SRRPs have been characterized from numerous streptococci and staphylococci inhabiting different environments.

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**Fig. 7.** AFM force spectroscopy histograms of SRRP\textsubscript{53608}BR interacting with DNA. Quantification of adhesion values of SRRP\textsubscript{53608}BR interacting with DNA in 137 mM NaCl or 1 M NaCl PBS (A) or after the addition of free DNA (B) or free nucleotides dGTP (C), dATP (D), dCTP (E), all four nucleotides (dGTP, dATP, dTTP, and dCTP) (F), or two nucleotides (dGTP and dTTP) (Inset in F).
riches and contributing to pathogenesis due to their role in host cell adhesion and biofilm formation. FapP1 from *S. parasanguinis* (72, 81, 82) as well as the sialoglycan-binding SRR adhesins Hsa and GspB from *S. gordoni* strains CH1 and M99, respectively, are involved in dental plaque formation and periodontal disease via attachment to salivary components. They are also a virulence factor for infective endocarditis initiated by their binding to sialylated glycoproteins. *S. parasanguinis* and *S. aureus* are other pathogens causing bacterial endocarditis in which their SRR adhesins, SrpA and SraP, respectively, also mediate binding to salivated receptors on human platelets (63, 83). Srr-1 and Srr-2 adhesins from the *S. agalactiae* pathogen, causing neonatal meningitis, bind to human fibrinogen and keratin 4. Keratin 4 binding mediates colonization of the female genital tract, leading to neonatal infection, and fibrinogen binding mediates adhesion to human brain microvascular endothelial cells (47, 55). PspF from *S. pneumoniae*, causing streptococcal pneumonia, facilitates biofilm formation on lung epithelial cells via self-oligomerization, and by DNA binding and adhesion to keratin 10, both facilitated by its BR (50, 59, 62, 78). It is worth noting that SRRPs also occur in commensal streptococci based on sialic acid binding and adhesion to keratin and DNA (9145). 274. *Streptococcus cristatus* was recently shown to play a major role in host colonization, although no structural information is available (1-4).

Here, we show that SRRP34353BR from *L. reuteri* binds to the epithelium and dietary components in a pH-dependent mechanism, which may favor persistence in the GI tract. In addition to its role in adhesion to polysaccharides and glycoproteins, SRRP34353BR could bind DNA in a specific manner, as shown by AFM. DNA from autolysed bacterial cells is a component of many biofilms, helping form an extracellular network to which live cells can attach. This therefore may contribute to the ability of *L. reuteri* rodent strains (and perhaps other *Lactobacillus* sp., harboring SRRPs identified by bioinformatics analysis) to form biofilm on the murine forestomach in vivo (6). While pathogenic biofilms contribute to states of chronic inflammation, biofilm formation by probiotic bacteria such as *Lactobacillus* spp. causes a negligible immune response and is considered a beneficial property by promoting colonization and longer permanence in the host mucosa and limiting colonization by pathogenic bacteria. Understanding at the molecular level the contribution of lactobacillus SRRPs in biofilm formation is needed to fully exploit the functions of this intra- and interspecies family of adhesins across Gram-positive bacteria. These molecular findings may help the rational selection of probiotic strains of lactobacilli that can compete with the SRRP-mediated adhesion of pathogenic streptococci and staphylococci.

**Methods**

**Crystallography of SRRP34353BR and SRRP150-23BR.** Cloning, purification, cryo-

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**Binding Assays.** Binding of SRRP34353BR wild-type and mutants to PGA, RGI, pectins, and chondroitin sulfate A was performed by bio-layer interferometry. Binding of SRRP34353BR to mucins, DNA, and PGA was assessed by force spectroscopy using AFM. Immunofluorescence was used to monitor SRRP34353BR binding to mouse intestinal tissue sections as described in *SI Appendix.*

**Full details of all experimental procedures used are described in SI Appendix.**

Serine-rich repeat protein 1 from Streptococcus agalactiae reveals a novel interaction with fibrinogen and vaginal colonization.


