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Lactobacillus helveticus MIMLh5-Specific Antibodies for Detection of S-Layer Protein in Grana Padano Protected-Designation-of-Origin Cheese

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Single-chain variable-fragment antibodies (scFvs) have considerable potential in immunological detection and localization of bacterial surface structures. In this study, synthetic phage-displayed antibody libraries were used to select scFvs against immunologically active S-layer protein of *Lactobacillus helveticus* MIMLh5. After three rounds of panning, five relevant phage clones were obtained, of which four were specific for the S-layer protein of *L. helveticus* MIMLh5 and one was also capable of binding to the S-layer protein of *L. helveticus* ATCC 15009. All five anti-S-layer scFvs were expressed in *Escherichia coli* XL1-Blue, and their specificity profiles were characterized by Western blotting. The anti-S-layer scFv PolyH4, with the highest specificity for the S-layer protein of *L. helveticus* MIMLh5, was used to detect the S-layer protein in Grana Padano protected-designation-of-origin (PDO) cheese extracts by Western blotting. These results showed promising applications of this monoclonal antibody for the detection of immunomodulatory S-layer protein in dairy (and dairy-based) foods.

Lactobacillus helveticus is a non-spore-forming Gram-positive thermophilic homofermentative lactic acid bacterium which has a long history of use in the production of cheese types where high cooking temperatures are required (for instance, Grana Padano, Parmesan, Emmental, Gruyere, Comte, and Romano cheeses). Besides its well-known technological importance in cheese making, a growing number of studies are demonstrating that *L. helveticus* strains can also exhibit significant health-promoting properties, and it is now therefore included among the bacterial species that are generally considered to be probiotic (1).

L. helveticus MIMLh5 is a dairy bacterial strain isolated from the natural whey starter of Grana Padano, a protected-designation-of-origin (PDO) cheese (2, 3). MIMLh5 was included in previous studies for its metabolic, biotechnological, and probiotic features (2, 4, 5) and, interestingly, for its immunomodulatory properties (5–7). Particularly, *L. helveticus* MIMLh5 was proposed as a potential pharyngeal probiotic because of its ability to adhere to human epithelial cell lines and to efficiently antagonize group A streptococci on these cells (6). Furthermore, *L. helveticus* MIMLh5 appeared to be a promising modulator of the immune system which is able to reduce NF-κB activation, to influence cytokine secretion at the epithelial level, and potentially to skew the immune system toward a Th1 response (5–7). It has been documented that immunomodulatory properties of *Lactobacillus* spp. (e.g., *Lactobacillus acidophilus* NCFN [8, 9], *Lactobacillus brevis* ATCC 8287 [10], and *L. helveticus* strain M92 [11]) are due to their surface layer (S-layer) protein. Recently it was demonstrated that the immunostimulatory properties of *L. helveticus* MIMLh5 are mainly mediated by its S-layer protein as well (12). Specifically, it was shown that the S-layer protein mediates the activation of the innate immune system, as demonstrated by *in vitro* and *ex vivo* experiments performed on human intestinal epithelial cells and primary and tissue-specialized human and murine macrophages. As a consequence of the above-mentioned importance of the MIMLh5 S-layer protein, we decided to undertake this study, aimed at the development of a strategy to sensitively and selectively identify and track this protein.

Antibodies are a powerful tool to study protein function, protein localization, and protein-protein interactions, and they are also widely used for diagnostic and therapeutic purposes (13–16). Collections of antibody fragments can be displayed as a fusion to the coat protein of the filamentous phage of *Escherichia coli*, and from such repertoires, numerous target-specific antibodies have been extracted for both protein and hapten targets (17). Most commonly, the antibodies are displayed on the phage as single-chain variable fragment antibodies (scFvs). These antibodies carry only the variable domains, the minimal fragments needed for antigen recognition by a full-length IgG, linked to each other with a flexible glycine-serine linker (18). Their relatively small size allows easy genetic manipulation and construction of large libraries in the range of 10^{10} members (19). The antibody phage display technique for monoclonal antibody generation is much faster than the conventional hybridoma method and does not require any sophisticated equipment (17). Once antigen-positive scFv binders have been identified, they can be produced as soluble antibodies for use as immunological reagents (20).

The aim of the present study was to generate a monoclonal *L. helveticus* MIMLh5 S-layer-specific scFv antibody using phage display technology. As an application of this technology, the recom-

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binant scFv, expressed in *E. coli*, was used to detect this protein in Grana Padano PDO cheese.

MATERIALS AND METHODS

Reagents, bacterial strains, and growth conditions. All chemicals and reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. *L. helveticus* strains were grown in De Man, Rogosa, and Sharpe (MRS) broth (Sigma-Aldrich) supplemented with 1% Tween 80 at 42°C, whereas *L. acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were grown in the same medium at 37°C. *Lactobacillus* strains were inoculated from frozen glycerol stocks and subcultured twice in MRS medium using 1% inoculum.

Extraction of S-layer protein from *L. helveticus* MIMLh5. Extraction of the S-layer protein from *L. helveticus* MIMLh5 was performed with 5 M LiCl as described previously (21–24) and detailed in reference 12. Protein concentration was determined by the Bradford microassay method (25) using bovine serum albumin (BSA) as a standard.

Coating procedure. S-layer protein was used to coat microtiter wells by passive adsorption. Freeze-dried S-layer protein was dissolved in 5 M LiCl solution in MilliQ water to 50 μg ml^{-1} and later diluted to 10 μg ml^{-1} with 5 M LiCl. MaxiSorp and PolySorp microtitration plates in 12-strip well formats were from Nunc (Roskilde, Denmark). The normal coating procedure for S-layer protein is briefly described below. S-layer protein was diluted in the coating buffer (100 mM Tris-HCl, pH 9.0) to a final concentration of 5.0 μg ml^{-1} (or 1.0 μg ml^{-1}). Then 200 μl of the coating solution was dispensed into each well, giving 1 μg (for pannings) and 0.2 μg (for immunoassays) S-layer protein per well. The plates were closed in a humidified box and incubated overnight at 25°C. The plates were washed twice in a Delfia Platewash (Perkin-Elmer Life Sciences, Turku, Finland) with Delfia wash solution supplemented with Tween 20 to the final concentration 0.05%. After washing, 250 μl of saturation solution (50 mM Tris-HCl, pH 7.0; 150 mM NaCl; 0.05% NaN_3; 6% D-sorbitol) with BSA (0.2% bovine serum albumin fraction V powder, gamma globulin free; Sigma-Aldrich) or skim milk (1%) was added per well. The plates were saturated overnight at 25°C. The saturation solution was aspirated in a Delfia Platewash, and the plates were dried (25°C, under the laminar hood) for 4 h. Finally the plates were packed with moisture adsorbent into an aluminum zip bag and stored dry at 4°C. The control plates were prepared by coating microtiter wells with only saturation solution, containing BSA or skim milk.

Synthetic human antibody libraries, vectors, and helper phage. The synthetic human single-framework antibody libraries ScFvP and ScFvM are fully synthetic man-made antibody repertoires (26). They were created from a single-germ-line antibody sequence consisting of a human immunoglobulin G (IgG), the most stable heavy chain domain (V_H3), and the most stable light chain domain (Vκ3). Partly different positions in the complementarity-determining region (CDR) loops were randomized, and the diversity of content was modified. The displayed ScFvP and ScFvM repertoires were combined and used for selecting antibodies against the S-layer protein of *L. helveticus* MIMLh5. The scFvs were expressed in the library as a fusion to the truncated p3 of the filamentous phage from chloramphenicol-resistant phagemid pEB32x (see Fig. S1A in the supplemental material) and displayed monovalently by VCS M13 (Stratagene, La Jolla, CA) superinfection. For screening, the enriched scFv genes were cloned from the phagemid pEB32x into the ampicillin-resistant expression vector pLK06H with SfiI restriction sites (26). In pLK06H, the scFv sequences are fused to the gene for bacterial alkaline phosphatase (PhoA) (see Fig. S1B in the supplemental material). A 6-His tag at the C terminus of PhoA allows affinity purification using a nickel-nitrilotriacetic acid (Ni-NTA) matrix, and the induction of scFv genes in both pEB32x and pLK06H vectors was controlled with the Lac promoter. Fusion proteins were directed to the periplasmic space of *E. coli* by the *pelB* signal sequence. Primer pAKfor (5′-TGAAATACCTATTGCCTACG-3′) was used for sequencing of scFv fragment from the vector pLK06H.

Panning of microtiter wells. Panning is the process of the selection and isolation of specific antibodies by their binding activity (27). S-layer-coated microtiter wells were panned with a 1:1 mixture of pEB32x ScFvP and ScFvM libraries (26). This phage library mix (2.5×10^{12} CFU ml^{-1}, dilution in Tris-buffered saline [TBS] supplemented with 0.1% BSA [TBT] and 0.05% Tween 20 [TBT-0.05]) was first incubated in a well (200 μl $well^{-1}$) not coated with S-layer protein for 1 h at 25°C with slow shaking (Delfia Plateshake, low mode; Perkin-Elmer), after which the unbound fraction was transferred to S-layer-coated wells. After 2 h (for the first panning) and 1 h (for the subsequent pannings) of incubation, the wells were washed 3 times (for the first panning) and 4 times (subsequent pannings) with TBT-0.1 and then once with TBS–0.1% Tween 20. The bound phages were eluted with 200 μl of trypsin solution (60 μg ml^{-1} in TBS) for 15 min at 25°C and then neutralized with 1/10 volume of trypsin inhibitor (1 mg ml^{-1} in TBS).

The eluate was amplified by infection of *E. coli* XL1-Blue. The phages were purified by precipitation with 1/3 volume of polyethylene glycol 8000 (PEG-8000; 16%, wt/vol)-NaCl (12%, wt/vol) on ice for 10 min and titrated using LB agar plates with tetracycline (10 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}). The titrated phage was then diluted to 1×10^{11} CFU ml^{-1} in TBT-0.05 and applied as the input phage for subsequent pannings.

Screening for the binding specificity of scFv antibodies to purified S-layer protein. To screen for binding specificity of phage library stocks (scFv-p3 fusions), they were diluted 1:10,000 in red Kaivogen assay buffer (Kaivogen, Turku, Finland) and analyzed by a Delfia time-resolved fluorescence immunoassay. In the assay, an antigen, the S-layer protein, was first bound to MaxiSorp and PolySorp microtitration wells, 0.2 μg/well, as described above. Then phage samples were incubated for 1 h with slight agitation (Delfia Plateshake, low mode; PerkinElmer), and after washing, the plate Eu-N1-labeled anti-phage monoclonal antibody (University of Turku, Turku, Finland) was bound. The time-resolved fluorescence signal of Eu^{3+} was measured with Victor 1420 multilabel counter (PerkinElmer) after 10 min development with Delfia enhancement solution.

To screen for binding specificity of individual phage antibody clones (scFv-PhoA fusions), the enriched library was cloned into vector pLK06H (through SfiI restriction sites). Individual clones were inoculated in SB medium (0.05% glucose, 10 μg ml^{-1} tetracycline, 100 μg ml^{-1} ampicillin) onto a 96-well V-bottom culture plate (Corning Life Sciences, Pittston, PA) covered with breathable sealing tape (Nunc). Clones were grown for 4 to 6 h at 37°C, 700 rpm, and 70% humidity. The cells were induced with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated overnight at 26°C and 700 rpm. For periplasmic extraction, 1/5 volume of freshly prepared 5× lysis buffer (350 mM Tris [pH 8.0], 10 mM EDTA, 10 mg ml^{-1} lysozyme) was added. Plates were incubated 10 min at 30°C and 700 rpm and subsequently centrifuged for 10 min at 4°C and 3,220 × *g*. Supernatants were analyzed by alkaline phosphatase (AP) chromogenic enzyme-linked immunosorbent assay (ELISA). In the assay, samples, diluted 1:10 with red Kaivogen assay buffer were incubated for 1 h in the microtitration wells coated with S-layer protein. After the plate had been washed four times, the substrate, a *para*-nitrophenyl phosphate (pNPP) solution (50 mM Tris [pH 9.0], 200 mM NaCl, 1 mM $MgCl_2$, 5 mM pNPP), was added. Absorbance of *p*-nitrophenolate at 405 nm was measured with a Victor 1420 multilabel counter (PerkinElmer) after 1 h of color development. A clone was considered positive if the specific signal and relative absorbance [A_{405}(S-layer-coated well) − A_{405}(non-S-layer-coated well)] were above 0.5 (A_{405}).

The test of specificity of the *L. helveticus* MIMLh5 S-layer protein-binding clones was based on phage binding to different S-layer-containing lactic acid bacterial (LAB) strains in suspension, and it was performed as described below.

Screening for the binding specificity of scFv antibodies to S-layer protein-containing LAB strains. To screen for the MIMLh5 strain S-layer-specific phage antibodies, the MIMLh5 S-layer-positive scFv binder clones were inoculated in 5 ml of SB medium (0.05% glucose, 10 μg ml^{-1}

tetracycline, 100 μg ml^{-1} ampicillin) in culture tubes and cultivated at 37°C with 300 rpm shaking to an optical density at 600 nm (OD_{600}) of 0.8. The cultures were induced with 100 μM IPTG and grown overnight at 26°C with 250 rpm shaking. Cells were harvested by centrifugation at 3,220 × *g* for 10 min at 4°C. The pellet was resuspended in 1 ml (i.e., concentrated 5×) of modified Kaivogen assay buffer (mKAB; 50 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.01% Tween 40, 0.5% BSA [fraction V powder; Sigma-Aldrich]). Cells were disrupted by sonication and by two subsequent freeze (−70°C)-thaw cycles. The lysate was centrifuged at 15,700 × *g* for 5 min at 4°C, and the supernatant containing soluble anti-S-layer-scFv–PhoA binders was aliquoted and stored at −20°C before AP chromogenic immunoassay in suspension. In the assay, lactobacillus cells were grown overnight in MRS medium, harvested by centrifugation at 3,200 × *g* for 5 min at 4°C, resuspended in mKAB, and incubated for 30 min at a bacterial growth temperature. Then cell pellets were washed with mKAB and resuspended in the same buffer up to approximately 10^9 cells ml^{-1}. Portions (100 μl) of cell suspensions were aliquoted (approximately 10^8 cells/tube). The number of cells was selected empirically by testing different cell concentrations (e.g., 10^8 and 10^9 cells ml^{-1}). Portions (100 μl) of 5× diluted mKAB lysates, containing anti-S-layer-scFv-PhoA binders, were added. LAB cell and scFv-PhoA binder suspensions were incubated for 1 h at 25°C in rotational agitation (13 rpm, Grant-bio PTR-30 rotator; Grant Instruments, Cambridge, United Kingdom). After two washes with cold mKAB, a substrate (pNPP) solution was added, and the suspension was incubated at 25°C for 2 h with rotational agitation (13 rpm). Cells were precipitated by centrifugation at 15,700 × *g* for 5 min at 4°C, and the absorbance of *p*-nitrophenolate in the supernatant was measured as described above. A clone was considered positive for binding to S-layer protein if the specific signal was above 3.0 or the relative absorbance [A_{405}(MIMLh5 − A_{405}(Lb11842)] was above 1.0 (OD_{405}).

Preparation of anti-S-layer binders (anti-S-layer-scFv–PhoA–6×His fusions) for Western blotting. Anti-S-layer-scFv–PhoA–6×His fusion-expressing supernatants were prepared under native conditions as described below. Briefly, cultures in 5 ml SB (10 μg ml^{-1} tetracycline, 100 μg ml^{-1} ampicillin, 0.05% glucose) were inoculated with precultures to an OD_{600} of 0.05 and grown at 37°C with 300 rpm shaking to an OD_{600} of 0.8. The cultures were induced for periplasmic expression of anti-S-layer-scFv–PhoA binders with 200 μM IPTG and grown overnight at 26°C with 300 rpm shaking. One mg ml^{-1} lysozyme, 25 U ml^{-1} Benzonase (Merck KGaA, Darmstadt, Germany), and 1 mM $MgCl_2$ were added. Suspensions were incubated for 1 h at 25°C in rotational agitation and further subjected to a single freeze-thaw cycle. Samples were subsequently centrifuged at 3,200 × *g* for 10 min at 4°C. The supernatants (soluble fractions) were transferred to fresh tubes and stored on ice before use.

Preparation of LAB total protein extracts. LAB cells were cultivated overnight in MRS medium, harvested by centrifugation at 3,220 × *g* for 15 min at 4°C. Cell pellets were washed, resuspended in cold PBS buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1:5 (wet weight of biomass versus volume of resuspension buffer), and subjected to disruption in a French press (E 1061; Constant Systems, Daventry, United Kingdom) at a pressure of 35,000 lb/in^2 3 times consecutively. Cell lysates were collected, aliquoted, and stored at −70°C before being loaded onto a polyacrylamide gel.

Preparation of cheese extracts. Samples of commercial Grana Padano PDO cheese (i.e., at least 9 months old), designated 30 to 39, were kindly supplied by the Consorzio per la tutela del Formaggio Grana Padano with registration numbers indicating the manufacturer and the production site (see Table S1 in the supplemental material).

Samples were defatted by Soxhlet extraction with diethyl ether (28) and lyophilized. Subsequently, they were extracted with 5 M LiCl: 1.3 g of lyophilized cheese per 10 ml 5 M LiCl. Extractions were performed at 25°C for 1 h in rotational agitation. Samples were centrifuged at 5,000 × *g* at 4°C for 15 min. The supernatant was filtered through a 0.2-μm filter and exhaustively dialyzed for 36 h at 8°C against distilled water using 12-kDa-cutoff membranes (Sigma-Aldrich), which were prepared for dialysis by boiling in 2% $NaHCO_3$ and 1 mM EDTA solution. Each time the water was changed, 0.001% protease inhibitor cocktail was added. The dialysates were collected, and the total protein concentration was determined by the Bradford microassay method using bovine serum albumin (BSA) as a standard. Cheese extract dialysates were lyophilized and kept at −20°C.

SDS-PAGE and Western blotting. S-layer protein, total bacterial lysates, and Grana Padano PDO extracts were resuspended in SDS-PAGE sample buffer (29) (Bio-Rad Laboratories, Richmond, CA), boiled for 5 min, and separated on 10% SDS-PAGE (total bacterial lysates) or 4 to 20% mini-Protean TGX precast gels (Bio-Rad) in Tris-glycine-SDS buffer on a mini-Protean 3 system (Bio-Rad). Gels were stained with Coomassie brilliant blue G-250. Proteins from SDS-PAGE gels were transferred to polyvinylidene fluoride (PVDF) membranes (Hybond-P; Amersham Biosciences, Buckinghamshire, United Kingdom) on a Trans-Blot Turbo blotting system (Bio-Rad) according to the manufacturer's instructions. Membranes were blocked overnight in 5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS, pH 7.5) at 4°C. Blotting was then done using anti-S-layer binder expression supernatant (anti-S-layer-scFv–PhoA fusion) at a 1:100 dilution (in TBS with 0.05% Tween-20) as the primary antibody (incubation overnight at 8°C or for 1 h at 25°C in rotational agitation) and horseradish peroxidase (HRP)-conjugated anti-His mouse monoclonal IgG1 (5Prime; VWR International, Helsinki, Finland) at a 1:2,000 dilution (in TBS with 0.05% Tween-20) as the secondary antibody (incubation for 1 h at 25°C with rotational agitation). The membrane was visualized by chemiluminescent detection with the Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad) according to the manufacturer's instructions.

Detection of *L. helveticus* DNA and the S-layer gene in cheese samples. Total DNA from cheese was extracted by the lytic method as described by Quigley et al. (30). *L. helveticus*-specific PCR was performed using primers PeC_f and PeC_r for the amplification of *pepC* locus as described by Fortina et al. (31). The PCR conditions were as follows: predenaturation at 94°C for 2 min; 40 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min; and a single final extension at 72°C for 7 min. The S-layer coding gene was detected by PCR as described by Ventura et al. (32) using the specific primers SLY ex F (CTGCAACTGCTATGCCTGT) and SLY ex R (ATACGCTTAGTACCATCGA) (D. Mora, unpublished data). The PCR conditions were as indicated above except that the primer annealing temperature was increased up to 61°C and the elongation step was prolonged to 1.5 min. All amplification reactions were performed in a MyCycler thermal cycler (Bio-Rad). PCR products were loaded on 1.5% Tris-acetate-EDTA–agarose gels. GeneRuler DNA ladder mix (Thermo Scientific, Vilnius, Lithuania) was used as a fragment size marker.

RESULTS AND DISCUSSION

Preparation of *L. helveticus* MIMLh5 S-layer protein and similarity search. LiCl extraction was applied for the isolation of S-layer protein from *L. helveticus* MIMLh5. The purity and identity of the *L. helveticus* MIMLh5 S-layer protein were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reverse-phase high-pressure liquid chromatography–electrospray ionization-mass spectrometry (RP-HPLC/ESI-MS) analysis (12).

A similarity search using the deduced amino acid sequence of the *L. helveticus* MIMLh5 S-layer protein (EMBL database accession number HE993893) employing the algorithm BLASTP (33) revealed a high level of sequence homology to the other *L. helveticus* S-layer proteins (11) and to *L. acidophilus* S-layer proteins (8, 24) (Fig. 1).

Selection and basic characterization of single chain variable fragment (scFv) antibodies against *L. helveticus* MIMLh5 S-layer protein from phage-displayed libraries. scFv antibodies for *L. helveticus* MIMLh5 S-layer protein were obtained by

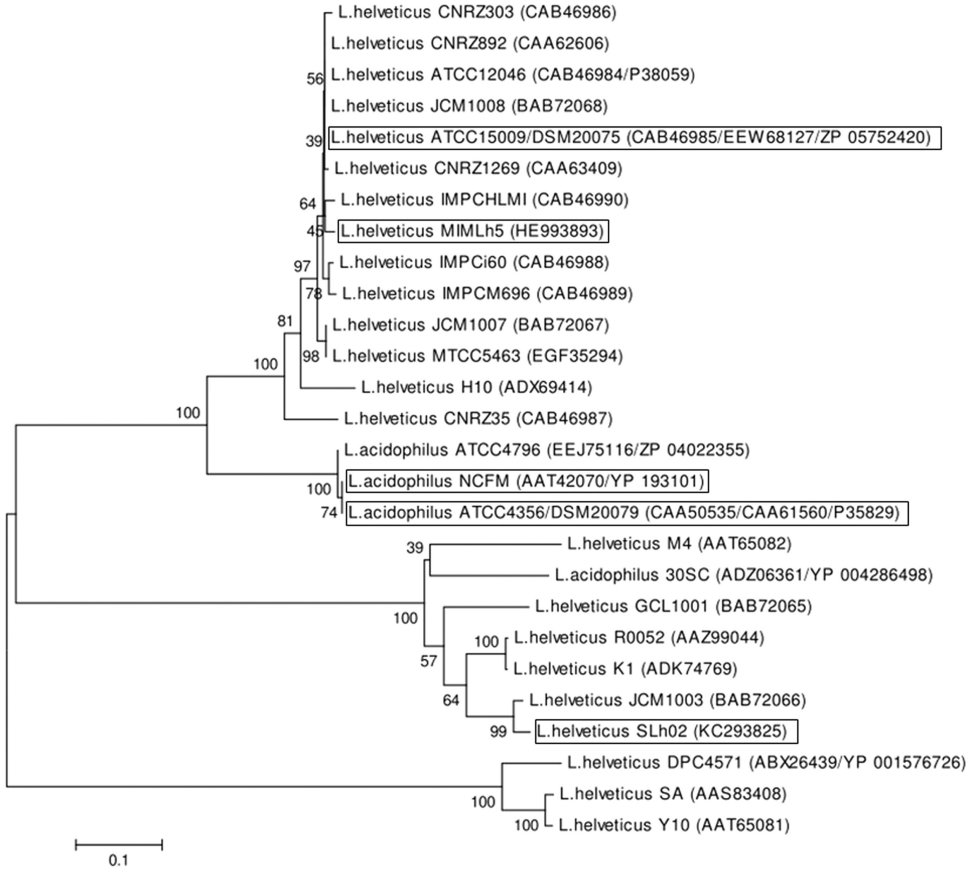

FIG 1 Neighbor-joining dendrogram generated from the ClustalW alignment of the mature S-layer proteins most closely related to the S-layer protein of *L. helveticus* MIMLh5. Database accession numbers are in parentheses. Bar, 0.1 substitution/site. Bootstrap values of the main internodes (500 replicates) are shown. Proteins used in this study are boxed.

phage display technology. To generate antibodies to *L. helveticus* MIMLh5 S-layer protein, we employed solid-phase panning.

The first step of scFv selection against *L. helveticus* MIMLh5 S-layer protein was immobilization of the purified protein on the surface, i.e., coating of microtiter wells. Two different surfaces, MaxiSorp and PolySorp, were employed for coating. The first one is a modified, highly charged polystyrene surface with high affinity for molecules with polar or hydrophilic groups. It has a high capacity to bind proteins. The PolySorp surface is more hydrophobic, and it is particularly suited to nonprotein antigens. As the S-layer protein of MIMLh5 contains 36.3% of amino acids with hydrophobic side chains (see Table S2 in the supplemental material), we used the PolySorp surface as well. For S-layer protein coating, we applied a passive coating strategy, which was expected to be advantageous because of the exceptional ability of the S-layer protein to self-assemble on surfaces in its native conformation (21, 34). The insolubility of the protein in aqueous solutions introduced additional difficulties in the coating procedure, as for coating the protein should be soluble. For this reason, we completely solubilized the protein in 5 M LiCl and then diluted it with Tris-HCl buffer at pH 9.0, which is compatible with LiCl and possesses a pI similar to that (9.39) of the MIMLh5 S-layer protein (35). Subsequently, plates were blocked with two different agents, fat-free milk and bovine serum albumin (BSA), in order to avoid the possible cross-reaction of selected binders to other cheese proteins. There are no means of monitoring the efficacy of binding to the microtiter well of an unknown target in the coating stage. Therefore, the comparison of the efficacy of binding to two different surfaces, MaxiSorp and PolySorp, was performed only in the subsequent stages, after the preselection of S-layer protein binders.

A mix of ScFvP and ScFvM repertoires was used for the selection of scFvs against the S-layer protein. A preselection step (subtractive panning) on BSA-blocked plates was used to remove the BSA- and plastic-binding phages. Three rounds of panning were performed. The panning scheme is presented in Fig. 2. The first panning was done on MaxiSorp and PolySorp plates blocked with BSA. The second round was performed on MaxiSorp and PolySorp plates blocked with fat-free milk. The third round was on MaxiSorp and PolySorp plates blocked with milk or BSA, in order to have the maximal variety of binders.

Phages that were applied to (input) and eluted out from (output) the panning were titrated in each round to monitor the enrichment of the panning process (Table 1). The titer of the eluted phages dropped from the first-round outputs of 10^8 CFU to less than 10^7 CFU on the second round. On the third round of panning with antigen-coated MaxiSorp plates, the number of eluted phage was 10- to 100-fold higher than after the second round, whereas only 7-fold-higher titers were obtained with the antigen on PolySorp wells with milk as the blocking agent. The eluted-phage

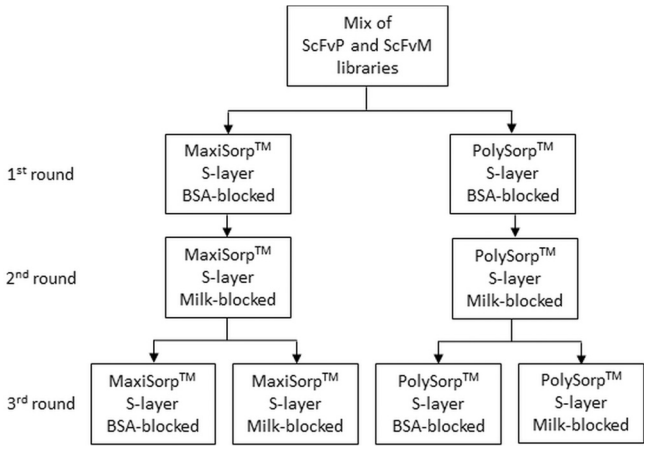

FIG 2 Panning scheme for the selection of scFvs, specific for the *L. helveticus* MIMLh5 S-layer protein, from ScFvP and ScFvM libraries.

count from the BSA-blocked PolySorp wells was even smaller than on the second round.

Panning was performed in parallel on S-layer- and milk-coated wells on the second round and S-layer- and BSA- or milk-coated wells on the third round. In particular, the eluted phage counts from the third round of panning on PolySorp-surface indicated clear enrichment, as 67- and 178-fold more clones were obtained from the antigen wells than the background wells (Table 1). On the other hand, only a 3-fold difference at the best was seen in the output counts from the MaxiSorp wells.

Further evidence for the enrichment was obtained from a phage immunoassay (see Fig. S2 in the supplemental material), in which 2.86×10^{11} CFU phage were bound per antigen- and BSA- or milk-coated well and washed, and the bound phage were detected with a dissociation-enhanced lanthanide fluoroimmunoassay (Delfia) by measuring the Eu^{3+} signal from a labeled anti-phage antibody by time-resolved fluorescence (TRF). Signal-to-background ratios of 155- and 327-fold were obtained from the third-round phage stocks of the experiments using MaxiSorp+BSA- and MaxiSorp+milk-coated plates, respectively. Signal-to-background ratios of 637- and 229-fold were obtained from the third-round phage stocks of experiments using PolySorp+BSA-coated and PolySorp+milk-coated plates, respectively.

The immunoassay results are in agreement with the phage count data, except that the signal-to-background ratios observed in the phage immunoassay of the MaxiSorp-derived stocks indicate a more efficient enrichment than the eluted phage counts of the same panning imply. This is explained by the fact that the assay resolution is higher in phage immunoassay than the phage counts, which are subject to more variation due to growth conditions, incubation times, dilution errors, and the state of susceptibility of the *E. coli* strain to infection. Furthermore, there are additional washing steps in the phage immunoassay, which efficiently reduce the background signal.

The four third-round phage stocks were similar in their ability to recognize the S-layer protein based on the phage immunoassay. Therefore, individual clones were characterized further by subcloning scFv genes into the pLK06H screening vector from one MaxiSorp-BSA and one PolySorp-BSA phage population (Fig. 3).

For the primary screening, 84 single clones were cultivated and expressed on 96-well microtiter plates as alkaline phosphatase fusions. The specificity of the selected clones was evaluated on a 96-well plate assay with the purified S-layer protein of *L. helveticus* MIMLh5 as the target and BSA as the background control (see Fig. S3 in the supplemental material). The bound scFv-PhoA fusions were detected with the chromogenic alkaline phosphatase substrate pNPP, and 37 clones (44%) were considered positive, with the cutoff set at 0.5 unit of absorbance at 405 nm. None of the clones were able to bind BSA.

A similar alkaline phosphatase-based chromogenic ELISA was performed with 37 selected scFv-PhoA binders to evaluate their specificity for nonpurified S-layer protein present on intact bacterial cells of *L. helveticus* MIMLh5. To this end, we developed an assay in which antibodies were added to a suspension of bacterial cells (in-suspension assay) (Fig. 4). Assay buffer (mKAB) and lysates of *E. coli* XL1-Blue cells (which do not express any binder) were included as negative controls to demonstrate the pNPP background absorbance. StrepA G09-scFv and Tropo37-scFv binders, which were obtained from pannings with proteins not related to the S-layer protein, were used as negative controls to demonstrate the preliminary background binding signal of pNPP chromogenic assay. *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, a strain phylogenetically closely related to *L. helveticus* but lacking the S-layer protein (Slp^-), was used as an S-layer-negative control.

It was found that 15 out of 37 scFv binders (40.5%) bound

TABLE 1 Overview of antibody selection against the *L. helveticus* MIMLh5 S-layer protein

Panning round	Plate	Blocking agent	No. of phages			% phage recovery	Antigen output/ background output[c]
			Applied (input)[a]	Eluted (output)	Background control[b]		
1	MaxiSorp	BSA	6.48×10^{12}	1.33×10^{8}		0.21×10^{-4}	
	PolySorp	BSA	6.48×10^{11}	9.30×10^{7}		0.14×10^{-4}	
2	MaxiSorp	Milk	2.58×10^{11}	2.33×10^{6}	3.82×10^{6}	0.90×10^{-5}	0.5
	PolySorp	Milk	4.20×10^{11}	9.74×10^{6}	1.08×10^{7}	2.31×10^{-5}	0.9
3	MaxiSorp	BSA	3.42×10^{11}	2.27×10^{7}	6.81×10^{6}	0.66×10^{-4}	3.3
	MaxiSorp	Milk	3.42×10^{11}	1.35×10^{8}	1.51×10^{8}	3.95×10^{-4}	0.9
	PolySorp	BSA	2.30×10^{11}	5.71×10^{6}	8.54×10^{4}	2.48×10^{-5}	66.9
	PolySorp	Milk	2.30×10^{11}	6.86×10^{7}	3.85×10^{5}	2.98×10^{-4}	178.2

[a] Calculated empirical input (obtained by measuring infectivity of the stocks).
[b] Panning on BSA- or milk-coated surface.
[c] Ratio of eluted phage from antigen-coated wells to that from BSA- or milk-coated wells.

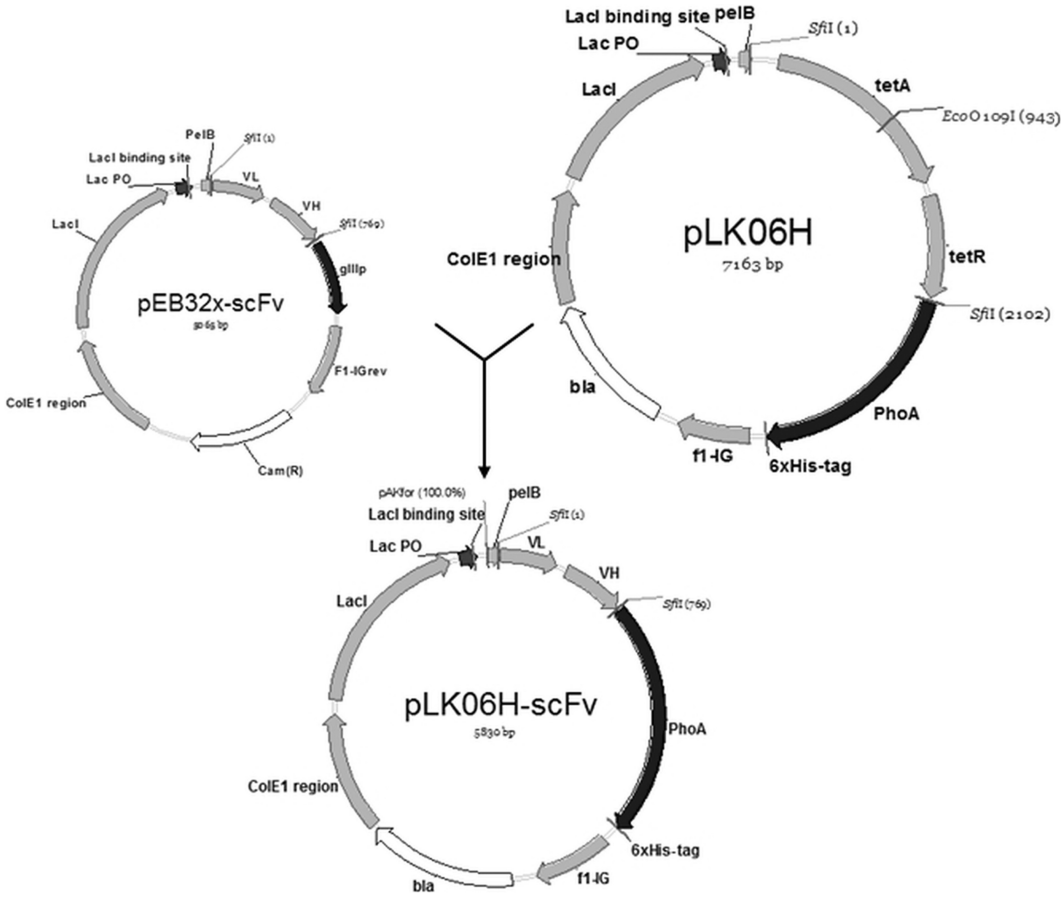

FIG 3 Cloning scheme to obtain individual anti-S-layer-scFv binders (PhoA fusion).

specifically to *L. helveticus* MIMLh5 (Slp^+) but not to *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (Slp^-). Fourteen of them had high relative expression levels.

Finally, an in-suspension assay was performed with diverse S-layer-containing bacterial cells: *L. helveticus* MIMLh5 (containing our analyzed S-layer protein), *L. helveticus* ATCC 15009 (having the S-layer protein which differs from MIMLh5 in only five amino acids; see Fig. S4 in the supplemental material), *L. helveticus* SLh02 (harboring the S-layer protein distantly related to the S-layer protein of *L. helveticus* MIMLh5) (Fig. 1), *L. acidophilus* ATCC 4356 and NCFM (having two identical S-layer proteins, phylogenetically related to that of *L. helveticus* MIMLh5) (Fig. 1). *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 was used as an S-layer-negative control. Fifteen Slp^+ bacterial-cell-specific scFv binders were analyzed (Fig. 5). The assay demonstrated that 14 scFv antibodies recognized only *L. helveticus* MIMLh5 cells. One antibody, anti-S-layer scFv-PhoA-6×His PolyF5, was less specific and recognized both *L. helveticus* MIMLh5 and *L. helveticus* ATCC 15009 S-layer proteins present on bacterial cells. It is worth noting that the binder PolyH4 had a very low background binding level.

DNA of the 15 monoclonal binders in which anti-S-layer-scFv-PhoA exhibited the best interaction with *L. helveticus* MIMLh5 S-layer protein was sequenced: MaxiA9, MaxiB2, MaxiB3, MaxiB9, MaxiB11, MaxiC5, MaxiC10, MaxiC11, MaxiD4, MaxiD5, PolyE9, PolyG10, PolyH4, PolyH5, and PolyF5. Six of them were different: MaxiC5, MaxiC11, PolyH4, PolyH5, PolyF5, and a group of 10 identical binders (MaxiA9, MaxiB11, MaxiB2, MaxiB3, MaxiB9, MaxiC10, MaxiD4, MaxiD5, PolyE9, and PolyG10) represented by MaxiB9, which, according to their specificity for bacterial cells harboring phylogenetically related S-layer proteins, seems to show the highest affinity and the highest expression level. MaxiC5 was discarded, because the phagemid formed a concatemer (data not shown). The deduced amino acid sequences were aligned with the library template. The framework gene and anti-S-layer scFvs differed in CDR1 and CDR3 of both heavy and light chains and CDR2 of the heavy chain (Table 2), which corresponds to the phage library design rules (19). Only one clone, PolyF5, originated from the ScFvP repertoire based on the presence of a tryptophan (W) as the last amino acid in the CDR-L3 loop (26). Accordingly, the remaining selected binders originated from the ScFvM repertoire.

A more detailed analysis of the anti-S-layer scFv binding pattern was achieved by determining anti-S-layer scFv binders' specificity by Western blotting. For this, five selected scFvs (MaxiB9, MaxiC11, PolyH4, PolyH5, and PolyF5) were expressed in liquid cultures as 6×His-tagged PhoA fusions. LAB lysates (selected as described above) were separated by SDS-PAGE and blotted on a PVDF membrane. Expression supernatants containing the scFv-PhoA proteins were applied to the PVDF membrane as the primary antibody. The binding of scFvs was detected by an anti-His HRP conjugate (Fig. 6).

The purified S-layer protein from *L. helveticus* MIMLh5 was

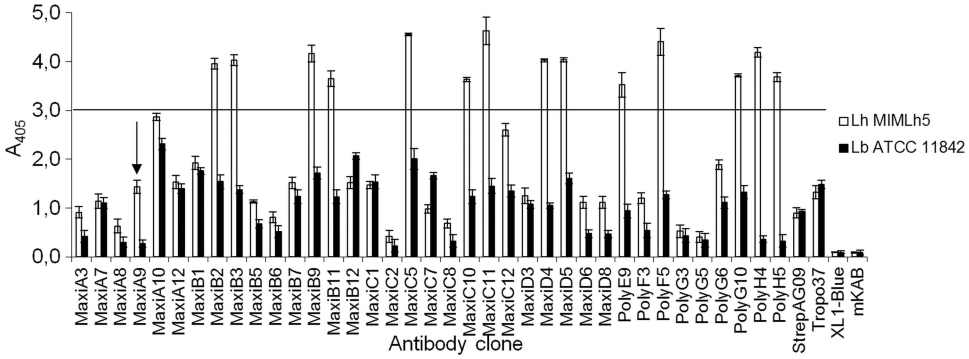

FIG 4 Characteristics of individual anti-S-layer protein scFv binders. White columns represent the pNPP chromogenic in-suspension assay A_{405} of monoclonal anti-S-layer-scFv–PhoA–6×His particles bound to *L. helveticus* MIMLh5 cells; black columns represent binding to *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (Lb11842; Slp$^-$) cells. The antibody selection threshold (as described in Materials and Methods) is indicated with a horizontal line and an arrow.

separated in the SDS-PAGE as a single band (Fig. 6, lane 2). LAB cell lysates were separated into a mixture of bands (Fig. 6, lanes 3 to 8), representing the total bacterial protein content. Western blotting revealed that the binders MaxiB9, MaxiC11, PolyH4, and PolyH5 strongly bound to the purified S-layer protein of *L. helveticus* MIMLh5 (Fig. 6, lane 2) as well as to the approximately 44-kDa band in the lysates, representing the S-layer protein of the MIMLh5 strain (Fig. 6, lane 3). scFv PolyF5 bound not only to the purified S-layer protein of *L. helveticus* MIMLh5 and MIMLh5 cell lysate but also to the lysate of *L. helveticus* ATCC 15009 (Fig. 6, PolyF5, lane 4). This result is in agreement with the data obtained previously with the in-suspension assay (Fig. 5). All binders (especially MaxiB9, MaxiC11, and PolyH5) showed relatively high background binding. This is not surprising, since antibodies selected in pannings by phage display technology normally do not demonstrate very high binding affinity. To increase the binders' affinity, the subsequent step of affinity maturation is required (36–37). However, this was not a goal of the present study.

PolyH4 was chosen for further experiments due to the fact that it had the lowest background binding and the highest specificity for the S-layer protein of MIMLh5.

Cheese analysis by anti-S-layer scFv PolyH4. To apply the technique described here, the anti-S-layer scFv PolyH4 antibody was used to detect the presence of the *L. helveticus* MIMLh5 S-layer protein in commercial cheese samples.

L. helveticus MIMLh5 was isolated from natural whey starter (NWS) used in the production of Grana Padano PDO cheese (2). The dynamics of the bacterial population during Grana Padano PDO cheese production and ripening was monitored (38), and the persistence of *L. helveticus* strains in NWS and during early cheese ripening was demonstrated (39–43). However, it is not known whether the immunologically active MIMLh5 S-layer-like protein persists in the commercial Grana Padano PDO cheese, i.e., in 9-month-old cheese at least.

Before the occurrence of the S-layer protein was assessed, the presence in cheese of *L. helveticus* DNA and DNA encoding the S-layer protein was ensured. For this purpose, total DNA was extracted from cheese samples, and the presence of *L. helveticus* was confirmed in all 10 Grana Padano PDO samples by species-specific PCR (31) (see Fig. S5 in the supplemental material). The presence of the S-layer protein-encoding gene was also confirmed in the same cheese DNA samples (see Fig. S6 in the supplemental material). On these bases, the Grana Padano PDO cheese samples were assessed for the presence of the *L. helveticus* MIMLh5 S-layer protein. To this end, cheese extracts were loaded on SDS-PAGE (Fig. 7A) and blotted onto the PVDF membrane. The dilution

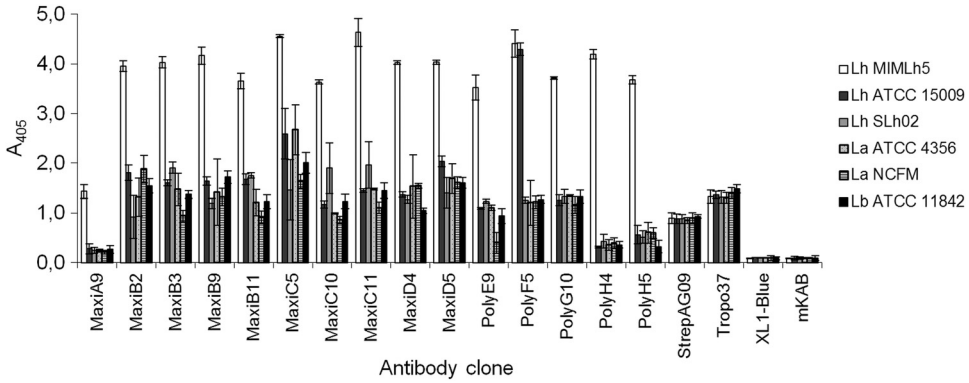

FIG 5 Characteristics of individual anti-S-layer protein scFv binders. White columns represent the pNPP chromogenic-assay A_{405} of monoclonal anti-S-layer-scFv–PhoA–6×His particles bound to *L. helveticus* MIMLh5 cells; black columns represent binding to *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (Slp$^-$) cells; gray columns represent the A_{405} of monoclonal anti-S-layer-scFv–PhoA–6×His particles bound to bacterial cells harboring phylogenetically related S-layer proteins (Fig. 1; also, see Fig. S4 in the supplemental material).

TABLE 2 Sequence alignment of the CDRs of selected S-layer protein-specific antibodies[a]

Name	Sequence					No. of identical selected antibodies
	Light chain		Heavy chain			
	CDR-L1	CDR-L3	CDR-H1	CDR-H2	CDR-H3	
Framework	SLA	QQMHSTPW	SDVMH	AISDLNGSTY	ARGSASGFYYFDY	
MaxiB9[b]	YLN	LQDNYIPY	SYLMD	QITPSGGSTD	TTDMYY	10
MaxiC5	YLN	LQHNYVPP	SYLMH	EINPSGGSTY	AREWYPSWGDY	1
MaxiC11	YLN	LQDNYVPY	SYLMD	EINPSGGSTD	ASDMYY	1
PolyH4	YLN	LQDNYYPY	SYLMH	EINPSGGSTD	ATGWYLYL	1
PolyH5	YLS	LQDTYVPL	SYAMD	EINSSGGSTY	ARNSYVMDY	1
PolyF5	NLA	QQSSSLPW	DYSMH	AIRPVTGNTY	AARYWGMDY	1

[a] Amino acid sequences relative to a framework gene in randomized complementarity-determining regions (CDRs) of scFv light (L) and heavy (H) chains are indicated.
[b] The anti-S-layer scFvs MaxiA9, MaxiB11, MaxiB2, MaxiB3, MaxiB9, MaxiC10, MaxiD4, MaxiD5, PolyE9, and PolyG10 are identical and are represented by MaxiB9.

series from 0.5 to 500 ng of purified *L. helveticus* MIMLh5 S-layer protein were used as positive controls and to preliminarily determine the detection limit for the S-layer protein. Expression supernatant containing the anti-S-layer-scFv–PhoA PolyH4 was applied as the primary antibody. The binding of scFvs was detected by an anti-His HRP conjugate and visualized with an ECL substrate by chemiluminescent detection (Fig. 7B). The Western blot revealed the presence of the *L. helveticus* MIMLh5 S layer starting from 5 ng of protein (Fig. 7B, lane 13). A band in the electropherogram of cheese extract corresponding to the molecular weight of *L. helveticus* MIMLh5 S-layer protein was detected in nine samples (30 to 35 and 37 to 39) (Fig. 7B), and they could be divided according to the amount of intact S-layer observed on the blot by comparing them to the pure S-layer gradient. In 50 μg of cheese extract loaded per lane, there was over 50 ng of intact S layer in sample 37 (Fig. 7B, lane 9), 5 to 50 ng in samples 32 and 35 (Fig. 7B, lanes 4 and 7, respectively), and 0.5 to 5 ng in samples 30, 31, 33, 34, 38, and 39 (Fig. 7B, lanes 2, 3, 5, 6, 10, and 11, respectively). The different signal intensities could be explained by the presence of different quantities of *L. helveticus* MIMLh5-related strains as well as different ratios of *L. helveticus* strains in general in the NWS used for cheese making by the various cheese factories from which the samples originated. The negative result obtained for sample 36 (Fig. 7B, lane 8) was likely because the amount of MIMLh5-like S-layer protein was under the detection limit of the assay or because of the absence of an *L. helveticus* MIMLh5-related strain. The different microbial characteristics of unprocessed milk from which both NWS and Grana Padano cheeses derived could account for a small amount of or no presence of an *L. helveticus* MIMLh5-related strain. Nonetheless, further studies are required to elucidate the impact of NWS and cheese origin.

Conclusions. In this study, we isolated, for the first time,

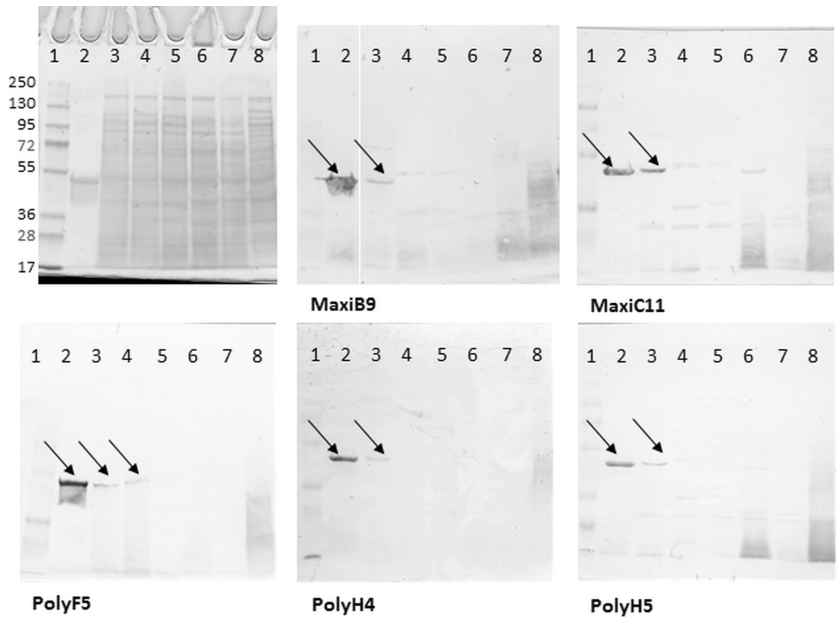

FIG 6 Specificity of anti-S-layer scFvs for purified S-layer protein of *L. helveticus* MIMLh5 and S-layer protein-containing LAB lysates under denaturing conditions as revealed by Western blotting. The SDS-PAGE gel was stained with Coomassie brilliant blue G-250 to reveal the whole protein profile. Binding of five scFvs was tested: MaxiB9, MaxiC11, PolyF5, PolyH4, and PolyH5. Lane 1, protein molecular weight ladder; lane 2, *L. helveticus* MIMLh5 purified S-layer protein; lanes 3 to 7, lysates of the Slp^+ strains *L. helveticus* MIMLh5 (lane 3), *L. helveticus* ATCC 15009 (lane 4), *L. helveticus* SLh02 (lane 5), *L. acidophilus* ATCC 4356 (lane 6), and *L. acidophilus* NCFM (lane 7); lane 8, lysate of Slp^- *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, used as a negative control. Sharp bands revealed by scFvs are indicated by arrows.

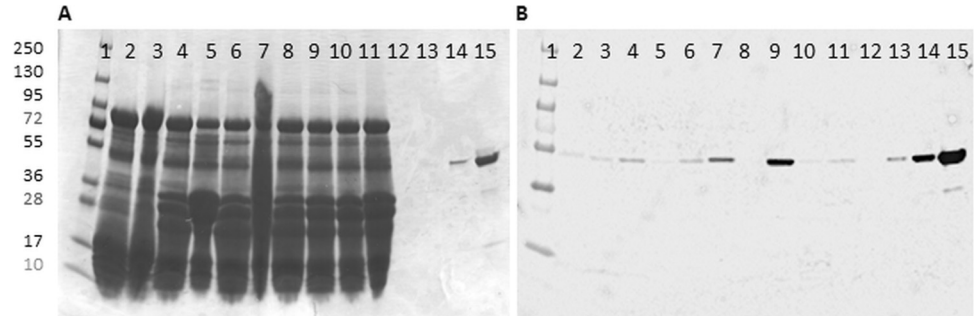

FIG 7 Detection of *L. helveticus* MIMLh5 S-layer protein in Grana Padano PDO cheese by Western blotting. The gel was stained with Coomassie brilliant blue G-250 to reveal the whole protein profile (A). Chemiluminescent detection was used to visualize the binding of anti-S-layer scFv PolyH4 (B). Lane 1, protein molecular weight ladder; lanes 2 to 11, Grana Padano PDO cheese extracts 30 to 39 (sample designations are in Table S1 in the supplemental material; 50 μg was loaded per lane); lanes 12 to 15, *L. helveticus* MIMLh5 S-layer protein at 0.5 ng (lane 12), 5 ng (lane 13), 50 ng (lane 14), and 500 ng (lane 15).

highly specific scFv antibodies against the immunologically important *L. helveticus* MIMLh5 S-layer protein from synthetic libraries by the phage display technique. The scFv PolyH4 construct showed high specificity and sensitivity in detecting this protein in Grana Padano PDO cheese samples in nanogram quantities. Taken together, the results show that the isolated scFv antibody is promising for the development of a rapid and accurate ELISA-based detection assay for the *L. helveticus* MIMLh5 S-layer protein to characterize the potential immunomodulatory properties of dairy (and dairy-based) foods. The high specificity of the scFv PolyH4 antibody may also facilitate an *in vivo* animal study addressing the fate of the S-layer protein during gastrointestinal tract transit.

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M.S., U.L., I.D.N., S.G. and D.M. designed the research; M.S., E.-C.B. and T.H. performed phage display experiments; M.S. and T.H. performed Western blot analyses; V.T. extracted the S-layer protein; S.A. performed the detection of DNA in cheese samples; M.S. wrote the manuscript; I.D.N., U.L., T.H., E.-C.B., S.G., V.T., D.M., and S.A. commented on the manuscript. U.L. supervised the development process of phage display antibodies, and I.D.N. supervised the cheese part of the research.

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