DOI: 10.17344/acsi.2018.4422 *Acta Chim. Slov.* **2019,** *66,* 18–27

Scientific paper

Surface Anchoring on *Lactococcus lactis* by Covalent Isopeptide Bond

Tina Vida Plavec[1,2] and Aleš Berlec[2,*]

[1] *Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, Ljubljana, Slovenia*

[2] *Department of Biotechnology, Jožef Stefan Institute, Jamova 39, Ljubljana, Slovenia*

* *Corresponding author: E-mail: ales.berlec@ijs.si*
+ 386 1477 3754

Received: 04-24-2018

Dedicated to the memory of Prof. Dr. Igor Kregar

Abstract

Display of recombinant proteins on the bacterial surface is an emerging research area with wide range of potential biotechnological applications. Because of its GRAS (generally recognized as safe) status, lactic acid bacterium *Lactococcus lactis* represents an attractive host for surface display and promising vector for *in situ* delivery of bioactive proteins. The present study focused on finding a new alternative approach for surface display on *Lactoccocus lactis*. We developed a system that enables the formation of irreversible isopeptide bonds on the surface of *Lactococus lactis*. This was achieved through the following two protein/peptide pairs, SpyCatcher/SpyTag and SnoopCatcher/SnoopTag.[1–3] Attachment of tagged model protein B domain to the cell surface of *Lactococccus lactis* displaying the corresponding catcher protein was demonstrated using flow cytometry. We demonstrated effective use of aforementioned protein anchors which thus represent a promising alternative to established approaches for surface display on *Lactoccocus lactis*.

Keywords: Surface display; *Lactococcus lactis*; isopeptide bond

1. Introduction

Display of recombinant proteins on bacterial surface offers a variety of possible biotechnological applications. Proteins-displaying bacteria can act as bioadsorbents, biosensors, biocatalysts or oral vaccines. They can be used in antibody production and in peptide screening.[4–6] Several lactic acid bacteria (LAB) are probiotics and are therefore considered valuable hosts in biotechnology due to their beneficial influence on health.[7,8] Because of the "generally recognized as safe" (GRAS) status which confirms their safety, LAB are attractive not only for industrial application but also therapeutically.[9] Display of heterologous proteins on the surface of LAB has already been exploited in therapy for the preparation of mucosal vaccines.[10–12] Moreover, beneficial effects in inflammatory bowel disease could be achieved when displaying binding molecules directed against pro-inflammatory molecules such as TNFα.[13–17]

Different approaches can be exploited for displaying a protein on the bacterial surface. The protein to be displayed is usually fused to an anchoring motif.[14,18] Five different types of surface anchoring domains have been described for LAB: transmembrane domains, LPXTG-type domains, lysin motif (LysM) domains, surface layer proteins and lipoprotein anchors.[19–21] The most frequently applied surface anchoring domains in prototype LAB, *Lactococcus lactis*, are the C-terminal part of endogenous AcmA, enabling non-covalent anchoring through peptidoglycan binding LysM repeats,[22–25] and the LPXTG sequence of M6 protein of *Streptococcus pyogenes* enabling covalent anchoring.[26,27] Despite these available options, alternative surface display approaches are being sought.

Recently, two peptide/protein pairs known as SpyTag/SpyCatcher, from *Streptococcus pyogenes,* and SnoopTag/SnoopCatcher, from *Streptococcus pneumoniae* have been developed.[1–3] Interaction between the peptide and the protein leads to the formation of an irreversible isopeptide bond. The reaction is high-yielding and fast while the bond is highly stable. It can survive extreme pH, high ionic strength and exposure to detergents.[1,28,29] Stable bond formation enables combinatorial assembly of multi-

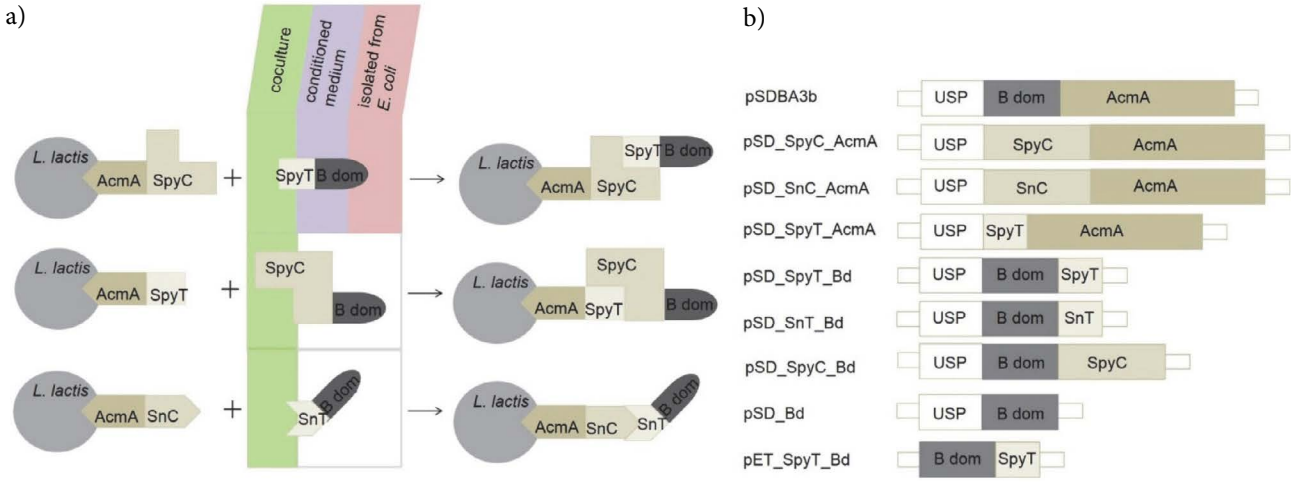

Fig. 1. The principle of surface display of B domain on *L. lactis* by the formation of isopeptide bond (A), and the gene constructs prepared for its implementation (B). A: Surface displayed SpyCatcher (SpyC), SpyTag (SpyT), or SnoopCatcher (SnC), all anchored via AcmA on *L. lactis,* bind fusion proteins consisting of SpyT, SpyC, or SnoopTag (SnT), respectively, and B domain. The fusion proteins were produced by *L. lactis* co-culture, were extracted from *L. lactis* conditioned medium, or were isolated from *E. coli*. B: Gene constructs for lactococcal surface display and isopeptide bond formation. USP: gene for Usp45 signal peptide for secretion to the growth medium (84 bp). B dom: gene for reporter protein B domain of staphylococcal protein A (174 bp). SpyC: gene for protein SpyCatcher which binds SpyT (348 bp). SnC: gene for protein SnoopCatcher which binds SnT (336 bp). SpyT: gene for peptide SpyTag (39 bp). SnT: gene for peptide SnoopTag (36 bp). AcmA: gene for C-terminal part of AcmA protein-containing 3 LysM repeats for surface anchoring to *L. lactis* (642 bp).

protein constructs, and opens an opportunity to use this approach in vaccine production, enzyme substrate channeling, antibody polymerization, cell signaling activation, and biomaterials.[1,30] The key properties of isopeptide binding are simple and fast procedure, irreversible and stable bond, specificity, and cysteine independence; the latter offering the possibility to use the approach in reducing environment.[3]

The goal of the present study was to develop a system for surface display on recombinant LAB *L. lactis* by applying the isopeptide bond formation (Fig. 1a). This was achieved by preparing genetic constructs consisting of surface anchor, elements of SpyTag/SpyCatcher or SnoopTag/SnoopCatcher pairs and model passenger protein B domain (Fig. 1b).[13] B domain is one out of five antibody-binding domains of staphylococcal protein A that can bind antibodies via their Fc region. [13] The fusion proteins were expressed either in *L. lactis* or in *E. coli,* and assembled on the surface of *L. lactis*, as confirmed by surface localization of B domain.

2. Experimental

2. 1. Bacterial Strains, Media and Culture Conditions

Bacterial strains used in this study are listed in Table 1. *E. coli* strains DH5α and BL21 (DE3) were grown at 37 °C, with aeration in lysogeny broth (LB) medium supplemented with either ampicillin (100 μg/mL) or kanamycin (50 μg/mL). *L. lactis* NZ9000 was grown in M-17 medium (Merck) supplemented with 0.5% glucose (GM-17) and chloramphenicol (10 μg/mL) at 30 °C without aeration.

2. 2. Molecular Cloning

Plasmid DNA was isolated with NucleoSpin Plasmid (Macherey and Nagel, Düren, Germany), with an additional lysozyme treatment step for *L. lactis*. Lactococci were transformed with electroporation using a Gene Pulser II apparatus (Biorad, Hercules, USA) according to the MoBiTec GmbH (Goettingen, Germany) instructions. Nucleotide sequencing was performed by GATC (Constance, Germany).

Gene for SpyTag in fusion with B domain for expression in *E. coli* was amplified from pSDBA3b by PCR using primers B-F-NcoI-Spy and B-R-XhoI, cloned to pGEM-T Easy and then to pET28a via restriction enzymes NcoI/XhoI, yielding pET_SpyT_Bd. Gene for SpyTag in fusion with B domain for secretion from *L. lactis* was amplified from pSDBA3b by PCR using primers B-F-BamHI and B-R-Kpn-Sy-Xba. Gene for SnoopTag in fusion with B domain was prepared likewise using primers B-F-BamHI and B-R-Kpn-So-Xba. Both were first cloned to plasmid pGEM-T Easy and then to plasmid pSDBA3b via restriction enzymes BamHI/XbaI, yielding pSD_SpyT_Bd and pSD_SnT_Bd. Gene for B domain for secretion from *L. lactis* was amplified from pSDBA3b by PCR using primers B-F-BamHI and B-R-Xba, first cloned to plasmid pGEM-T Easy and then to plasmid pSDBA3b via restriction enzymes BamHI/XbaI, yielding pSD_Bd (Table 2–4).

Genes for SpyCatcher and SnoopCatcher were designed and synthesized *de novo* as gBlocks (Table 4) by

Table 1. Strains used in this study

Strain	Relevant features or sequence	Reference
E. coli		
DH5α	endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Invitrogen
L. lactis		
NZ9000	MG1363 *nisRK* Δ*pepN*	NIZO

Table 2. Plasmids used in this study

Plasmid	Relevant features or sequence	Reference
pET28a	Kan^r, E. coli expression vector	Novagen
pGEM-T Easy	Ap^r, cloning vector for PCR products	Promega
pSDBA3b	pNZ8148 containing gene fusion of $sp_{Usp45,}$ *b-dom* and *acmA3b*	[31]
pNZ8148	pSH71 derivative, P_{nisA}, Cm^r, nisin-controlled expression	[32–34]
pET_SpyT_Bd	pET28a containing gene fusion of *spytag* and *b-dom*	This work
pSD_SpyC_AcmA	pNZ8148 containing gene fusion of sp_{Usp45}, *spycatcher* and *acmA3b*	This work
pSD_SnC_AcmA	pNZ8148 containing gene fusion of sp_{Usp45}, *snoopcatcher* and *acmA3b*	This work
pSD_Bd	pNZ8148 containing gene fusion of sp_{Usp45} and *b-dom*	This work
pSD_SpyT_Bd	pNZ8148 containing gene fusion of sp_{Usp45}, *spytag* and *b-dom*	This work
pSD_SnT_Bd	pNZ8148 containing gene fusion of sp_{Usp45}, *snooptag* and *b-dom*	This work
pSD_SpyC_Bd	pNZ8148 containing gene fusion of sp_{Usp45}, *spycatcher* and *b-dom*	This work
pSD_SpyT_AcmA	pNZ8148 containing gene fusion of sp_{Usp45}, *spytag* and *acmA3b*	This work

Table 3. Primers used in this study

Primer	Relevant features or sequence	Reference
B-F-NcoI-Spy	5'-CCATGGCTCATATTGTAATGGTCGATGCATATAAACCAACCAAAGCTGATAA CAAATTCAACAAAGAAC-3'	This work
B-R-XhoI	5'-CTCGAGTTTTGGTGCTTGTGCATC-3'	This work
B-F-BamHI	5'-AGGATCCGCTGATAACAAATTCAAC-3'	This work
B-R-Kpn-Sy-Xba	5'-TTCTAGATTATTTGGTTGGTTTATATGCATCGACCATTACA ATATGAGCGGTACCTTTTGGTGCTTGTGCATC-3'	This work
B-R-Kpn-So-Xba	5'-TTCTAGATTATTTGTTAACTTTAATAAATTCGATGTCACCCA ACTTGGTACCTTTTGGTGCTTGTGCATC-3'	This work
B-R-Xba	5'-TTCTAGATTATTTTGGTGCTTGTGCATC-3'	This work
SpyC-F-Kpn	5'-AGGTACCGGAGCTATGGTTGATACATTG -3'	This work
SpyC-R-Xba	5'-TTCTAGATTAAATATGAGCATCACCTTTTGTTG-3'	This work
AcmA-F-Bam-SpyT	5'-AGGATCCGCTCATATTGTAATGGTCGATGCATATAAACCAACCAAA TCTGGTGGCTCGACAACC-3'	This work
AcmA-R-Xba	5'-TTCTAGATTATTTTATTCGTAGATACTGACC-3'	This work
Spy-F-Bam	5'-AGGATCCGGAGCTATGGTTGATACATTG-3'	This work
Spy-R-Eco	5'-AGAATTCAATATGAGCATCACCTTTTGTTG-3'	This work
Sno-F-Bam	5'-AGGATCCAAACCTTTGCGTGGTGCAG-3'	This work
Sno-R-Eco	5'-AGAATTCCTTTGGTGGGATTGGTTCGTTC-3'	This work

IDT (Leuven, Belgium). Gene for secretion of SpyCatcher-B domain fusion from *L. lactis* was amplified from gBlock by PCR using primers SpyC-F-Kpn and SpyC-R-Xba, cloned to pGEM-T Easy and then to pSD_SpyT_Bd via restriction enzymes KpnI/XbaI, yielding pSD_SpyC_Bd (Table 1–4).

Genes for the surface display of SpyCatcher and SnoopCatcher were amplified from gBlocks using primer pairs Spy-F-Bam/Spy-R-Eco and Sno-F-Bam/Sno-R-Eco, respectively, and were cloned first to pGEM-T Easy and then to plasmid pSDBA3b via restriction enzymes EcoRI/BamHI, yielding pSD_SpyC_AcmA and pSD_SnC_AcmA, respectively. Gene for the display of SpyTag on the *L. lactis* surface was amplified from pSDBA3b by PCR using primers AcmA-F-Bam-SpyT and AcmA-R-Xba, cloned to pGEM-T Easy and then to pSD_SpyC_AcmA via re-

Table 4. Genes used in this study

Gene	Relevant features or sequence	Reference
spycatcher	GGATCCGGAGCTATGGTTGATACATTGTCAGGTTTATCATCAGAACAA GGACAAAGTGGAGATATGACTATTGAAGAAGATTCTGCTACACATATTAAA TTTTCAAAACGTGATGAAGATGGAAAAGAATTAGCAGGTGCTACTATGGA ATTGCGTGATTCATCAGGTAAAACAATTTCAACTTGGATTTCAGATGGACAA GTTAAAGACTTTTATCTGTACCCTGGAAAATATACTTTCGTTGAAACAGCAGCA CCTGACGGATACGAAGTTGCTACTGCTATCACTTTTACAGTTAACGAACAAGG TCAAGTTACAGTTAATGGTAAAGCAACAAAAGGTGATGCTCATATTGAATTC	This work
snoopcatcher	GGATCCAAACCTTTGCGTGGTGCAGTCTTCTCATTACAAAAACAACATCC AGACTACCCTGATATTTATGGTGCCATTGATCAAAATGGTACTTATCAGAA TGTTCGAACTGGTGAAGACGGAAAATTGACTTTTAAGAATTTGAGTGACGG TAAATATCGTTTATTCGAAAACAGTGAACCAGCTGGATATAAGCCAGTA CAAAATAAACCTATTGTCGCATTTCAAATTGTAAACGGTGAAGTTAGAGACG TTACTTCTATTGTACCTCAGGATATTCCTGCTACTTATGAATTTACTAA TGGAAAACATTATATTACGAA CGAACCAATCCCACCAAAGGAATTC	This work
spytag	GCTCATATTGTAATGGTCGATGCATATAAACCAACCAAA	This work
snooptag	AAGTTGGGTGACATCGAATTTATTAAAGTTAACAAA	This work

striction enzymes BamHI/XbaI, yielding pSD_SpyT_AcmA (Table 2–4).

2. 3. Expression of SpyTag-B Domain Fusion in *E.coli*

100 µL of overnight culture of *E. coli* BL21 (DE3) harboring plasmid pET_SpyT_Bd was diluted (1:100) in 10 mL of fresh LB medium and, to determine optimal expression conditions, various parameters were tested: incubation temperature 37 °C or 25 °C, induction at optical densities (A_{600}) 0.5 or 1.0, induction with IPTG in concentration of 0.5 and 1.0 mM.

Large-scale expression of SpyTag-B domain fusion was performed by diluting 10 mL of overnight culture of *E. coli* BL21 (DE3) harboring plasmid pET_SpyT_Bd in 1 L of fresh LB medium. The culture was grown to optical density A_{600} = 0.5 at 37 °C. At that point, the expression of SpyTag-B domain fusion, additionally tagged with hexa-histidine (H6), was induced by the addition of 0.5 mM IPTG for 3 h at 37 °C; the conditions that were found to be the most effective in preliminary screen.

2. 4. Purification of SpyTag-B Domain With Hexa-histidine (H6) Tag

The *E. coli* culture expressing SpyTag-B domain with hexa-histidine (H6) tag was centrifuged at 5000 × g for 20 min and the pellet resuspended in 20 mL of equilibration/wash (Eq/W) buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0). The cells were lysed with a cycle of freezing and thawing, and with 3 cycles of 5 min sonication with a UPS200S sonifier (Hielscher, Teltow, Germany). After cell lysis, the suspension was centrifuged at 15000 × g for 20 min and the supernatant stored. SpyTag-B domain with H6 tag was isolated with BD Talon metal affinity resin (BD Biosciences), using batch/gravity-flow column purification and imidazole elution (elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, pH 7.0) according to the manufacturer's instructions. Eluted fractions were analyzed by SDS-PAGE, pooled and concentrated by ultrafiltration using Amicon Ultra 1 KDa cut off (Merck Millipore; Darmstadt, Germany). Purified fusion protein was dialyzed against PBS.

2. 5. Expression of Fusion Proteins in *L. lactis*

Overnight cultures of *L. lactis* NZ9000 harboring pSD_SpyC_AcmA, pSD_SnC_AcmA, pSD_SpyT_AcmA, pSD_SpyT_Bd, pSD_SnT_Bd, pSD_SpyC_Bd, or pSD_Bd were diluted (1:100) in 10 mL of fresh GM-17 medium and grown to optical density A_{600} = 0.8–1.0. Fusion protein expression was induced with 25 ng/mL nisin (Fluka AG, Buchs, Switzerland) for 3 h at 30 °C. After incubation, 1 mL of culture was stored at 4 °C for flow cytometric analysis. The remaining cell culture was centrifuged at 5000 × g for 10 min.

2. 6. Formation of Isopeptide Bond Between SpyTag and SpyCatcher / SnoopTag and SnoopCatcher

In order to enable binding of *E. coli*-expressed SpyTag-B domain to *L. lactis* with surface displayed SpyCatcher, we centrifuged 20 µL of the cell culture of *L. lactis* with surface displayed SpyCatcher for 5 min at 5000 × g at 4 °C, resuspended the pellet in 500 µL of purified *E. coli*-expressed SpyTag-B domain with concentration of 0.4 mg/mL and incubated for 2 h at RT with constant shaking.

To enable binding of SpyTag-B domain from *L. lactis* conditioned medium to *L. lactis* with surface displayed

SpyCatcher, we separately cultured the SpyTag-B domain-secreting *L. lactis* and SpyCatcher-displaying *L. lactis*. The producer cells of SpyTag-B domain were removed and the conditioned medium containing SpyTag-B domain fusion protein was stored. 20 µL of *L. lactis* cell culture with surface displayed SpyCatcher was centrifuged for 5 min at 5000 × g at 4 °C, resuspended in 500 µL of conditioned medium containing SpyTag-B domain and incubated overnight at RT with constant shaking.

Binding of SpyTag-B domain secreted from *L. lactis* to SpyCatcher displayed on *L. lactis* was also achieved during co-culturing of the two strains. 100 µL of overnight cultures of *L. lactis* NZ9000 harboring pSD_SpyT_Bd and pSD_SpyC_AcmA were concomitantly added to 10 mL of fresh GM-17 medium. Simultaneous expression of the two fusion proteins was induced with nisin. Similarly, binding of SpyCatcher-B domain secreted from *L. lactis* to SpyTag displayed on *L. lactis* was achieved by co-culturing *L. lactis* NZ9000 harboring pSD_SpyC_Bd and pSD_SpyT_AcmA, as well as binding of SnoopTag-B domain secreted from *L. lactis* to SnoopCatcher displayed on *L. lactis* by co-culturing *L. lactis* NZ9000 harboring pSD_SnC_AcmA and pSD_SnT_Bd.

2. 7. SDS-PAGE and Western Blot

SDS PAGE was performed with a Mini-Protean II apparatus (Bio-Rad, Hercules, USA). Samples were thawed in an ice bath, briefly sonicated with UPS200S sonicator (Hielscher, Teltow, Germany), mixed with 2× Laemmli Sample buffer and DTT, and denatured by heating at 100 °C before loading. Page Ruler Plus (Fermentas, St. Leon-Rot, Germany) pre-stained standard was used for molecular weight comparison. Proteins were stained with Coomassie Brilliant Blue or transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore) using wet transfer at 100 V for 90 minutes. Membranes were blocked in 5% non-fat dried milk in TBS with 0.05% Tween-20 (TBST; 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) and incubated overnight at 4 °C with goat anti-protein A antibody (1:2000, Abcam) in 5% non-fat dried milk in TBST. Following three washes with TBST, membranes were incubated for 2 h with HRP conjugated secondary donkey anti-goat IgG (1:5000, Jackson ImmunoResearch) in 5% non-fat dried milk in TBST. After three further washes with TBST, membranes were incubated with Lumi-Light chemiluminescent reagent (Roche). Images were acquired using ChemiDoc MP Imaging System (BioRad).

2. 8. Flow Cytometry

For flow cytometry 20 µL of cell culture in stationary phase was added to 500 µL of Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and centrifuged for 5 min at 5000 × g at 4 °C. The pellet was resuspended in 500 µL of TBS with 1 µL of fluorescein-5-isothiocyanate (FITC)-conjugated human IgG antibody (Jackson ImmunoResearch, West Grove, USA) that binds the B domain via Fc region. After 2 h of incubation at RT with constant shaking at 100 rpm, cells were washed three times with 200 µL 0.1% TBST and finally resuspended in 500 µL TBS. Samples were analyzed with a flow cytometer (FACS Calibur; Becton Dickinson, Franklin Lakes, USA) using excitation at 488 nm and emission at 530 nm in the FL1 channel. The geometric mean fluorescence intensity (MFI) of at least 20 000 bacterial cells in the appropriate gate was measured. The average of at least three independent experiments was considered. All the samples went through the same procedures of preparation for flow cytometry analysis.

2. 9. Statistical Analyses

Statistical analyses were performed with GraphPad Prism 5.0 software. Student's t test was used to compare the significance of differences between B domain-displaying bacteria and control.

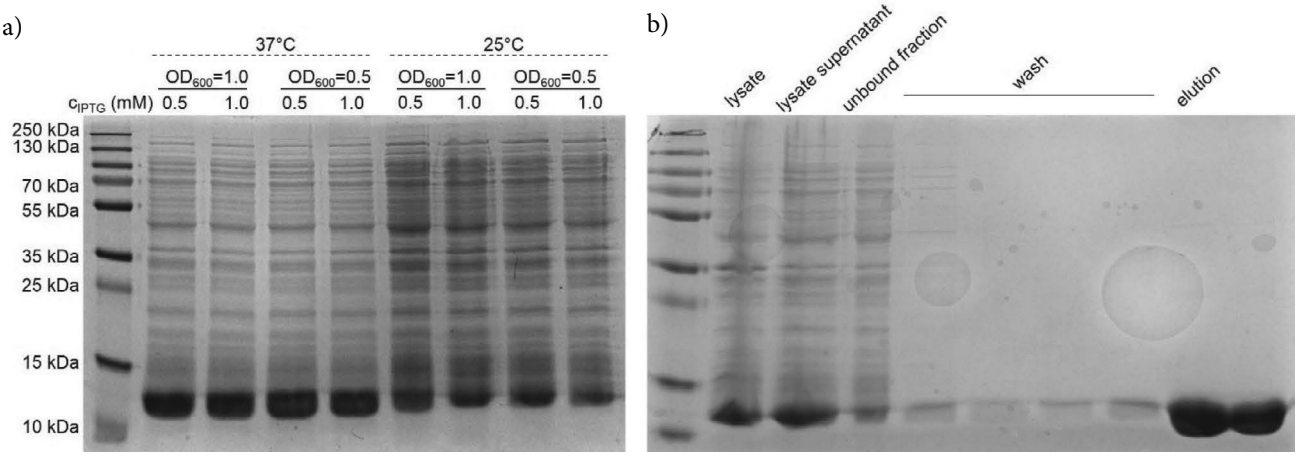

Fig. 2. Coomassie-stained SDS-PAGE of *E. coli* expressing SpyTag-B domain under different culturing conditions (A) and fractions obtained after IMAC purification of SpyTag-B domain from cell lysate (B).

3. Results

3. 1. Expression and Purification of Recombinant SpyTag-B Domain from *E. coli*

Gene for SpyTag-B domain, possessing affinity for the Fc region of human IgG, was cloned into the pET28a plasmid (Fig. 1b) in order to express the protein in *E. coli* and obtain it in sufficient amount and purity. Recombinant SpyT-B domain fusion protein with H6 tag was produced in *E. coli* BL21 DE3. Various expression conditions (growth at 37 °C and 25 °C, induction at optical densities (A_{600}) 0.5 and 1, induction with IPTG concentration of 0.5 and 1.0 mM) were tested. The highest total amount of SpyT-B domain expression was achieved by growing the bacteria at 37 °C to A_{600} = 0.5 or 1.0, followed by induction with 0.5 or 1 mM IPTG for 3 h at 37 °C (Fig. 2a). The majority of the fusion protein was produced in the soluble form as it could be detected in the soluble fraction (supernatant) of the cell lysate (Fig. 2b). SpyTag-B domain was isolated with immobilized metal affinity chromatography (IMAC) (Fig. 2b).

3. 2. Binding of *E. coli*-expressed SpyTag-B Domain to *L. Lactis* with Surface Displayed SpyCatcher

SpyCatcher in fusion with Usp45 secretion signal[26] and the surface anchoring C-terminal domain of AcmA was displayed on the surface of *L. lactis* as previously reported for other proteins.[13,35–37] Binding of SpyTag-B domain, isolated from *E. coli*, to recombinant *L. lactis* with surface displayed SpyCatcher was evaluated by flow cytometry using antibody recognizing B domain. Statistically significant increase in MFI was observed when SpyT-B domain was incubated with *L. lactis* with induced SpyCatcher expression, in comparison to control non-induced *L. lactis* cells, or induced *L. lactis* cells without the addition of SpyT-B domain (19.9%; Fig. 3).

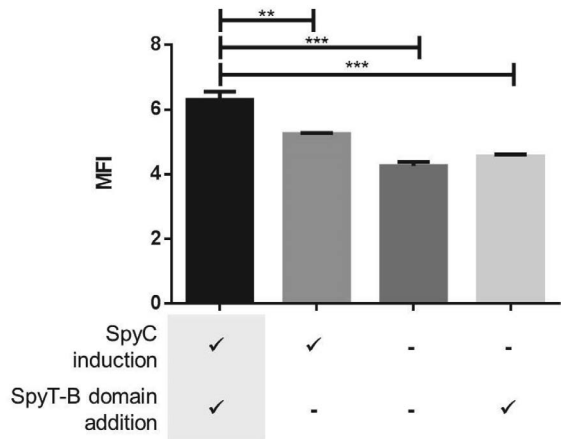

Fig. 3. Flow cytometric analysis of binding of SpyTag (SpyT)-B domain, isolated from *E. coli,* to *L. lactis* cells displaying SpyCatcher (SpyC) on their surface. FITC-conjugated human IgG was used for detection. Mean fluorescence intensity (MFI) value of SpyC-displaying bacteria with added SpyT-B domain was compared to those of the controls by using Student's t test. ** p<0.01, *** p<0.001.

3. 3. Binding of SpyTag-B Domain from *L. lactis* Conditioned Medium to *L. lactis* with Surface Displayed SpyCatcher

SpyTag in fusion with B domain and Usp45 secretion signal (plasmid pSD_SpyT_Bd) was expressed in *L. lactis* under the control of NisA promoter[38] and secreted to the growth medium. The producer cells were removed and the conditioned medium containing SpyTag-B domain fusion protein was incubated with *L. lactis* cells with surface displayed SpyCatcher (plasmid pSD_SpyC_AcmA). Low extent of binding was observed with flow cytometry using antibody recognizing B domain. Small statistically significant increase in MFI was reported when SpyTag-B domain was incubated with SpyCatcher-displaying *L. lactis*, in comparison to empty plasmid pNZ8148-containing control. No difference was observed when non-tagged B domain was incubated with SpyCatcher-displaying *L. lactis* (Fig. 4).

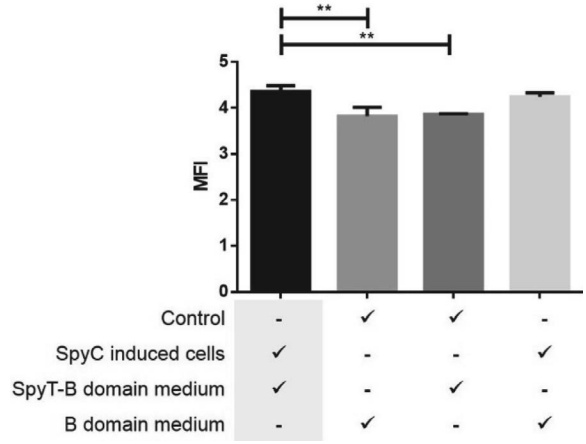

Fig. 4. Flow cytometric analysis of binding of *L. lactis*-secreted SpyTag (SpyT)-B domain to *L. lactis*-displaying SpyCatcher (SpyC). FITC-conjugated human IgG was used for detection. Mean fluorescence intensity (MFI) values of SpyC-displaying bacteria with added SpyT-B domain-containing medium were compared to those of the controls by using Student's t test. * p<0.05, ** p<0.01.

3. 4. Binding of SpyTag-B Domain Secreted from *L. lactis* to SpyCatcher Displayed on *L. lactis* During the Co-culturing of the Two Strains

We co-cultured *L. lactis* secreting SpyTag-B domain fusion (plasmid pSD_SpyT_Bd) with *L. lactis* displaying SpyCatcher (plasmid pSD_SpyC_AcmA) to achieve immediate bond formation between SpyCatcher and SpyTag protein/peptide pair. Binding was evaluated with flow cy-

tometry using antibody recognizing B domain. Statistically significant increase in MFI (40.1%) was observed, when Spy-tagged B domain producing *L. lactis* was co-cultured with SpyCatcher-displaying *L. lactis* cells, in comparison to SpyCatcher-displaying *L. lactis* cells (Fig. 5).

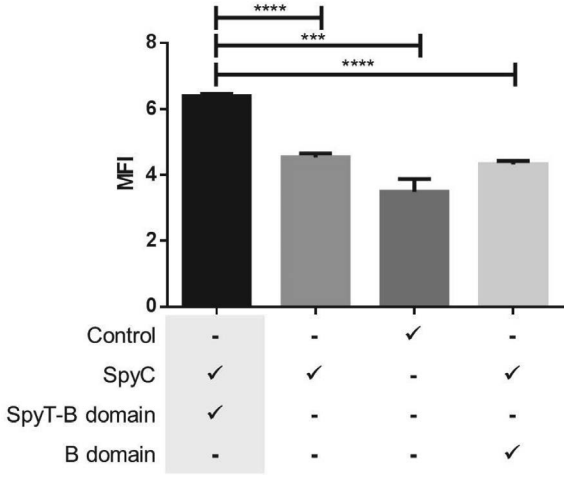

Fig. 5. Flow cytometric analysis of binding of *L. lactis*-secreted SpyTag (SpyT)-B domain to *L. lactis* cells displaying SpyCatcher (SpyC) on their surface after co-culturing of the two strains. FITC-conjugated human IgG was used for detection. Mean fluorescence intensity (MFI) values were compared to those of controls using Student's t test. *** p<0.001, **** p<0.0001.

3. 5. Replacing Tag and Catcher: Binding of SpyCatcher-B Domain Secreted from *L. lactis* to SpyTag Displayed on *L. lactis* During Co-culturing of the Two Strains

The location of interacting protein/peptide pair was reversed by engineering a strain of *L. lactis* to secrete the SpyCatcher fusion protein, and another strain to display the SpyTag. SpyCatcher-B domain fusion-secreting *L. lactis* (plasmid pSD_SpyC_Bd) was co-cultured with SpyTag-displaying *L. lactis* (plasmid pSD_SpyT_AcmA). Statistically significant increase in MFI (22.0%) was observed, when SpyCatcher-B domain producing *L. lactis* was co-cultured with SpyTag-displaying *L. lactis* cells, in comparison to SpyCatcher-displaying *L. lactis* cells (Fig. 6).

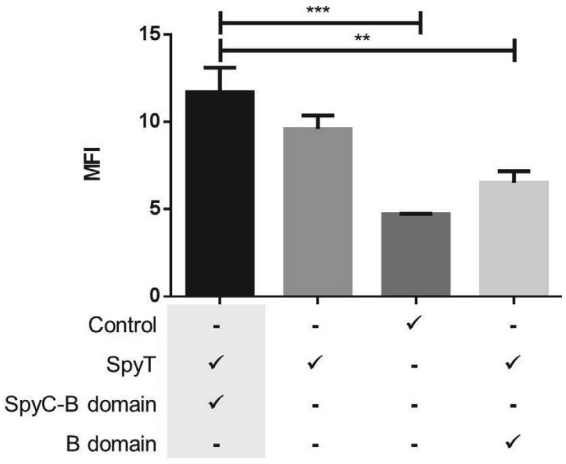

Fig. 6. Flow cytometric analyses of binding of *L. lactis*-secreted SpyCatcher (SpyC)-B domain to *L. lactis* cells displaying SpyTag (SpyT) on their surface. FITC-conjugated human IgG was used for detection. MFI: Mean fluorescence intensity. MFI values were compared to those of controls using Student's t test. ** p < 0.01, *** p < 0.001.

3. 6. Introducing SnoopCatcher and Tag: Binding of SnoopTag-B Domain Secreted from *L. lactis* to SnoopCatcher Displayed on *L. lactis* During Co-culturing of the Two Strains

We co-cultured *L. lactis* secreting SnoopTag-B domain fusion (plasmid pSD_SnT_Bd) with *L. lactis* displaying SnoopCatcher (plasmid pSD_SnC_AcmA), respectively, to achieve immediate bond formation, as demonstrated previously for SpyCatcher/SpyTag pair. Binding was evaluated with flow cytometry using antibody recognizing B domain. Statistically significant increase in MFI (21.1%) was observed, when Snoop-tagged B domain-producing *L. lactis* was co-cultured with SnoopTag-displaying *L. lactis* cells, in comparison to SnoopCatcher-displaying *L. lactis* cells (Fig. 7).

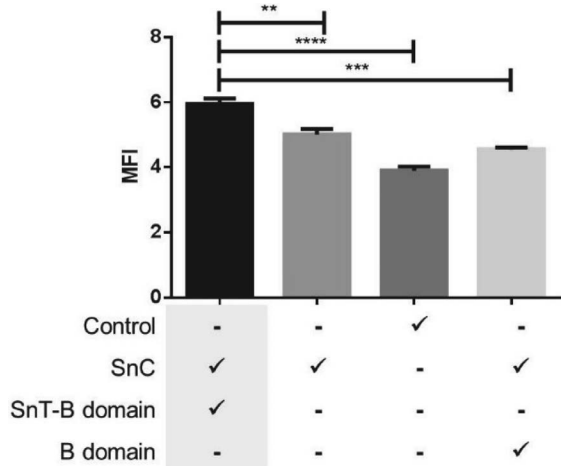

Fig. 7. Flow cytometric analysis of binding of *L. lactis*-secreted SnoopTag (SnoopT)-B domain to *L. lactis* cells displaying SnoopCatcher (SnoopC) on their surface after co-culturing of the two strains. FITC-conjugated human IgG was used for detection. Mean fluorescence intensity (MFI) values were compared to those of controls using Student's t test. ** p<0.01, *** p<0.001, **** p<0.0001.

4. Discussion

Isopeptide bond formation was applied to develop alternative surface display systems for LAB *L. lactis* by enabling a stable covalent bond between a peptide SpyTag

and a protein SpyCatcher or, similarly, between a peptide SnoopTag and a protein SnoopCatcher.[1,2] The peptide/protein pair has already been employed to stabilize enzymes, for modular vaccine production, vaccine optimization and formation of catalytic biofilms.[29,39]

To test the feasibility of the isopeptide bond formation, we anchored one of the binding partners to the surface of *L. lactis,* by fusing it with Usp45 secretion signal and peptidoglycan-binding C-terminus of AcmA protein, as previously reported.[13,35] The second binding partner was fused to a reporter protein B domain that we previously applied for the assessment of surface display.[13,40] B domain fusion was isolated from *E. coli* or secreted from another recombinant *L. lactis* species (Fig. 1). Formation of the isopeptide bond resulted in the attachment of the B domain to the surface of *L. lactis* and was assessed by flow cytometry. We obtained statistically significant display of B domain on the surface of *L. lactis* with almost all the systems that were constructed.

Firstly, we expressed a fusion peptide SpyTag-B domain-His Tag in *E. coli* to obtain sufficient amount of the fusion protein following IMAC purification. Purified SpyTag-B domain-His Tag was added to SpyCatcher-displaying recombinant *L. lactis.* Statistically significant display of B domain, in comparison to the control, was determined. However, the binding was relatively weak and partially unspecific.

Secondly, we expressed SpyTag-B domain in *L. lactis* and directed it to the growth medium. After removal of the producer cells, the conditioned medium was incubated with SpyCatcher-displaying recombinant *L. lactis*. The display of B domain was lower than that achieved with SpyTag-B domain from *E. coli* and was not statistically significant. This could be due to the lower amount of the fusion protein in the conditioned medium. Moreover, there are several other factors in the medium that could hinder binding, for example pH value of the medium, and the presence of numerous other proteins and peptides. Even though SpyCatcher-SpyTag bond is claimed to be stable under a range of pH values (5–8)[29] it is possible that low pH of the conditioned medium hinders the bond formation. Additionally, numerous peptides in the conditioned medium might non-specifically interact with SpyCatcher.

Thirdly, we expected the formation of the isopeptide bond to be more probable if the Spy-tagged B domain was available immediately after induction of the surface display of SpyCatcher, as this would decrease the probability of unspecific interactions. Availability of Spy-tagged B domain was provided by co-culturing two species of *L. lactis*: one displaying SpyCatcher, and the other secreting SpyTag-B domain fusion. Thus achieved surface display of B domain was indeed higher than that achieved by the addition of SpyTag-B domain from *E. coli* or from the conditioned medium of *L. lactis.*

In the above examples the SpyCatcher was immobilized on the surface of *L. lactis*. To test the influence of the location of binding partners, we reversed the system by displaying SpyTag on *L. lactis*, and co-cultured the strain with *L. lactis* secreting SpyCatcher-B domain fusion protein. The display of B domain was again achieved; however due to relatively high unspecific binding of antibodies with SpyTag-displaying lactococci the display was not statistically significant.

Apart from SpyCatcher/SpyTag pair, the isopeptide bond can also be formed by combining SnoopCatcher and SnoopTag. We applied similar experimental setup as previously described for SpyCatcher/SpyTag by displaying SnoopCatcher on the surface of *L. lactis* and co-culturing the bacteria with a strain of recombinant *L. lactis* secreting SnoopTag-B domain fusion protein. Significant surface display of B domain was again observed; however there was no improvement over SpyCatcher/SpyTag pair.

5. Conclusion

In the present study we demonstrated, for the first time, the surface display of reporter protein on *L. lactis* by exploiting isopeptide bond-forming partners SpyCatcher and SpyTag, as well as SnoopCatcher and SnoopTag. The most effective display was obtained by anchoring SpyCatcher to the bacterial surface, and co-culturing the bacteria with a lactococcal strain that secreted Spy-tagged reporter protein. This represents a proof-of-principle for a new, highly flexible surface display system for *L. lactis* that warrants further studies with an intention to improve the extent of surface display.

6. Acknowledgments

This study was funded by the Slovenian Research Agency (grant number P4-0127).

7. References

1. G. Veggiani, T. Nakamura, M. D. Brenner, R. V. Gayet, J. Yan, C. V. Robinson, M. Howarth, *Proc Natl Acad Sci U S A* **2016**, *113*, 1202–7. **DOI:** https://doi.org/10.1073/pnas.1519214113
2. G. Veggiani, B. Zakeri, M. Howarth, *Trends Biotechnol* **2014**, *32*, 506–12. **DOI:** https://doi.org/10.1016/j.tibtech.2014.08.001
3. B. Zakeri, J. O. Fierer, E. Celik, E. C. Chittock, U. Schwarz-Linek, V. T. Moy, M. Howarth, *Proc Natl Acad Sci U S A* **2012**, *109*, E690–7. **DOI:** https://doi.org/10.1073/pnas.1115485109
4. S. Y. Lee, J. H. Choi, Z. Xu, *Trends Biotechnol* **2003**, *21*, 45–52. **DOI:** https://doi.org/10.1016/S0167-7799(02)00006-9
5. S. Stahl, M. Uhlen, *Trends Biotechnol* **1997**, *15*, 185–92. **DOI:** https://doi.org/10.1016/S0167-7799(97)01034-2
6. G. Georgiou, C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson, R. Curtiss, 3rd, *Nat Biotechnol* **1997**, *15*, 29–34. **DOI:** https://doi.org/10.1038/nbt0197-29

7. V. Laroute, H. Tormo, C. Couderc, M. Mercier-Bonin, P. Le Bourgeois, M. Cocaign-Bousquet, M. L. Daveran-Mingot, *Microorganisms* **2017**, *5*.
DOI: https://doi.org/10.3390/microorganisms5020027
8. A. A. Song, L. L. A. In, S. H. E. Lim, R. A. Rahim, *Microb Cell Fact* **2017**, *16*, 55.
DOI: https://doi.org/10.1186/s12934-017-0669-x
9. M. de Vrese, J. Schrezenmeir, *Adv Biochem Eng Biotechnol* **2008**, *111*, 1–66. **DOI:** https://doi.org/10.1007/10_2008_097
10. A. Berlec, T. Malovrh, P. Zadravec, A. Steyer, M. Ravnikar, J. Sabotic, M. Poljsak-Prijatelj, B. Strukelj, *Appl Microbiol Biotechnol* **2013**, *97*, 4333–42.
DOI: https://doi.org/10.1007/s00253-013-4722-3
11. A. Berlec, M. Ravnikar, B. Strukelj, *Pharmazie* **2012**, *67*, 891–8.
12. M. de Azevedo, M. Meijerink, N. Taverne, V. B. Pereira, J. G. LeBlanc, V. Azevedo, A. Miyoshi, P. Langella, J. M. Wells, J. M. Chatel, *Vaccine* **2015**, *33*, 4807–12.
DOI: https://doi.org/10.1016/j.vaccine.2015.07.077
13. M. Ravnikar, B. Strukelj, N. Obermajer, M. Lunder, A. Berlec, *Appl Environ Microbiol* **2010**, *76*, 6928–32.
DOI: https://doi.org/10.1128/AEM.00190-10
14. C. Michon, P. Langella, V. G. Eijsink, G. Mathiesen, J. M. Chatel, *Microb Cell Fact* **2016**, *15*, 70.
DOI: https://doi.org/10.1186/s12934-016-0468-9
15. S. Kosler, B. Strukelj, A. Berlec, *Curr Pharm Biotechnol* **2017**, *18*, 318–326.
DOI: https://doi.org/10.2174/1389201018666170210152218
16. K. Vandenbroucke, H. de Haard, E. Beirnaert, T. Dreier, M. Lauwereys, L. Huyck, J. Van Huysse, P. Demetter, L. Steidler, E. Remaut, C. Cuvelier, P. Rottiers, *Mucosal Immunol* **2010**, *3*, 49–56. **DOI:** https://doi.org/10.1038/mi.2009.116
17. A. Berlec, M. Perse, M. Ravnikar, M. Lunder, A. Erman, A. Cerar, B. Strukelj, *Int Immunopharmacol* **2017**, *43*, 219–226.
DOI: https://doi.org/10.1016/j.intimp.2016.12.027
18. K. Leenhouts, G. Buist, J. Kok, *Antonie Van Leeuwenhoek* **1999**, *76*, 367–76. **DOI:** https://doi.org/10.1023/A:1002095802571
19. G. R. Visweswaran, K. Leenhouts, M. van Roosmalen, J. Kok, G. Buist, *Appl Microbiol Biotechnol* **2014**, *98*, 4331–45.
DOI: https://doi.org/10.1007/s00253-014-5633-7
20. P. Zadravec, A. Mavric, B. Bogovic Matijasic, B. Strukelj, A. Berlec, *Protein Eng Des Sel* **2014**, *27*, 21–7.
DOI: https://doi.org/10.1093/protein/gzt059
21. E. K. Call, T. R. Klaenhammer, *Front Microbiol* **2013**, *4*, 73.
DOI: https://doi.org/10.3389/fmicb.2013.00073
22. S. H. Lim, F. Jahanshiri, R. A. Rahim, Z. Sekawi, K. Yusoff, *Lett Appl Microbiol* **2010**, *51*, 658–64.
DOI: https://doi.org/10.1111/j.1472-765X.2010.02950.x
23. K. Okano, Q. Zhang, S. Kimura, J. Narita, T. Tanaka, H. Fukuda, A. Kondo, *Appl Environ Microbiol* **2008**, *74*, 1117–23.
DOI: https://doi.org/10.1128/AEM.02012-07
24. G. Andre, K. Leenhouts, P. Hols, Y. F. Dufrene, *J Bacteriol* **2008**, *190*, 7079–86.
DOI: https://doi.org/10.1128/JB.00519-08
25. G. Buist, H. Karsens, A. Nauta, D. van Sinderen, G. Venema, J. Kok, *Appl Environ Microbiol* **1997**, *63*, 2722–8.
26. Y. Dieye, S. Usai, F. Clier, A. Gruss, J. C. Piard, *J Bacteriol* **2001**, *183*, 4157–66.
DOI: https://doi.org/10.1128/JB.183.14.4157-4166.2001
27. A. S. Wieczorek, V. J. Martin, *Microb Cell Fact* **2010**, *9*, 69.
DOI: https://doi.org/10.1186/1475-2859-9-69
28. L. Li, J. O. Fierer, T. A. Rapoport, M. Howarth, *J Mol Biol* **2014**, *426*, 309–17. **DOI:** https://doi.org/10.1016/j.jmb.2013.10.021
29. S. C. Reddington, M. Howarth, *Curr Opin Chem Biol* **2015**, *29*, 94–9. **DOI:** https://doi.org/10.1016/j.cbpa.2015.10.002
30. S. Thrane, C. M. Janitzek, S. Matondo, M. Resende, T. Gustavsson, W. A. de Jongh, S. Clemmensen, W. Roeffen, M. van de Vegte-Bolmer, G. J. van Gemert, R. Sauerwein, J. T. Schiller, M. A. Nielsen, T. G. Theander, A. Salanti, A. F. Sander, *J Nanobiotechnology* **2016**, *14*, 30.
DOI: https://doi.org/10.1186/s12951-016-0181-1
31. K. Skrlec, A. Pucer Janez, B. Rogelj, B. Strukelj, A. Berlec, *Microb Biotechnol* **2017**, *10*, 1732–1743.
DOI: https://doi.org/10.1111/1751-7915.12781
32. P. G. de Ruyter, O. P. Kuipers, W. M. de Vos, *Appl Environ Microbiol* **1996**, *62*, 3662–7.
33. O. P. Kuipers, M. M. Beerthuyzen, R. J. Siezen, W. M. De Vos, *Eur J Biochem* **1993**, *216*, 281–91.
DOI: https://doi.org/10.1111/j.1432-1033.1993.tb18143.x
34. I. Mierau, M. Kleerebezem, *Appl Microbiol Biotechnol* **2005**, *68*, 705–17.
DOI: https://doi.org/10.1007/s00253-005-0107-6
35. P. Zadravec, B. Strukelj, A. Berlec, *Appl Environ Microbiol* **2015**, *81*, 2098–106.
DOI: https://doi.org/10.1128/AEM.03694-14
36. A. Steen, G. Buist, K. J. Leenhouts, M. El Khattabi, F. Grijpstra, A. L. Zomer, G. Venema, O. P. Kuipers, J. Kok, *J Biol Chem* **2003**, *278*, 23874–81.
DOI: https://doi.org/10.1074/jbc.M211055200
37. A. Steen, G. Buist, G. J. Horsburgh, G. Venema, O. P. Kuipers, S. J. Foster, J. Kok, *FEBS J* **2005**, *272*, 2854–68.
DOI: https://doi.org/10.1111/j.1742-4658.2005.04706.x
38. J. N. Ahmad, J. Li, L. Biedermannova, M. Kuchar, H. Sipova, A. Semeradtova, J. Cerny, H. Petrokova, P. Mikulecky, J. PoP. Sebo, P. Maly, *Proteins* **2012**, *80*, 774–89.
DOI: https://doi.org/10.1002/prot.23234
39. Z. Liu, H. Zhou, W. Wang, W. Tan, Y. X. Fu, M. Zhu, *Sci Rep* **2014**, *4*, 7266. **DOI:** https://doi.org/10.1038/srep07266
40. T. Bratkovic, A. Berlec, T. Popovic, M. Lunder, S. Kreft, U. Urleb, B. Strukelj, *Biochem Biophys Res Commun* **2006**, *349*, 449–53. **DOI:** https://doi.org/10.1016/j.bbrc.2006.08.078

Povzetek

Predstavitev rekombinantnih proteinov na bakterijski površini postaja pomembno raziskovalno področje s številnimi možnostmi uporabe na področju biotehnologije. Zaradi statusa GRAS (generally recognized as safe – splošno priznana kot varna) predstavlja mlečnokislinska bakterija *Lactococcus lactis* privlačen gostiteljski organizem za površinsko predstavitev in obetaven vektor za *in situ* dostavo proteinov. Opisana raziskava se je osredotočila na iskanje novega alternativnega pristopa za površinsko predstavitev na bakteriji *Lactococcus lactis*. Razvili smo sistem, ki omogoča tvorbo ireverzibilne izopeptidne vezi na površini bakterije *Lactococcus lactis*. To smo dosegli s pomočjo dveh parov protein/peptid, SpyCatcher/SpyTag in SnoopCatcher/SnoopTag.[1–3] Pritrditev modelnega proteina domene B na površino bakterij *Lactococcus lactis*, ki so imele na površini ustrezen lovilni protein, smo potrdili s pretočno citometrijo. V raziskavi smo prikazali učinkovito uporabo omenjenih proteinskih sidrnih domen, ki tako predstavljajo potencialno alternativo obstoječim načinom površinske predstavitve na bakteriji *Lactococcus lactis*.