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RAZVOJ GENSKO SPREMENJENIH MLEČNOKISLINSKIH BAKTERIJ S SPOSOBNOSTJO VEZAVE TOKSINA ŠIGA

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KAZALO

POVZETEK

Okužbe z bakterijami, ki tvorijo toksine šiga, povzročajo različne črevesne bolezni, ki se lahko razvijejo v potencialno smrtne sistemske zaplete. Glavni virulentni dejavnik je toksin šiga (Stx), ki ga bakterije proizvajajo v različicah Stx1 in Stx2, in je sestavljen iz encimsko aktivne A podenote in pentamerne B podenote, odgovorne za vezavo na glikolipidni Gb_3 receptor. Zaradi pomanjkanja učinkovitih načinov zdravljenja teh bolezni je aktualen razvoj novih načinov, med katerimi prednjačijo analogi Stx receptorja, spojine, ki vplivajo na transport, procesiranje in funkcijo toksina ter monoklonska protitelesa proti toksinu. Mlečnokislinske bakterije (MKB) s površinsko predstavljenimi vezavnimi proteini za toksin šiga, bi lahko bile učinkovit sistem za vezavo toksinov šiga v črevesju bolnikov. MKB so zaradi dolgotrajne uporabe v prehrambeni industriji in potencialnega dobrodejnega delovanja v prebavnem traktu primerne kot dostavni sistemi za biološko aktivne snovi. S tem namenom so se razvili različni sistemi površinske predstavitve na MKB, med katerimi se največkrat uporablja sistem na osnovi C-končnega dela laktokoknega proteina AcmA (cA), ki vsebuje ponovitve lizinskega motiva (LysM) in omogoča nekovalentno vezavo na peptidoglikan. Na površini MKB lahko predstavimo različne terapevtske proteine. Mednje uvrščamo načrtovane vezavne proteine, kot so npr. načrtovani proteini z ankirinskimi ponovitvami (DARPini) ali albumin-vezavna domena (ABD), ki so zaradi majhnosti in visoke stabilnosti potencialno zanimivi za površinsko predstavitev na MKB s ciljem terapevtskega delovanja. Zaradi stroge zakonodaje na področju gensko spremenjenih organizmov (GSO) je v prehrani in terapiji zaželena uporaba nerekombinantih pristopov. Nespremenjene bakterije z nekovalentno pripetimi rekombinantimi proteini na površini bi s stališča zakonodaje lahko predstavljale sprejemljivejši način površinske predstavitve.

V prvem delu doktorske disertacije smo se osredotočili na razvoj gensko spremenjene prehransko sprejemljive mlečnokislinske bakterije *L. lactis* s površinsko predstavljenimi novimi vezavnimi proteini proti B podenoti toksina šiga (Stx1B). Kot tarčo za selekcijo novih vezavnih proteinov smo pripravili rekombinanten protein Stx1B v bakteriji *E. coli* in njegovo aktivnost potrdili z vezavo na sintezni Gb_3 receptor in internalizacijo v HeLa celice. Z uporabo visoko kompleksne knjižnice različic ABD, pridobljene z randomizacijo sekvence ABD streptokoknega proteina G, smo v petih ciklih predstavitve na ribosomih izselekcionirali 17 različic ABD, ki smo jih imenovali S1B vezalci. Tri najobetavnejše S1B vezalce (S1B9, S1B22 in S1B26) smo podrobneje ovrednotili z ELISA in površinsko plazmonsko resonanco. Konstanta afinitete vezalcev S1B22 in S1B26 do Stx1B je znašala 0.70 oz. 1.00 μM. Izbrane

vezavne proteine smo uspešno predstavili na površini bakterije *L. lactis* v obliki fuzije z Usp45 signalnim peptidom za izločanje in s peptidoglikan-vezavnim zaporedjem cA. Vezavo Stx1B smo dokazali s pomočjo pretočne citometrije in ELISE s celimi celicami. Bakterije s površinsko predstavljenimi vezavnimi proteini proti Stx1B so potencialno uporabne za odstranitev toksina šiga iz črevesja.

Za predstavitev heterolognih proteinov na površini MKB *L. lactis* smo uporabljali dva različna sistema za površinsko predstavitev, in sicer kovalentno predstavitev z nosilnim bazičnim proteinom A (BmpA) in nekovalentno predstavitev na osnovi cA zaporedja. Oba sistema smo dodatno optimizirali v drugem oz. tretjem poglavju. Sistem površinske predstavitve z BmpA smo v drugem delu skušali izboljšati s krajšanjem BmpA in dodajanjem vmesnika med skrajšano različico BmpA in potniškim proteinom (B domeno stafilokoknega proteina A). V primerjavi z BmpA smo pri različici Bmp1 opazili občutno povečanje količine B domene na površini bakterije *L. lactis*. Skrajšani različici Bmp2 in Bmp3 sta prav tako izboljšali površinsko predstavitev, pri nadaljnjih krajšanjih pa je prišlo do zmanjšanja površinske predstavitve ali celo njene odsotnosti. Bližina N- in C-konca v Bmp1 modelu in neučinkovitost vmesnika kažeta na to, da razdalja potniškega proteina od membrane ni bistvenega pomena pri površinski predstavitvi. Proteina BmpA in Bmp1 predstavljata učinkovito alternativo ostalim nosilnim proteinom, ki se uporabljajo za površinsko predstavitev na bakteriji *L. lactis*.

Namen tretjega dela doktorata je bila optimizacija cA-posredovane heterologne nerekombinantne površinske predstavitve na bakteriji *L. lactis* in desetih izbranih sevih iz rodu *Lactobacillus*. Na površini bakterije *L. lactis* smo v obliki fuzije z Usp45 in cA nosilnim proteinom uspešno predstavili dva DARPina (I_07 in I_19), ki imata sposobnost vezave Fc regije človeških imunoglobulinov (Ig) G. Heterologno vezavo v gojišče izločenega fuzijskega proteina I_07-cA na seve iz rodu *Lactobacillus* smo testirali s pretočno citometrijo, ELISO s celimi celicami in fluorescenčno mikroskopijo. Fuzijski proteini so se vezali na površino vseh laktobacilov. Pri večini sevov je bila vezava šibka do zmerna, pri sevu *Lactobacillus salivarius* ATCC 11741 pa je bila vezava izjemno močna. Dodatno smo pokazali koncentracijsko odvisno 15-kratno povišanje nerekombinantne površinske predstavitve z gojenjem bakterij v prisotnosti subletalnih koncentracij antibiotikov kloramfenikola in eritromicina. V raziskavi smo optimizirali nerekombinantno površinsko predstavitev na MKB z izborom seva *Lactobacillus salivarius* ATCC 11741 kot optimalnega gostiteljskega organizma in z uvedbo antibiotikov kot dodatkov za izboljšanje površinske predstavitve na

bakteriji *L. lactis*. Učinkovita predstavitev DARPinov na površini nerekombinantnih MKB odpira številne nove možnosti uporabe v terapiji.

V zadnjem poglavju doktorata smo podrobneje opisali in ovrednotili heterologno nerekombinanto površinsko predstavitev. Predstavili smo pogled nanjo iz stališča regulative, pri čemer smo se še posebej osredotočili na veljavno Evropsko zakonodajo.

ABSTRACT

Infections with shiga toxin producing bacteria cause several gastrointestinal diseases, which can develop into potential life threatening complications. The major virulence factor is Shiga toxin, which is produced by bacteria as variant Stx1 or Stx2, and is composed of enzymatically active A subunit and pentameric B subunit, responsible for binding to glycolipid receptor Gb_3. Because no effective treatment is available for these diseases, there is a need for the development of novel therapies. Among them Shiga toxin receptor analogs, compounds that interfere with toxin transport, processing or function, and monoclonal antibodies against toxin predominate. Lactic acid bacteria (LAB) with surface displayed proteins capable of binding Shiga toxin could be an effective system for removal of Shiga toxin from human intestine. LAB are appropriate delivery systems for biologically active compounds due to their long history of safe usage in the food industry and, potentially, due to their health promoting effects in human gastrointestinal tract. To achieve this goal, several surface display systems have been developed. Of those, surface display system using C-terminal part of the lactococcal protein AcmA (cA) with lysine motif (LysM) repeats has been the most commonly used. Different therapeutic proteins can be displayed on the surface of LAB. Engineered binding proteins, such as designed ankyrin repeat proteins (DARPins) and albumin-binding domain (ABD), are potentially interesting therapeutic proteins for surface display on LAB considering their small size and high stability. The non-recombinant approach to surface display is preferred for therapeutic and food application due to strict regulatory requirements regarding genetically modified organisms. From regulatory point of view, unmodified bacteria wih recombinant proteins non-covalently attached to their surface could represent a more acceptable system for surface display.

In the first part of the doctoral thesis we focused on the development of geneticaly modified food-grade LAB *L. lactis* with surface displayed novel engineered binding protein against Shiga toksin 1 B subunit (Stx1B). Stx1B as a target for selection of novel binding proteins was produced in *E. coli* and its functionality was confirmed by binding to synthetic Gb_3 receptor and internalization into HeLa cells. Using high-complexity combinatorial library of albumin-binding domain (ABD) variants, derived from ABD scaffold of streptococcal protein G, and five cycles of ribosome display, we selected 17 ABD variants (called S1B binders). Three most promising S1Bs (S1B9, S1B22 in S1B26) were characterized into more details by ELISA and surface plasmon resonance. The affinity constants of S1B22 and S1B26 for Stx1B were 0.70 and 1.00 μM, respectively. Selected binding proteins were successfully displayed

on the surface of *L. lactis* as a fusion with the Usp45 secretion signal and peptidoglycan-binding cA. The binding of Stx1B was confirmed by flow cytometry and whole cell ELISA. Bacteria with surface displayed binding proteins against Stx1B are potentially useful for the removal of Shiga toxin from human intestine.

For display of heterologous proteins on the surface of LAB *L. lactis* we used two different surface display systems, which were further optimized in second and third chapters of the thesis. We have attempted to optimize the surface display system based on basic membrane protein A (BmpA) as a carrier protein, by truncating the BmpA and by adding spacer between BmpA variants and passenger protein, B domain of staphylococcal protein A. Significantly larger amount of B domain on the surface of *L. lactis* was displayed with Bmp1 in comparison to BmpA. Bmp2 and Bmp3 variants also increased surface display ability, but further shortening resulted in the stepwise decrease or the abolition of the surface display ability. The closeness of the N- and C-terminals in the Bmp1 model and the inefficiency of the spacer suggest that the distance of the passenger from the membrane is not of prime importance in surface display. BmpA and Bmp1 represent an effective alternative to others carrier proteins used in the surface display on *L. lactis.*

The purpose of the third part of doctoral thesis was optimization of cA-mediated heterologous non-recombinant surface display on *L. lactis* and ten selected species from *Lactobacillus* genus. We successfully displayed two DARPins (I_07 and I_19), capable of binding Fc region of human IgG, on the surface of *L. lactis* by fusion to Usp45 and to cA. Heterologous binding of secreted fusion protein I_07-cA from the growth medium to the surface of *Lactobacillus* species was tested by flow cytometry, whole cell ELISA and fluorescence microscopy. The fusion proteins bound to the surface of all *Lactobacillus* tested. Weak to moderate binding was observed for the majority of species; however, *Lactobacillus salivarius* ATCC 11741 exhibited exceptionally strong binding. Additionally, 15-fold concentration-dependent increase in non-recombinant surface display on *L. lactis* was demonstrated by growing bacteria with sublethal concentrations of the antibiotics chloramphenicol and erythromycin. Non-recombinant surface display on LAB, based on the cA protein, was optimized by selecting *Lactobacillus salivarius* ATCC11741 as the optimal host, and by introducing antibiotics as additives for increasing surface display on *L. lactis*. Effective display of DARPins on the surface of non-recombinant LAB has opened up several new therapeutic possibilities.

In the last part of doctoral thesis heterologous non-recombinant surface display was described and evaluated in more detail. Its possible regulatory implications were discussed, with a special focus on European Union regulations.

SEZNAM KRATIC IN OKRAJŠAV

A_{280}	absorbanca pri 280 nm
ABD	albumin-vezavna domena (ang. albumin-binding domain)
AcmA	N-acetilmuramidaza
cA	C-končni del laktokoknega proteina AcmA
ATCC	Ameriška banka mikroorganizmov in celičnih kultur
B dom.	B domena
BLP	bakterijam podobni delci (ang. bacteria-like particles)
BmpA	bazični membranski protein A (ang. basic membrane protein A)
BSA	goveji serumski albumin (ang. bovine serum albumine)
DARPin	načrtovan protein z ankirinskimi ponovitvami (ang. designed ankyrin repeat protein)
EFSA	Evropska agencija za varnost hrane (ang. European food safety authority)
ELISA	encimsko imunski test (ang. enzyme-linked immunosorbent assay)
EMA	Evropska agencija za zdravila (European medicines agency)
FITC	fluorescein izotiocianat (ang. fluorescein isothiocyanate)
FPLC	tekočinska kromatografija za hitro ločevanje proteinov (ang. fast protein liquid chromatography)
Gb_3	globotriaozil ceramid
GI	gastrointestinalni trakt
GRAS	splošno priznani kot varni (ang. generally recognized as safe)
GSO	gensko spremenjeni organizmi
HRP	hrenova peroksidaza (ang. horseradish peroxidase)
HSA	človeški serumski albumin (ang. human serum albumine)
HUS	hemolitično uremični sindrom (ang. hemolytic uremic syndrome)
IMAC	afinitetna kromatografija z imobiliziranimi kovinskimi ioni
LysM	domena z lizinskim motivom (ang. lysine motif domain)
MKB	mlečnokislinske bakterije (ang. LAB, lactic acid bacteria)
NICE	z nizinom nadzorovano izražanje genov (ang. Nisin-controlled gene expression)
A_{450}	absorbanca pri 450 nm
PBS	fosfatni pufer (ang phosphate buffer saline)
PBST	fosfatni pufer s Tween-om 20 (ang. phosphate buffer saline with Tween 20)
PCR	verižna reakcija s polimerazo (ang. polymerase chain reaction)
RPM	vrtljaji na minuto (ang. revolutions per minute)
SDS	natrijev dodecil sulfat (ang. sodium dodecyl sulphate)
SDS-PAGE	poliakrilamidna gelska elektroforeza v prisotnosti SDS
SPR	površinska plazmonska resonanca (ang. surface plasmon resonance)
STEC	*E. coli*, ki proizvajajo toksin šiga (ang. Shiga toxin producing *E.coli*)
Stx	toksin šiga
Stx1B	B podenota različice 1 toksina šiga
T_m	talilna temperatura (ang. melting temperature)
Usp45	signalni peptid za izločanje

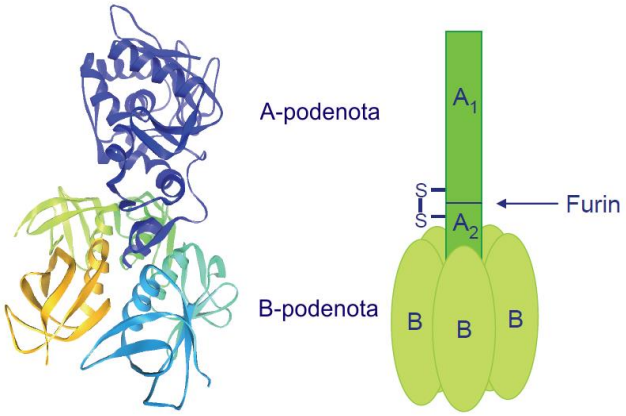
UVOD

Okužbe z bakterijami *Shigella dysenteriae* in *Escherichia coli*, ki tvorijo toksin šiga (STEC), povzročajo pri ljudeh različne črevesne bolezni. Bakterije STEC in *S. dysenteriae* so pri ljudeh povzročiteljice driske in hemoragičnega kolitisa, ki se lahko razvijeta v življenjsko nevaren hemolitično uremični sindrom (HUS) s kliničnimi znaki kot so mikroangiopatska hemolitična anemija, trombocitopenija, akutna odpoved ledvic ter različni nevrološki zapleti (1, 2). Do okužbe pride največkrat po zaužitju okužene vode ali hrane, kot je mleta govedina, surova zelenjava, nepasterizirano mleko in sokovi, ali s stikom okuženih živali in ljudi (3, 4). Zaradi možnosti hitrega prenosa, se lahko okužba zelo hitro razširi med ljudmi in povzroči masovni izbruh STEC bolezni. Največ primerov izbruha STEC bolezni v razvitih državah je povezanih z okužbo z enterohemoragično bakterijo *E. coli* (EHEC) serotipa O157:H7, katerega vir so prebavila prežvekovalcev, večinoma goveda in ovc (5). Z epidemijami so povezani tudi drugi sevi EHEC, kot so na primer O145:H$^-$, O111:H$^-$, O103:H2, O121:H19 in O26:H11 (6). Zadnji večji izbruh enterohemoragičnega kolitisa in HUS v Evropi se je pojavil leta 2011 v Nemčiji, kjer je prišlo do okužbe z okuženimi kalčki in prenosa na ljudi, kot povzročitelj pa je bil identificiran enteroagregativni sev *E. coli* (EAEC) serotipa O104:H4, ki je pridobil zmožnost produkcije šiga toksina 2, najverjetneje preko horizontalnega genskega prenosa (7, 8). V primeru okužb z EAEC O104:H4 so zboleli predvsem odrasli ljudje srednjih let, medtem ko pri okužbah z EHEC sevi najpogosteje obolevajo mlajši otroci in starejši ljudje, ki so tudi najbolj rizična skupina (5). V primeru izbruha epidemije z EAEC O104:H4 je bilo identificiranih več kot 4000 okužb in zabeleženih 50 smrtnih primerov (8). Glavni povzročitelj zapletov, ki nastanejo ob okužbah z opisanimi bakterijami, je toksin šiga, ki bo predstavljen v nadaljevanju.

Toksin šiga in njegovo delovanje

Toksin šiga (Stx), imenovan tudi vero toksin, bakterije proizvajajo v različicah Stx1 (Stx1 in Stx1c) in Stx2 (Stx2, Stx2c, Stx2d, Stx2e) (3). STEC, ki povzročajo obolenja pri ljudeh, lahko tvorijo Stx1, Stx2 ali kombinacijo večih različic (9). Stx so v STEC bakterijah večinoma kodirani na lizogenih lambdoidnih fagih, ki izražajo Stx v velikih količinah, ko pride do aktivacije litičnega cikla fagov (3). Stx je pripadnik družine AB_5 toksinov, sestavljen iz encimsko aktivne podenote A (StxA, molekulska masa približno 32 kDa) in nekovalentno vezane pentamerne podenote B (StxB, vsaka podenota B ima molekulsko maso 7.7 kDa), ki je odgovorna za vezavo toksina na receptor (Slika 1) (4). Skoraj vse različice Stx se vežejo na

glikolipidni receptor globotriaozilceramid (Gb_3), ki je izražen v membranah tarčnih celic. Izjema je le različica Stx2e, ki povzroča prašičjo vodenico, tako da se veže na receptor globotetraozilceramid (Gb_4) (10). Stx, ki ga proizvaja *S. dysenteriae*, je skoraj identičen Stx1 iz *E. coli*, razlikuje se le v eni aminokislini v podenoti A. Stx1 in Stx2 se kljub podobni strukturi in vezavi na skupni receptor med sabo imunološko razlikujeta in imata le približno 56 % identične aminokislinske sestave (11). HUS se z večjo verjetnostjo razvije ob prisotnosti Stx2, čeprav Stx1 izkazuje višjo afiniteto do receptorja in je do desetkrat bolj citotoksičen *in vitro* (3, 4).



Slika 1: Struktura in shema strukture toksina šiga. Prirejeno po (4).

Prisotnost Stx ni zadosten pogoj za nastanek in razvoj bolezni, ampak gre za večstopenjski proces, ki vključuje uspešno kolonizacijo gastrointestinalnega (GI) trakta, absorpcijo toksina s črevesnimi epitelijskimi celicami, interakcijo toksina z Gb_3 receptorjem in prenos toksina z retrogradnim transportom v citosol, kjer pride do aktivacije toksina in posledičnega delovanja. Po zaužitju STEC morajo bakterije preživeti potovanje skozi kislo okolje želodca do črevesja, kjer pride do kolonizacije preko adhezije na črevesne epitelijske celice in izločanja Stx v lumen črevesja (1). Toksin se nato absorbira skozi črevesne epitelijske celice v krvni obtok, preko katerega doseže celice tkiv in organov, ki izražajo Gb_3 receptor, npr. ledvice, možgani, jetra, trebušna slinavka, srce in krvne celice (12). Po vezavi na receptor, Stx vstopi v tarčne celice preko klatrin-odvisne in neodvisne endocitoze in se preko retrogradnega transporta (zgodnjih endosomov, trans-Golgijeve mreže in Golgijevega aparata) prenese v endoplazmatski retikulum (ER). Na poti pride do cepitve podenote A z endoproteinazo furinom (Slika 1) na encimsko aktiven fragment A_1 in neaktiven fragment A_2, ki ostaneta povezana s StxB preko disulfidnega mostička (3, 13, 14). Po cepitvi disulfidnega mostička v ER se encimsko aktiven fragment A_1 premesti v citosol, kjer izkazuje N-glikozidazno aktivnost in odstranjuje adenine od adenozinov v ohranjeni zanki 28S ribosomalne RNK na

60S ribosomalni podenoti ter s tem zavira sintezo proteinov ter povzroča celično smrt (4, 12, 14).

Načini zdravljenja okužb z bakterijami, ki tvorijo toksin šiga

Kljub dolgoletnemu raziskovanju okužb z bakterijami, ki tvorijo toksin šiga, specifično in učinkovito zdravljenje pacientov z razvitim HUS še vedno ni na voljo. Možnosti zdravljenja so trenutno zelo omejene in so usmerjene predvsem v podporno terapijo, ki vključuje nadomeščanje izgubljene tekočine, uravnavanje ravnotežja elektrolitov, dodajanje hranil, zniževanje visokega krvnega tlaka, dializo in transplantacijo ledvic, če je potrebno (1, 12). Razvoj hitrih in natančnih metod za zgodnjo identifikacijo okužbe s STEC, še preden pride do pojava znakov HUS, je eden od pomembnih preventivnih pristopov, saj omogoča hitro prepoznavanje vira okužbe in s tem preprečitev širjenja med ljudmi ter izbruha epidemije. Laboratorijska identifikacija STEC sevov temelji na detekciji prisotnosti toksina šiga ali *stx* genov v fekalnih izločkih ter izolaciji STEC bakterij (15). Konvencionalno protimikrobno zdravljenje okužb z bakterijami, ki tvorijo toksin šiga, se je v preteklosti izkazalo za neučinkovito, saj je dokazano, da nekateri antibiotiki povečajo izražanje Stx in izločanje le tega v črevesje, kar lahko povzroči težji potek bolezni in s tem večjo verjetnost razvoja HUS (12).

Zaradi pomanjkanja učinkovitih načinov zdravljenja okužb z bakterijami, ki tvorijo Stx, je aktualen razvoj novih načinov zdravljenja. Nekateri novi pristopi zdravljenja, ki so v različnih fazah razvoja, so podrobneje prikazani v preglednici 1. Med njimi je največ analogov Stx receptorja, spojin, ki vplivajo na transport, procesiranje in funkcijo toksina ter monoklonskih protiteles proti Stx1 in Stx2, ki so trenutno najdlje v razvoju (16). Princip delovanja analogov Stx receptorja je vezava prostega toksina, še preden se ta veže na receptor in povzroči razvoj bolezni. Zaradi takega načina delovanja se je analog Stx receptorja SYNSORB Pk izkazal kot neučinkovit pri zdravljenju pacientov z že razvitim z drisko-povezanim HUS-om (17). Cilj delovanja druge večje skupine terapevtikov pri okužbah s STEC je zaviranje procesov transporta, procesiranja in funkcije toksina. Med njimi je največ zaviralcev retrogradnega transporta, ki lahko zavirajo različne stopnje transporta kot so izstop iz zgodnjih endosomov, vstop v trans-Golgijevo mrežo, vstop v Golgijev aparat in ER ali izstop A podenote iz ER (16). Testirani so bili tudi različni inhibitorji furina, vendar so se izkazali za neučinkovite pri višjih koncentracijah toksina. Inhibitorji furina namreč ne preprečijo popolnoma delovanje toksina in razvoja bolezni, saj furin ni edini encim, ki je zmožen cepitve A podenote toksina (16). Uporaba monoklonskih protiteles za nevtralizacijo Stx je pomemben terapevtski pristop

za zdavljenje STEC okužb. Različna monoklonska protitelesa proti Stx1 ali/in Stx2 so se izkazala kot učinkovita v živalskih in celičnih modelih, nekatera med njimi pa so že bila testirana v prvi in drugi fazi kliničnih študij (16). Za humanizirano monoklonsko protitelo proti Stx2, urtoxazumab, so tako dokazali varnost uporabe pri odraslih in otrocih (18). Čeprav so monoklonska protitelesa lahko učinkovita pri zdravljenju STEC okužb, je njihova proizvodnja drag in dolgotrajen proces (19). Proti okužbam s STEC se testirajo tudi različne majhne spojine, kot je LED 209 in divalentni cinkovi ioni, ki delujejota proti samim STEC bakterijam, tako da zmanjšajo adhezijo na celice in hkrati povzročijo zmanjšanje izražanja *stx* genov (16). Primer spojine proti STEC je tudi spremenjen baktericidni protein piocin, ki je dokazano učinkovit za selektivno ubijanje O157:H7 sevov, brez da bi pri tem sprožil povečano izločanja Stx (20).

Kljub znanim kontraindiciranim učinkom antibiotikov pri zdravljenju HUS, so v razvoju novejši načini uporabe različnih antibiotikov, ki imajo pozitiven učinek na zdravljenje STEC okužbe in ne povečajo verjetnosti razvoja HUS. Pri uporabi antibiotikov je pomembno, da se najprej natančno določi povzročitelja okužbe, izbere specifično protimikrobno sredstvo ali kombinacijo le-teh in določi ustrezno koncentracijo, ki dokazano ne vpliva na povečano izražanje Stx in izločanje le-tega iz celic (8). Antibiotiki so zlasti neprimerni za uporabo pri STEC sevih, ki imajo Stx kodirane na bakteriofagih, saj njihovo delovanje povzroči sprožitev litične faze fagov in s tem posledično povečano produkcijo Stx (9, 16). V primerih STEC sevov, pri katerih so geni za Stx kodirani kromosomalno, ali pa imajo okvarjeno delovanje prisotnih bakteriofagov, je antibiotično zdravljenje lahko učinkovito, saj ne povzroči povečanega izražanja Stx (16).

Med možnimi alternativni načini zdravljena okužb s STEC se omenjajo tudi naravni produkti, kot je mlečna kislina, različni rastlinski produkti, čaji ter ekstrakti, ki so se izkazali kot obetavni v *in vitro* testih ali na živalskih modelih (8). Za zdavljenje STEC okužb se preizkušajo tudi različne kombinacije dveh ali več opisanih pristopov, kot npr. uporaba kombinacije antibiotika tigeciklina in nevtralizirajočih protitels proti Stx1 in Stx2 (21), ali kombinacija izvlečka zelenega čaja in antibiotika levofloksacina (22).

Številne *in vitro* raziskave poročajo tudi o pozitivnih učinkih nekaterih probiotikov na zaviranje rasti STEC ali zaviranje produkcije Stx. Mednje spadajo *E. coli* Nissle 1917 (23, 24), *E. coli* 1307 (25), bifidobakterije (26-28) in različni sevi iz rodu *Lactobacillus* (29-33). Učinkovitost probiotikov je odvisna od uporabljenega seva in povzročitelja okužbe. Probiotiki delujejo predvsem kot preventivno sredstvo, kar pomeni, da morajo biti aplicirani pred samo okužbo s STEC ali v prvih dneh po okužbi, še preden pride do izločanja Stx.

Preglednica 1: Pristopi k zdravljenju okužb z bakterijami, ki tvorijo toksin šiga, v razvoju. Prirejeno po (8, 16).

Pristop/tarča	**Ime učinkovine**	**Opis in delovanje**
Zaviralec sinteze Gb_3	C-9	Zavira zgodnjo stopnjo sinteze Gb_3 receptorja, in sicer pretvorbo ceramida v glukozilceramid.
Analogi Stx receptorja	SYNSORB Pk	Silicijev dioksid, ki vsebuje trisaharidne komponente Gb_3, veže prosti Stx in s tem prepreči vezavo na Gb_3.
	STARFISH	Petvalentna sladkorna spojina, ki ima na vsaki roki 2 analoga receptorja, veže dve StxB molekuli hkrati in nevtralizira Stx1 in Stx2 ter zaščiti pred smrtjo Stx1-okužene miši.
	Daisy	Izboljšan divalentni trisaharidni analog Gb_3, zaščiti pred smrtjo Stx1- in Stx2-okužene miši.
	SUPER TWIG	Spojina iz 6 trisaharidov, ki blokira vezavo Stx1 in Stx2 na Vero celice.
	Gb_3 polimeri	Veže Stx z višjo afiniteto kot SUPER TWIG, zaradi večjega števila vezavnih mest.
	Probiotik *E. coli* CWG308:pJCP-Gb3	Gensko spremenjena bakterija *E. coli* s površinsko izraženim analogom receptorja Gb_3, ki nevtralizira Stx *in vitro* in *in vivo*.
	HuSAP	Protein iz plazme, ki nevtralizira Stx2, ne pa tudi Stx1.
Protitelesa proti toksinu	poliklonska antiStx2	Poliklonski antiserum proti Stx2, ki zaščiti gnotobiotičnih prašiče pred infekcijo s sevom O157:H7.
	cαStx1	Humanizirano monoklonsko protitelo proti Stx1B podenoti (13C4), nevtralizira toksin na Vero celicah in zaščiti miši, testiranje v I in II fazi klinične študije.
	cαStx2	Humanizirano monoklonsko protitelo proti Stx2A podenoti (11E10), nevtralizira toksin na Vero celicah in zaščiti miši, vpliva na znotrajcelični transport toksina in zaščiti celice pred inhibicijo sinteze proteinov, testiranje v I in II fazi klinične študije.
	anti-Stx1 (5-5B)	Nevtralizira Stx1 preko B podenote.
	Urtoxazumab (TMA-15)	Humanizirano monoklonsko protitelesa, nevtralizira Stx1 in Stx2, tako da prepreči vezavo na receptor, nevtralizira Vero celice in izkazuje zaščitno delovanje na živalskih modelih, trenutno v fazi III klinične študije.
	monoklonska antiStx1	Nevtralizira Stx1, večinoma preko B podenote.
	anti-Stx2 (5C12)	Humanizirano monoklonsko protitelo, ki nevtralizira Stx2, tako da vpliva na znotrajcelični transport toksina in zaščiti celice pred inhibicijo sinteze proteinov.
	mišji ScFv in človeški Fab proti Stx2	Rekombinantni fragmenti protiteles, ki prepoznajo in nevtralizirajo Stx2 *in vitro* (19).
Zaviralec komplementa	Ekulizumab	Anti C5 monoklonsko protitelo, ki blokira pot komplementa in s tem ovira STEC (dokazano učinkovit za zdravljenje atipičnega HUS) (34).
Spojine, ki vplivajo na transport, procesiranje in funkcijo toksina	TVP (Ac-PP-tet)	Tetravalentni peptidi, ki so sposobni nevtralizacije Stx v Vero celicah, tako da ovirajo prenos toksina v ER.
	MMA-tet	Tetravalentni peptidi, ki zavirajo Stx1 in Stx2 v Vero celicah, predvidoma preprečijo prenos A_1 podenote iz ER v citosol.
	Exo2	Vpliva na transport Stx v Golgijev aparat, tako da uniči Golgijev aparat, problem toksičnosti za celice.
	Retro-2, Retro-2^{cycl}	Blokira pot Stx v trans-Golgijevo mrežo.
	klorokin	Zavira izstop toksina iz ER.
	Eeyarestatin 1	Vpliva na znotrajcelični transport toksina in preprečuje zaviranje sinteze proteinov v HeLa celicah.
	nitrobenzil-tioinozin	Ovira transport Stx1 in ga zadrži v zgodnjih endosomih.
	mangan	Zavira znotrajcelični transport StxB, zaščiti miši pred Stx1, ne pa tudi pred Stx2.
	inhibitor furina	Zavira cepitev A podenote na A_1 in A_2, neučinkovit pri višjih koncentracijah toksina.

Nadaljevanje		
Pristop/tarča	**Ime učinkovine**	**Opis in delovanje**
Spojine proti STEC	piocin AVR2-V10	Spremenjen bakteriocidni protein, učinkovit le proti sevom O157:H7.
	LED 209	Zaviralec QseC senzorja, pomembnega za adhezijo EHEC in izražanje Stx2.
	Cink	Divalentni kation, ki zmanjša pritrditev STEC na HeLa celice in izražanje Stx genov.
Novi pristopi uporabe antibiotikov	ciprofloksacin, meropenem, kloramfenikol in fosfomicin	Uporaba terapevtskih koncentracij naštetih antibiotikov, dokazano ni prišlo do povečanega izločanja Stx pri STEC O104:H4 (35).
	fosfomicin	Uporaba v prvih petih dneh bolezni zmanjša nevarnost razvoja HUS pri otrocih (36).
	ciprofloksacin	Zmanjšanje nevarnosti razvoja HUS pri bolnikih, okuženih z O104:H4.
	azitromicin	Skrajšanje časa prisotnosti povzročitelja okužbe O104:H4 v obolelem.
	rifampicin in gentamicin	Uporaba določene koncentracije rifampicina, ki zniža produkcijo Stx pri STEC O157:H7, nato sledi aplikacija gentamicina, ki pobije bakterije.
	tigeciklin in antiStx1/2	Kombinacija antibiotika in nevtralizirajočih protiteles proti Stx1 in Stx2 omogoča popolno zaščito Vero celic pred Stx in okužbo s STEC (21).
Naravni produkti proti STEC	mlečna kislina	Produkt mlečnokislinskih bakterij.
	različni rastlinski produkti ali ekstrakti	Testirani *in vitro* in na živalskih modelih.
	zeleni čaj z levofloksacinom	Vzajemni učinek delovanja zelenega čaja in antibiotika.
	baikalin	Flavonoidna spojina, uporaba v kitajski medicini, izboljšava funkcije ledvic in zmanjšanje smrtnosti miši po dodatku Stx2 (37).

Gensko spremenjene mlečnokislinske bakterije (MKB) s površinsko predstavljenimi vezavnimi proteini, ki bi imele sposobnost vezave toksina šiga, bi lahko bile potencialno uporabne za zdravljenje okužb z STEC. Združujejo namreč več različnih terapevtskih pristopov, in sicer proizvodnjo mlečne kisline, ki zavira rast STEC, splošno probiotično delovanje MKB in uporabo specifičnih vezalcev toksina šiga na površini bakterij kot alternativo analogom Stx receptorja. Podoben pristop so uporabili pri razvoju rekombinantne probiotične bakterije *E. coli*, ki je s pomočjo spremenjene biosintezne poti oligosaharidov na površini izražala analog receptorja za toksin šiga (38, 39).

V nadaljevanju so predstavljene MKB kot primerni dostavni sistemi za terapevtske proteine in različni načini površinske predstavitve heterolognih proteinov na MKB.

Uporaba mlečnokislinskih bakterij v terapevtske namene

MKB so del prehrane ljudi že stoletja in danes poleg kvasovk predstavljajo industrijsko najpomembnejše mikroorganizme. Zaradi dolgotrajne in varne uporabe so pridobile status GRAS (splošno priznane kot varne). Poznane so tudi po svojem koristnem delovanju v prebavnem traktu, zato se vedno pogosteje uporabljajo kot probiotiki (živi organizmi, ki

zaužiti v zadostni količini ugodno vplivajo na zdravje gostitelja) v prehrani in za različne terapevtske namene (40). Zaradi teh ugodnih lastnosti so MKB postale pomembne tudi kot gostiteljski organizmi v biotehnologiji in medicini, kjer jih lahko uporabimo kot dostavne sisteme za različne biološko aktivne snovi. V ta namen so razvili tehnike transformacije, številne sisteme za kloniranje, pomnoževanje in izražanje genov v MKB ter različne sisteme za površinsko predstavitev (41-43). Kot gostiteljski organizem se v raziskavah največkrat uporablja modelna MKB *Lactococcus lactis* (*L. lactis*), ki preživi prehod skozi prebavni trakt in je zato uporabna za peroralno dostavo bioloških molekul v prebavni trakt. Napogosteje uporabljena seva sta *L. lactis* subsp. *cremoris* MG1363 (44) in *L. lactis* subsp. *lactis* IL1403 (45). Za uporabo pa so zanimivi tudi različni sevi iz rodu *Lactobacillus*, ki imajo kot naravni prebivalci prebavnega trakta sposobnost njegove kolonizacije, kar omogoča ciljano dostavo biološko aktivnih molekul v prebavni trakt (46).

Površinska predstavitev heterolognih proteinov na MKB

Tehnologija predstavitve heterolognih proteinov na površini bakterij postaja vse bolj pomembna na različnih področjih biotehnologije in mikrobiologije. Bakterije z izbranimi površinsko predstavljenimi proteini so uporabne kot biosenzorji, bioadsorbenti, biokatalizatorji, dostavni vektorji in kot peroralna cepiva. Prav tako jih lahko uporabimo za proizvodnjo protiteles, rešetanje peptidnih knjižnic in detekcijo mutacij (47-50).

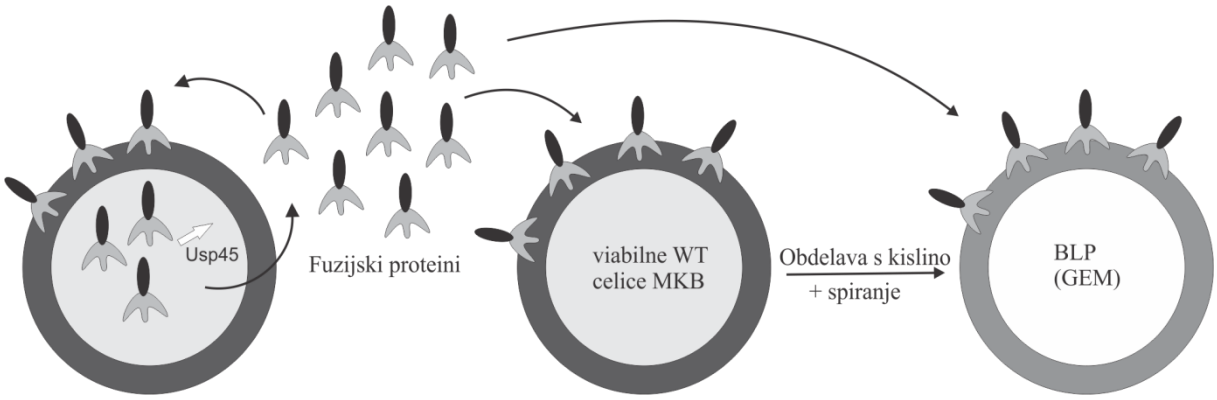
Predstavitev heterolognih proteinov na površini MKB se večinoma uporablja za pripravo cepiv (46, 51, 52), za dostavo vezavnih molekul v prebavni trakt (53), za sestavo makromolekularnih encimskih kompleksov (54) in bakterijsko imobilizacijo (55). Površinska predstavitev proteina (t. i. potniškega proteina) na bakterijah je omogočena s pomočjo fuzije s sidrnim proteinom, ki ga imenujemo tudi nosilni protein (47). Pri MKB je opisanih pet različnih vrst nosilnih proteinov: transmembranski proteini, lipoproteini, proteini z LPXTG motivom, proteini z lizinskimi motivi (LysM) in proteini površinskega (S) sloja (43). Pri modelni MKB *L. lactis* se kot nosilna proteina za površinsko predstavitev največkrat uporabljata del proteina M6 iz bakterije *Streptococcus pyogenes* z LPXTG sekvenco (54, 56, 57), ki se na peptidoglikan veže kovalentno, in C-končni del laktokoknega proteina AcmA (cA) z LysM ponovitvami, ki se veže nekovalentno (53, 58-60). Protein AcmA iz bakterije *L. lactis* subsp. *cremoris* MG1363 je avtolizin (N-acetilmuramidaza), ki ima pomembno vlogo pri celični delitvi bakterije *L. lactis* in je sestavljen iz encimske domene na N-koncu ter peptidoglikan-vezavne domene s tremi LysM ponovitvami na C-koncu (61-63). cA se uporablja kot fuzijski partner za nekovalentno pritrditev različnih heterolognih proteinov na

površino MKB v številnih raziskavah, kot so površinska predstavitev antigena hepatitisa A na bakteriji *L. lactis* (51), površinska predstavitev α-amilaze na bakterijah *Lb. plantarum* in *Lb. casei* (59), površinska predstavitev vezavnih proteinov za dejavnik tumorske nekroze alfa (TNF-α) na bakterijo *L. lactis* (53) in druge.

Kljub precejšnjemu številu razvitih sistemov za površinsko predstavitev na bakteriji *L. lactis,* je razvoj novih še vedno aktualen. Na podlagi raziskave površinskega proteoma *L. lactis* je bil identificiran bazični membranski protein A (BmpA) in predlagan kot potencialni nosilni protein za površinsko predstavitev IgG-vezavnih domen na bakteriji *L. lactis* (64). BmpA je lipoprotein z značilno lipoproteinsko sekvenco na N-koncu signalnega peptida, ki se po odcepitvi signalnega peptida s signalno peptidazo II pretvori v zrelo obliko in kovalentno pripne na lipidni dvosloj membrane s pomočjo procesa aminoacilacije (65). Sekvenca laktokoknega proteina BmpA ima 31 % identičnost s proteinom BmpA iz spirohete *Borrelia burgdorferi*, ki je imunodominanten protein z zmožnostjo vezave na laminin in je vključen v razvoj Lymske bolezni (66).

Heterologna površinska predstavitev na MKB:nerekombinanten pristop

Za terapevtske in prehrambne namene je zaradi stroge zakonodaje na področju gensko spremenjenih organizmov (GSO) in splošnega neodobravanaja GSO v Evropi zaželena uporaba nerekombinantnega pristopa površinske predstavitve na MKB. To lahko naredimo tako, da nekovalentno pripnemo rekombinantne proteine na površino živih gensko nespremenjenih MKB ali na nežive bakterijam podobne delce (BLP), prej poznane tudi kot delce GEM (Gram-positive enchancer matrix) (slika 2). Omenjen pristop lahko imenujemo tudi heterologna površinska predstavitev na bakterijah, saj na površini ene vrste bakterije predstavimo rekombinantne proteine, ki jih proizvede druga vrsta bakterije. Da lahko pripnemo rekombinantne proteine na površino bakterij, jih moramo pripraviti v fuziji z domenami, ki omogočajo nekovalento vezavo na celično steno bakterij. Različne domene, ki so sposobne nekovalentne vezave na celično steno bakterij, so opisane v PFAM podatkovni zbirki (67), in vključujejo domene Cpl-7, ChW, CW, LysM, PG 1-4, Sh3b, SLAP, SPOR in WxL (68). Za heterologno površinsko predstavitev na bakterijah se največkrat uporabljajo vezavne domene s ponovitvami LysM, ki se pripnejo na peptidoglikan na način neodvisen od vrste (63).



Slika 2: Shematični prikaz nekovalentne površinske predstavitve na mlečnokislinskih bakterijah. Na levi shemi rekombinantne bakterije tvorijo fuzijske proteine, ki se s pomočjo Usp45 signala izločajo iz celice, del pa se jih pripne na membrano. Izločene fuzijske proteine, ki ostajajo v gojišču, lahko uporabimo za heterologno površinsko predstavitev na ne-spremenjenih celicah MKB (sredina), ali na bakterijam podobnih delcih (BLP, imenovani tudi GEM delci; desno). GEM: Gram-positive enchancer matrix. Povzeto po (68).

C-konec laktokoknega proteina AcmA (cA) z LysM ponovitvami se uporablja kot nosilni protein za heterologno površinsko predstavitev različnih proteinov na številnih nerekombinantih MKB. Steen in sod. (63) so dokazali učinkovito vezavo malarijskega antigena (MSA2) v fuziji s cA na specifične dele površine bakterij *L. lactis, Lb. sake, Lb. buchneri, Lb. plantarum* in *Lb. casei*. V drugi študiji je bil na površini nerekombinantne bakterije *Lb. acidophilus* predstavljen plaščni protein virusa piščančje anemije v fuziji z cA, z namenom oralne vakcinacije piščancev (69). Za sidranje proteinov na površino nerekombinantih MKB so bili uporabljeni tudi drugi proteini, ki vsebujejo LysM ponovitve, kot je bakteriofagni endolizin Lyb5 (70) in muropeptidaza MurO iz *Lb. plantarum* (71). Pri različnih MKB so razvili tudi heterologno površinsko predstavitev s pomočjo fuzije s C-končnim delom proteina SlpB, ki je protein S-sloja bakterije *Lb. crispatus* K2-4-3 (72).

Tehnologijo heterologne površinske predstavitve na ne-viabilnih ali ne-živih MKB so razvili Bosma in sod. (73) na podlagi raziskav Steen in sod. (63), ki so odkrili, da obdelava živih celic MKB z vročo triklorocetno kislino močno poveča zmožnost vezave MSA2_cA fuzijskega proteina na površino obdelanih celic. Delce MKB, ki so nastali po enostavni obdelavi s trikloroocetno kislino in 30 min vrenjem, so najprej poimenovali delci GEM (Gram-positive enchancer matrix), kasneje pa bakterijam podobni delci (BLP) (73). Metoda priprave BLP delcev je primerna za uporabo na vseh MKB, čeprav se večinoma uporablja za laktokokne celice. V zadnjem času so bili razviti laktokokni delci BLP za pripravo različnih mukoznih cepiv proti virusom (gripa, respiratorni sincicijski virus in hepatitis B), bakterijam (*Streptococcus pnemoniae, Staphylococcus aureus, Yersinia pestis* in *Shigella flexneri*) in parazitom (*Plasmodium falciparum* in *Plasmodium berghei*) (pregledano v (74, 75)). Večina

naštetih cepiv je pripravljenih v fuziji z cA nosilnim proteinom, in so bila testirana v predkliničnih študijah. Kot učinkoviti delci za dostavo cepiva proti *Campylobacter jejuni* so se izkazali tudi BLP delci *Lb. salivarius* IBB3154 s površinsko predstavljenima antigenoma CjaA in CjaD v fuziji s cA (76). BLP delci so primerni za dostavo cepiv, saj izkazujejo imunostimulatorno aktivnost, lahko jih apliciramo skozi usta ali nos in omogočajo dostavo več različnih antigenov hkrati (75).

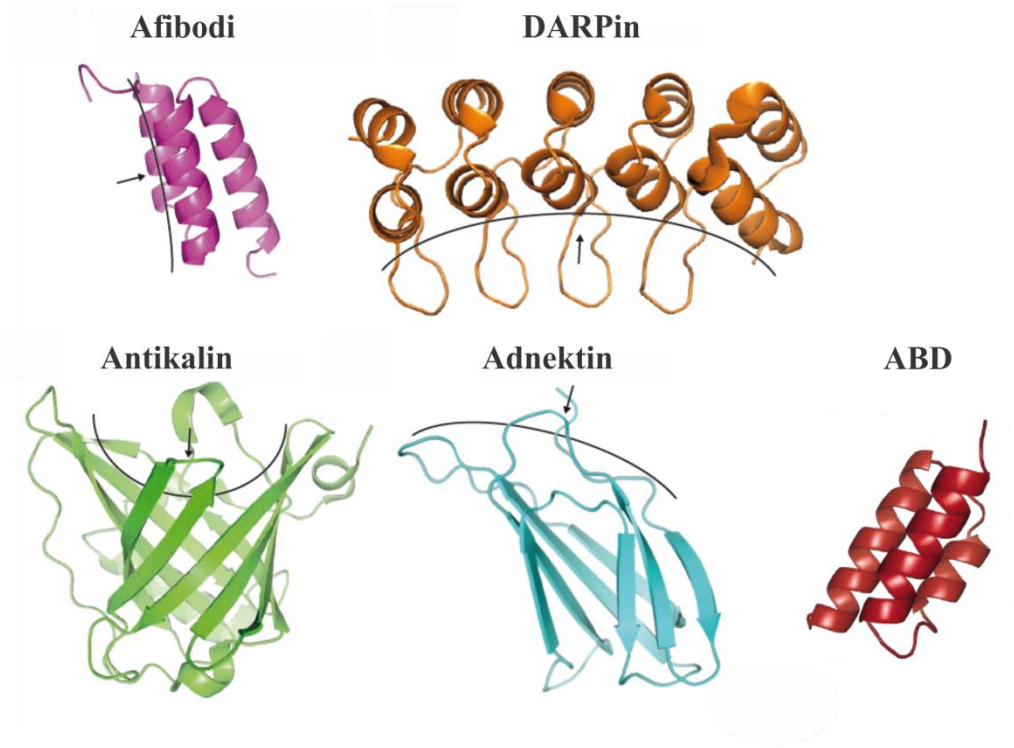
Optimizacija površinske predstavitve s cA nosilnim proteinom

Površina MKB je posejana z različnimi površinskimi strukturami, ki lahko vplivajo na uspešnost površinske predstavitve heterolognih proteinov v fuziji z cA nosilnim proteinom. Steen in sod. so pokazali, da je razporeditev fuzijskega proteina na površini bakterij odvisna od uporabljenega seva; bakterija *Lb casei* je vezala fuzijski protein le na celičnih polih, bakterija *Lb. sake* po celotni površini, medtem ko je bila vezava fuzijskega proteina zaradi prisotnosti proteinov S sloja pri bakteriji *Lb. helveticus* zelo šibka (63). Hkrati so dokazali, da se cA nosilni protein specifično veže na peptidoglikan, vendar je vezava ovirana na delih peptidoglikana, kjer so prisotne druge površinske strukture, kot je lipotehoična kislina (LTA). Po odstranitvi površinskih struktur s kislinsko obdelavo bakterijskih celic, pride do vezave fuzijskega proteina s cA nosilnim proteinom po celotni površini peptidoglikana. Obdelava bakterijskih celic s trikloroocetno kislino je eden od možnih načinov izboljšanja površinske predstavitve s cA nosilnim proteinom, pri katerem gre odstranitev motečih struktur na bakterijski površini.

Moteče strukture na bakterijski površini lahko spremenimo tudi z drugimi metodami, npr. z vnosom mutacij v dele genoma, ki so odgovorni za sintezo površinskih struktur, kot so LTA, teihoična kislina ali polisaharidi. Prav tako lahko pride do spremembe bakterijskega površinskega proteoma, če med gojenjem bakterij spremenimo rastne pogoje ali v gojišče vnesemo zunanje substance, ki lahko povzročijo stresni odziv na spremembe in s tem drugačen prepis genov. Med možnimi dodatki v gojišču, ki bi lahko vplivali na spremembo površinskega proteoma bakterij so tudi antibiotiki, ki lahko povzročijo značilne spremembe v prepisu genov že pri subinhibitornih koncentracijah in odziv na stres pri subletalnih koncentracijah (77). Eritromicin (78) in kloramfenikol (79) sta povzročila spremembo v transkripciji več kot 600 genov v bakteriji *Enterococcus faecalis*. Tudi obdelava bakterij *Lb. rhamnosus* z žolčem, ki ima prav tako protimikrobni učinek, je vplivala na prepis več kot 300 genov in povzročila spremembe v različnih funkcijah, povezanih s celično steno (80).

Vezavni proteini ne-imunoglobulinskega tipa

Na površini MKB lahko predstavimo različne heterologne proteine. V zadnjih letih je bilo razvitih več kot 20 različnih tipov majhnih enoverižnih vezavnih proteinov ne-imunoglobulinskega (ne-Ig) tipa proti več kot sto različnim tarčam (81). Ne-Ig vezavni proteini so majhni, stabilni, topni monomerni proteini, ki običajno nimajo disulfidnih vezi ali prostih cisteinov, ne potrebujejo post-translacijskih sprememb, se jih lahko enostavno in poceni proizvede v veliki količini v bakterijah, izkazujejo dobro penetracijo v tkiva, se v organizmu lahko hitro porazdelijo ali odstranijo, se lahko enostavno spremenijo s pomočjo konjugacije ali genske tehnologije in lahko izkazujejo večvalentnost ali večspecifičnost (81, 82). Zaradi teh lastnosti so postali pomembna alternativa protitelesom in so primerni potniški proteini za površinsko predstavitev na MKB. Vezavni proteini, ki temeljijo na ne-Ig proteinskem ogrodju, so potencialno široko uporabni v številnih terapevtskih in diagnostičnihe aplikacijah. Večinoma se uporabljajo za zdravljenje in diagnostiko raka ter protivnetnih bolezni, v bazični in aplikativni znanosti pa za določanje strukture proteinov, znotrajcelično detekcijo post-translacijskih sprememb, imunoafinitetno kromatografijo in regulacijo encimov (81). Med njimi so največkrat opisani in uporabljeni afibodiji (83), načrtovani proteini z ankirinskimi ponovitvami (DARPini) (84), antikalini (85, 86), adnektini (87) in albumin-vezavna domena (ABD) (88, 89), katerih strukture so prikazane na sliki 3. Večina vezavnih proteinov temelji na ne-Ig proteinskem ogrodju z znano strukturo, ki so ga s pomočjo naključnih mutacij aminokislin na površini proteinov spremenili v knjižnico različic vezavnih proteinov. Pri pripravi take knjižnice je pomembno, da se določijo primerna mesta za zamenjavo ali vstavljanje aminokisline, ki ne porušijo stabilnosti ali pravilnega zvitja proteina, pri tem pa mora biti število spremenjenih mest dovolj veliko, da se ustvari dovolj raznolika vezavna površina (90). Naključno spremenjena mesta so lahko prisotna na eni ali večih zankah proteinov (primer antikalini in adnektini) ali na bolj rigidnih sekundarnih strukturah, kot so α-vijačnice in β-ravnine (primer afibodiji, DARPini in ABD-ji) (91). Za selekcijo specifičnih vezavnih proteinov proti izbrani tarči iz pripravljenih proteinskih knjižnic se uporabljajo različni *in vitro* selekcijski sistemi, med njimi najpogosteje predstavitev na fagih, predstavitev na ribosomih, predstavitev na mRNA in predstavitev na bakterijah (kvasovkah ali *E. coli*) (90). V doktorskem delu smo za površinsko predstavitev na MKB kot potniška proteina uporabili dva različna vezavna proteina (ABD in DARPin), ki sta predstavljena v nadaljevanju.



Slika 3: Primeri alternativnih vezavnih proteinov in njihove strukture. Prirejeno po (90)

Albumin-vezavna domena (ABD)

Albumin-vezavna domena (ABD) je majhna proteinska domena zgrajena iz treh vijačnic, ki izvira iz streptokoknega proteina G in veže človeški serumski albumin (HSA) z nanomolarno afiniteto (92). ABD je po strukturi zelo podobna Z domeni, pridobljeni na osnovi spremenjenega zaporedja B domene stafilokoknega proteina A, ki je sposobna vezave imunoglobulinov preko Fc regije in predstavlja osnovo za vezavni protein afibodi (83). ABD je zaradi majhnosti in visoke stabilnosti primerno ogrodje za pripravo knjižnic različic ABD, ki lahko služijo za selekcijo vezavnih proteinov proti izbrani tarči. Kot vezavno ogrodje pri pripravi ABD knjižnice se največkrat uporablja 46 AK dolga, tretja albumin-vezavna domena proteina G iz bakterije *Streptococcus* G148 (88, 89, 93). Prva takšna knjižnica je bila pripravljena z naključnim spreminjanjem 15 AK na drugi in tretji vijačnici ABD in uspešno uporabljena za selekcijo novih ABD z izboljšano, femtomolarno afiniteto do HSA z uporabo predstavitve na fagih (89). Ahmad in sod. so z *in silico* metodami določili 11 mest na površini G148-ABD ogrodja, primernih za naključno spreminjanje, in pripravili ABD knjižnico s teoretično 10^{16} različicami, ki so imele površino za vezavo HSA zamenjano z novimi vezavnimi mesti (88). Pripravljeno knjižnico so uspešno uporabili za selekcijo visoko afinitetnih vezavnih proteinov človeškega interferona gama (IFN-γ) (88), človeškega receptorja za interlevkin 23 (IL-23) (94) in človeškega sekrecijskega proteina prostate 94

(PSP94), s pomočjo predstavitve na ribosomih (95). Pri pripravi tretje knjižnice so združili oba pristopa in konstruirali knjižnico bispecifičnih ABD tako, da so z naključnim spreminjanjem 11 AK na prvi in drugi vijačnici ABD vključili nova vezavna mesta in hkrati ohranili vezavna mesta za HSA (93). Knjižnico bispecifičnih ABD so uporabili za selekcijo vezavnih proteinov dimerne oblike proteina A (Z domene) z namenom čiščenja protiteles (93), vezavnih proteinov TNF-α (93) in vezavnih proteinov receptorja za epidermalni rastni faktor tipa 2 (96) ter tipa 3 (97) s pomočjo prestavitve na fagih.

Načrtovani proteini z ankirinskimi ponovitvami (DARPini)

DARPini so verjetno najbolj raziskani ne-Ig vezavni proteini. Imajo visoko afiniteto in specifičnost napram izbrani tarči, so majhni (14-21 kDa) in stabilni ter jih lahko proizvedemo v visokih količinah v bakteriji *E. coli* (98). Molekule DARPinov v knjižnici so sestavljene iz ankirinskih ponovitev; osrednji del tvori 33 AK dolg ankirinski modul, ki se običajno ponovi 2-4 krat in vsebuje 6-8 naključno spremenjenih mest na β-ravnini in prvi α-vijačnici ankirinske strukture, na N- in C- koncu pa je pripeta hidrofilna, tako imenovana pokrovna ponovitev (84, 99). Za selekcijo specifičnih DARPinov z visoko afiniteto se najpogosteje uporablja predstavitev na ribosomih in predstavitev na fagih (100). DARPini z visoko afiniteto (vse do pikomolarnega območja) so bili selekcionirani proti številnim tarčam za uporabo v strukturni biologiji, pri preusmerjanju virusov in v diagnostiki ter terapiji tumorjev (100, 101).

NAMEN DELA IN HIPOTEZE

Okužbe z bakterijami, ki tvorijo toksin šiga, povzročajo pri ljudeh različne črevesne bolezni, kot so driska in hemoragični kolitis, ki lahko vodijo v razvoj življensko nevarnega hemolitičnega uremičnega sindroma (HUS). Zaradi pomanjkanja učinkovitih načinov zdravljenja teh okužb je aktualen razvoj novih načinov zdravljenja. Glavni namen doktorske disertacije je bil razvoj gensko spremenjenih mlečnokislinskih bakterij (MKB) s površinsko predstavljenimi vezavnimi proteini, ki bodo imele sposobnost vezave toksina šiga. Da bi lahko pripravili take bakterije, je bil naš cilj pridobiti nove vezavne proteine proti rekombinantni podenoti B toksina šiga, jih ovrednotiti in nato uspešno predstaviti na površini MKB *L. lactis* ter dokazati njihovo učinkovitost.

Za predstavitev heterolognih proteinov na površini MKB so bili razviti različni sistemi površinske predstavitve, med katerimi se najpogosteje uporablja sistem nekovalente pritrditve s C-končnim delom laktokoknega proteina AcmA (cA). Kljub številnim razvitim sistemom za površinsko predstavitev na MKB sta še vedno zanimiva razvoj novih in optimizacija že obstoječih načinov predstavitve. V drugem in tretjem delu doktorske disertacije je bil naš namen optimizirati novejši sistem za površinsko predstavitev s pomočjo nosilnega proteina BmpA, ki smo ga identificirali kot potencialni nosilni protein za površinsko predstavitev na bakteriji *L. lactis* (64), in razvoj ter optimizacija nerekombinantne površinske predstavitve na izbranih sevih iz rodu *Lactobacillus* na osnovi obstoječega sistema z nosilnim proteinom cA. Hkrati je bil cilj ovrednotiti vpliv lipotehoične kisline (LTA) in antibiotikov kloramfenikol ter eritromicin na uspešnost površinske predstavitve s cA.

Zaradi stroge zakonodaje na področju gensko spremenjenih organizmov in splošnega neodobravanja javnosti je v prehrani in terapiji zaželena uporaba gensko nespremenjenih organizmov. V zadnjem delu doktorata je bil naš namen ovrednotiti in opisati možnosti heterologne nerekombinante površinske predstavitve in predstaviti njen regulatorni vidik.

V okviru doktorske disertacije smo postavili naslednje raziskovalne hipoteze:

- MKB s površinsko predstavljenimi vezavnimi proteini so uporabne za vezavo toksina šiga.
- Vezavni proteini DARPini in ABD-ji so primerni za površinsko predstavitev na mlečnokislinskih bakterijah.
- LTA na površini mlečnokislinskih bakterij vpliva na uspešnost predstavitve proteinov z nosilnim proteinom cA.
- Izbrani sevi iz rodu *Lactobacillus* so primerni za heterologno nerekombinantno površinsko predstavitev proteinov.

UPORABLJENE METODE

1. Tehnike genskega kloniranja
2. Mestno-specifična mutageneza
3. Določanje N-končnega zaporedja proteina
4. Adhezija na Caco-2 celice
5. Priprava kompetentnih celic *L. lactis* in elektroporacija
6. Izražanje proteinov na površini *L. lactis* z z nizinom nadzorovanim sistemom za izražanje proteinov (NICE)
7. Poliakrialamidna gelska elektroforeza ob prisotnosti natrijevega dodecil sulfata (SDS-PAGE) in prenos proteinov po Westernu (WB)
8. Encimsko imunski test (ELISA)
9. Pretočna citometrija
10. Konfokalna mikroskopija
11. Analize s površinsko plazmonsko resonanco
12. Izolacija proteinov z afinitetno kromatografijo z imobiliziranimi kovinskimi ioni (IMAC) ali tekočinsko kromatografijo za hitro ločevanje proteinov (FPLC) z uporabo HisTrap HP kolon
13. Analitska gelska filtracija
14. Selekcija vezavnih molekul s predstavitvijo na ribosomih
15. Določanje termodinamske stabilnosti proteinov s testom temperaturnega premika na podlagi fluorescence

PRVO POGLAVJE

Razvoj rekombinantnih bakterij *Lactococcus lactis* s površinsko predstavljenimi različicami albumin-vezavnih domen proti B podenoti toksina šiga 1

Development of recombinant *Lactococcus lactis* displaying albumin-binding domain variants against Shiga toxin 1 B subunit

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Poglavje napisano v obliki članka

POVZETEK

Okužbe z bakterijami, ki tvorijo toksin šiga, kot so enterohemoragične *Escherichia coli* in *Shigella dysenteriae,* predstavljajo resen zdravstveni problem. Učinkovito in specifično zdravljenje teh okužb ni na voljo, zato obstaja potreba po razvoju novih terapij. Rekombinantna prehransko sprejemljiva mlečnokislinska bakterija *Lactococcus lactis* je bila pripravljena za vezavo toksina šiga, s predstavitvijo novih načrtovanih albumin vezavnih domen (ABD) proti rekombinantni B podenoti toksina šiga (Stx1B) na njeni površini. Funkcionalna rekombinantna Stx1B je bila proizvedena v bakteriji *Escherichia coli* in uporabljena kot tarča za selekcijo 17 različic ABD (imenovanih S1B) iz knjižnice različic, pridobljene iz ABD ogrodja streptokoknega proteina G, z uporabo petih ciklov predstavitve na ribosomih. Tri najobetavnejše S1B različice (S1B9, S1B22 in S1B26) so bile podrobneje preučene z ELISA in površinsko plazmonsko resonanco. Konstanti afinitete do Stx1B sta pri različicah S1B22 in S1B26 znašali 0.70 oz. 1.00 μM. Vse tri ABD vezalce smo uspešno predstavili na površini bakterije *L. lactis* v obliki fuzije z Usp45 signalnim peptidom za izločanje in s peptidoglikan-vezavnim C koncem proteina AcmA. Vezava Stx1B z rekombinantnimi laktokoknimi celicami je bila potrjena z uporabo pretočne citometrije in ELISE s celimi celicami. Mlečnokislinske bakterije, pripravljene v tej študiji, so potencialno uporabne za odstranitev toksina šiga iz črevesja ljudi. Dodatno bi lahko nove Stx1B vezalce, pridobljene iz ABD ogrodja, uporabili v diagnostiki in bazičnih raziskavah okužb, povezanih s toksinom šiga.

Ključne besede:

toksin šiga, B podenota, albumin-vezavna domena, površinska predstavitev, *Lactococcus lactis*

Development of recombinant Lactococcus lactis displaying albumin-binding domain variants against Shiga toxin 1 B subunit

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Short title: Recombinant *Lactococcus lactis* displaying ABD variants against Stx1B

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Abstract

Infections with shiga toxin-producing bacteria, like enterohaemorragic Escherichia coli and Shigella dysenteriae, represent serious medical problem. No specific and effective treatment is available for patients with these infections, creating a need for the development of new therapies. Recombinant food-grade lactic acid bacterium *Lactococcus lactis* was engineered to bind shiga toxin by displaying novel designed albumin binding domains (ABD) against recombinant shiga toxin 1 B subunit (Stx1B) on their surface. Functional recombinant Stx1B was produced in *Escherichia coli* and used as a target for selection of 17 different variants of ABD (named S1B) from a library of variants derived from ABD scaffold of streptococcal protein G using five cycles of ribosome display. Three most promising S1Bs (S1B9, S1B22 and S1B26) were characterized into more details by ELISA and surface plasmon resonance. S1B22 and S1B26 had affinity constants for Stx1B of 0.70 and 1.00 μM, respectively. All three ABD binders were successfully displayed on the surface of *L. lactis* by fusion to the Usp45 secretion signal and to the peptidoglycan-binding C terminus of AcmA. Binding of Stx1B by engineered lactococcal cells was confirmed using flow cytometry and whole cell ELISA. Lactic acid bacteria prepared in this study are potentially useful for the removal of shiga toxin from human intestine. Additionally, novel ABD scaffold-derived Stx1B binders could be applied in diagnostics and basic research of shiga toxin-related infections.

Keywords

Shiga toxin, B subunit, engineered albumin binding domain, surface display, Lactococcus lactis

Introduction

Infections with shiga toxin (Stx)-producing bacteria, such as Stx-producing *Escherichia coli* (STEC) and *Shigella dysenteriae*, cause diarrhoea and haemorrhagic colitis in human, and may further develop into a life-threatening haemolytic uremic syndrome (HUS), characterized by microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure (1, 2). Shiga toxin, the major virulence factor of STEC, may be produced as Stx1 variants (Stx1 and Stx1c), Stx2 variants (Stx2, Stx2c, Stx2d, Stx2e, Stx2f) or both variants in different combinations (3). Stx produced by *S. dysenteriae* is almost identical to Stx1 produced by *E. coli*. All members of Stx family are composed of monomeric enzymatically active A subunit (StxA), which is non-covalently attached to pentameric B subunit (StxB). The latter is

responsible for binding to globotriaosylceramide receptor (Gb_3) on the cell surface, or to globotetraosylceramide (Gb_4) receptor in the case of Stx2e (3).

Human infection with STEC is mainly associated with ingestion of contaminated food, and can quickly spread among people causing massive outbreaks of STEC diseases. After ingestion, STEC pass through gastrointestinal (GI) tract, where they colonize the lower GI tract and release Stx into the gut lumen (1). Stx is absorbed by intestinal epithelium into the blood circulation and targets tissues expressing Gb_3 receptor, like kidney, brain, liver, pancreas, heart and hemopoetic cells (4). Once bound, toxin is very efficiently internalized into the cells by endocytosis and transported through retrograde pathway, whereby active A subunit is cleaved by furin, translocated to cytosol and functions as highly specific N-glycosidase, inhibiting protein synthesis and causing cell death (5, 6).

Presently, there is no specific treatment for patients with developed HUS. Recommended management relies on supportive therapy, that includes fluid and electrolyte balance, nutritional support, management of hypertension and renal transplantation (4). Because the conventional antibiotic treatment of STEC infections increases the risk of developing HUS by induction of Stx expression and toxin release into the gut, there is a need for the development of new therapies. Various potential therapeutic strategies are currently under development, and include compounds directed against STEC, Stx receptor analogs, receptor synthesis inhibitors, antitoxin antibodies, inhibitors of toxin transport, processing and function, natural products and also novel antimicrobial therapies (reviewed in (7, 8)). Monoclonal antibodies against Stx1 and Stx2 are currently the farthest in development (7).

As an alternative to conventional monoclonal antibodies, more than 20 different types of small single-domain non-immunoglobulin (non-IgG) binding proteins against more than hundred different targets have been introduced in recent years (9). Compared to antibodies, engineered non-IgG scaffolds are stable, robust and soluble monomeric proteins, which lack disulphide bonds, are easily and inexpensively produced in large amounts in bacteria, exhibit efficient tissue penetration, rapid distribution and elimination, can be easily modified via conjugation or gene fusion technology and can exert multivalency or multispecificity (9, 10). Among them, a highly complex combinatorial library, derived from three-helix bundle of albumin-binding domain (ABD) scaffold of streptococcal protein G has been successfully used for the selection of human interferon gamma binders (11), interleukin (IL)-23 receptor antagonist (12) and binders of human prostate secretory protein 94 (PSP94) (13). In the present research we applied ABD scaffold library for the selection of high affinity binders against Stx1B.

Probiotics, including *E. coli* Nissle 1917 (14), *E. coli* 1307 (15), and several *Lactobacillus strains* (16) were reported as efficient inhibitors of growth of STEC. Lactic acid bacteria (LAB) are often used as probiotics and are, due to their safety, also considered for genetic engineering and delivery of therapeutic proteins to the human intestine. We have previously demonstrated effective display of two non-Ig scaffolds, Affibodies (17) and DARPins (18), on the surface of recombinant or nonrecombinant lactic acid bacteria (LAB), by using C terminal part of the lactococcal AcmA protein (cA) containing lysine motif (LysM) domain as the cell wall anchor (19). Engineered probiotic LAB with surface displayed Stx-binding protein could be a promising candidate for the treatment of infections caused by STEC or *S. dysenteriae*. Similar approach was already used in the development of recombinant *E. coli* bacteria with engineered oligosaccharide biosynthesis pathway that resulted in the production of Stx receptor mimic on the bacterial surface (20, 21).

The goal of the present study was to engineer recombinant LAB *Lactococcus lactis* capable of binding Stx1B, by displaying binding proteins against Stx1B on the surface of *L. lactis*. We have chosen StxB as a target for selection of binding proteins against Stx, because it is capable for binding to the receptor and is not toxic by itself. In the first part of study we expressed, purified and characterized recombinant Stx1B and in the second part we focused on development of binding proteins against Stx1B, derived from ABD scaffold, their characterization and functional display on the surface of *L. lactis*.

Materials and Methods

Bacterial strains, media and culture conditions

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

Preparation of recombinant Stx1B subunit

Gene for Stx1B was designed (Table 1), de novo synthesized by ATG Biosynthetics (Merzhausen, Germany) and cloned to plasmid pET28b using NcoI/XhoI restriction sites, yielding pET28-Stx1B. Overnight culture of *E. coli* BL21 (DE3) harbouring plasmid pET28-Stx1B was diluted (1:100) in 1 L of fresh LB medium and grown to optical density A_{600}= 3.5-4.0. Expression of fusion protein Stx1B with hexa-histidine (his_6) tag was induced with

addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 28 °C. Culture was centrifuged at 5000 × g for 15 min and pellet was resuspended in 30 mL of equilibration/wash (Eq/W) buffer (50 mM NaH2PO4, 300 mM NaCl, pH 7.0). The cells were lysed with a cycle of freezing and thawing, and with 3 times 5 min sonication with UPS200S sonifier (Hielscher, Teltow, Germany). After cell lysis, the suspension was centrifuged at 15000 × g for 20 min and supernatant was stored. Inclusion bodies were dissolved in Eq/W buffers with increasing concentrations of guanidinium HCl (1M, 3M and 6M) for 6 h or overnight at 4 °C, followed in each step by centrifugation and supernatant removal. Stx1B-his_6 soluble in Eq/W with 6 M guanidinium HCl was isolated with BD Talon metal affinity resin (BD Biosciences) according to manufacturer's instructions, using batch/gravity-flow column purification and imidazole elution (elution buffer: 45 mM NaH2PO4, 270 mM NaCl, 5.4 M guanidinium HCl, 150 mM imidazole, pH 7.0). Fractions containing pure Stx1B were pooled and stored. We screened different refolding conditions according to (22-24). Recombinant Stx1B was efficiently refolded by 100-fold rapid dilution method in solubilisation buffer (50 mM Tris-HCl with 0.5 M arginine and 0.01 % Brij-35, pH 7.5).

Table 1. Strains, plasmids, gene and primers used in this study

Strain, plasmid, gene or primer	**Relevant features or sequence (5' – 3')**	**Reference**
Strains		
E. coli		
DH5α	endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR F- Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–	Invitrogen
BL21 (DE3)	F– ompT gal dcm lon hsdSB (rB- mB-) λ(DE3)	Novagen
BL21 (DE3) BirA	BL21 (DE3) with biotin ligase gene	
L. lactis NZ9000	MG1363 nisRK ΔpepN	NIZO
Plasmids		
pET28b(+)	Kanr, *E. coli* expression vector	Novagen
pNZ8148	pSH71 derivative, PnisA, Cmr, nisin-controlled expression	NIZO
pSDLBA3b	pNZ8148 containing gene fusion of spUsp-LEIS, b-dom and cA	(17)
pET28-Stx1B	pET28b containing Stx1B gene	This work
pET28-TolA-Avi	pET28b containing tolA gene with AviTag on C-terminus	
pET28-S1Bx-TolA-Avi	pET28b containing gene fusion of different variants of S1B clones with TolA and AviTag	This work
pET28-ABDwt-TolA-Avi	pET28b containing gene fusion of ABDwt with TolA and AviTag	
pSD-S1B9	pNZ8148 containing gene fusion of Usp45 signal peptide, S1B9 and cA	This work
pSD-S1B22	pNZ8148 containing gene fusion of Usp45 signal peptide, S1B22 and cA	This work
pSD-S1B26	pNZ8148 containing gene fusion of Usp45 signal peptide, S1B26 and cA	This work
pSD-ABDwt	pNZ8148 containing gene fusion of Usp45 signal peptide, ABDwt and cA	This work
pSD-His_6ABDwt	pNZ8148 containing gene fusion of Usp45 signal peptide, His_6 tag, ABDwt and cA	This work
Gene		
Stx1B	CCATGGCAAAAAAAACATTATTAATAGCTGCATCGCTTTCATTTTTTTCAGCA AGTGCGCTGGCGACGCCTGATTGTGTAACTGGAAAGGTGGAGTATACAAAAT ATAATGATGACGATACCTTTACAGTTAAAGTGGGTGATAAAGAATTATTTAC CAACAGATGGAATCTTCAGTCTCTTCTTCTCAGTGCGCAAATTACGGGGATG ACTGTAACCATTAAAACTAATGCCTGTCATAATGGAGGGGGATTCAGCGAAG TTATTTTTCGTCTCGAG	This work
Primers		
ABD-F	GGATCCCTGGCGGAAGCTAAAGTC	This work
S1B9-R	GAATTCAGGTAACGGAGCTAAAATACGATCTATC	This work
S1B22-R	GAATTCAGGTAAACGAGCTAAAATAGCATCTATC	This work
S1B26-R	GAATTCAGGTAACGCAGCTAAAATCCAATC	This work
ABDwt-R	GAATTCAGGTAATGCAGCTAAAATTTCATCTATC	This work
ABDhis_6-F	AGGATCCCATCACCATCACCATCAC	This work

Determination of molecular weight of Stx1B subunit

Molecular weight and oligomerization status of Stx1B was determined with analytical gel filtration chromatography (1.2 × 60 cm, 7 mL/h flow rate with 15 min fraction collection time) on a polyacrylamide gel Bio-Gel P-100 (Bio Rad, Hercules, USA) due to Stx1B cross-reaction with Superdex column (GE Healthcare) for size exclusion chromatography. A mixture of six proteins of 14.4-97 kDa (Amersham Low molecular weight Calibration Kit, GE Healthcare) was used as standards.

***In vitro* binding of Stx1B to globotriaosylceramide (Gb_3) receptor**

Binding of Stx1B to its natural receptor Gb_3 was determined by enzyme-linked immunosorbent assay (Gb_3 ELISA) as described previously (25). Receptor Gb_3 was purchased from Matreya LLC (PA, USA) and dissolved in chloroform/methanol (2:1). 100 µL of Gb_3 solution with concentration 10 µg/mL was used to coat Nunc PolySorp Strips (Thermo Fisher Scientific) overnight in laminar flow to evaporate chloroform/methanol. After washing with phosphate buffer saline with 0.05 % Tween 20 (PBST) and blocking with 2 % bovine serum albumin (BSA) in PBST, 100 µL of serial twofold dilutions of recombinant Stx1B in triplicate (starting with 1 µg/mL in 0.2 % BSA in PBST) were added to the wells and incubated for 1 h. Bound Stx1B-his_6 was detected with primary THE^{TM} His tag Antibody (GenScript, NJ, USA) (dilution 1:2000 in 0.2 % BSA in PBST) and with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Merck Millipore, Darmstadt, Germany) (dilution 1:5000 in 0.2 % BSA in PBST). The colour was developed with the addition of 100 µL substrate buffer (150 mM Na2HPO4, 50 mM citric acid, pH 6.0) and 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, MO, USA) for 15 minutes at room temperature. Reaction was terminated by the addition of 50 µL 2 M H_2SO_4 and absorbances were read at 450 nm using Infinite M1000 (Tecan, Salzburg, Austria). No Stx1B was added to 0.2 % BSA in PBST in control wells (zero concentration), while all the other steps were performed as described above.

Internalization of recombinant Stx1B into HeLa cells

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1 % GlutaMAX and pen-strep in 24-wells plates on coverslips. Fluorescein isothiocyanate (FITC)-labelled Stx1B (10 µg/mL in 300 µL of fresh medium) was added to the cells and incubated for 1 hour on 37 °C. Afterwards the cells were washed with PBS, fixed with 4 % paraformaldehyde in PBS for 15 min and permeabilized with 0.1 % triton X-100 in PBS for 6 min. Non-specific staining was blocked with 3 % BSA

in PBS for 30 min. Golgi apparatus was labelled with mouse monoclonal anti-human Golgin97 primary antibody (0.4 μg/ml in 3 % BSA for 1 h, Life Technologies) and with Alexa Fluor 555-conjugated donkey anti-mouse secondary antibody (1:1000 in 3 % BSA for 1 h, A-31570, Life technologies). Coverslips were mounted with ProlongGold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Cells were observed with Carl Zeiss LSM 710 confocal microscope.

Selection of Stx1B binders by ribosome display

Combinatorial DNA library of ABD protein was generated as described previously (11, 12). For the selection of binders five rounds of ribosome display were performed according to (12) with a few modifications as described by (13). After final round of selection, PCR product was cloned to pET28-TolA-Avi plasmid (Table 1) via NcoI/BamHI sites, yielding pET28-S1B-TolA-Avi. Resulting plasmid mixture was transformed into *E. coli* DH5α and plasmid DNA from randomly picked colonies was sequenced.

Sequence analysis and clustering of selected S1B binders

DNA sequencing of S1B variants was carried out by the Centre for DNA Sequencing of Institute of Microbiology of the ASCR, v.v.i. (Prague, Czech Republic) or GATC Biotech (Constance, Germany). Amino acid sequences of selected clones were aligned and similarity tree was constructed using Molecular Evolutionary Genetics Analysis tool (MEGA), version 6.0.6 (http://www.megasoftware.net/). Randomized sequences between residues 20 and 46 were compared, as the N-terminal amino acid positions 1–19 were non-randomized.

ELISA screening of Stx1B binders

S1B clones with unique (previously unreported) sequences were transformed into *E. coli* BL21 (DE3) BirA (Table 1) and expressed in 2.5 mL cultures with the addition of 1 mM IPTG and 50 μM D-biotin for 4 hours. Bacteria were pelleted at 15000 × g for 5 min and resuspended in 0.01 % PBST with 200 μg/mL lysozyme. Cell lysis was performed with three cycles of freezing at -80 °C and thawing at 37 °C for 30 min. Cell lysate was centrifuged for 15 min and 50 μL of supernatant was loaded to PolySorp microtiter plate (Nunc), previously coated with 10 μg/mL of recombinant Stx1B and blocked with 1 % BSA (Carl Roth GmbH, Karlsruhe, Germany). After 1 h of incubation at RT, the plate was washed five times with PBST, and ABD-binders were detected with HRP-conjugated streptavidin (1:5000 in 1 % BSA/PBST). The colour was developed with the addition of 0.5 mg/mL o-phenylenediamine (OPD, Sigma-Aldrich, St. Louis, USA) and 0.01 % H_2O_2 in 0.1 M citrate buffer (pH 5.0) for 5

min. The reaction was stopped with the addition of 2 M H_2SO_4 and absorbance was read at 492 nm. Lysate containing the wild-type albumin binding domain (ABDwt) in fusion with TolA protein was loaded to wells coated with 10 μg/mL of human serum albumin (HSA, Abcam, Cambridge, UK) to serve as a positive control. Negative background was recorded in wells loaded with 1 % BSA/ PBST without S1B-TolA lysate.

Expression and purification of selected S1B-TolA-Avi variants

Selected S1B-TolA-Avi variants were expressed in 200 mL cultures as described above. Cells were harvested by centrifugation at 5000 × g for 20 min, resuspended in 10 mL binding buffer (20 mM Na-phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4) and lysed by sonication for 3 min. Lysates were centrifuged at 10000 × g for 20 min and supernatants were kept. Purification of fusion proteins was performed by fast protein liquid chromatography (Äkta Purifier, GE Healthcare) using 1 mL HisTrap HP columns (GE Healthcare) or BD Talon metal affinity resin (BD Biosciences, Palo Alto, USA) using batch/gravity-flow column purification and imidazole elution according to manufacturer instructions. Eluted fractions were analysed by SDS-PAGE, pooled and concentrated by ultrafiltration using Amicon Ultra (Merck Millipore; Darmstadt, Germany). Purified S1B-TolA-Avi fusion proteins were dialysed against PBS and further used for ELISA tests and SPR analysis.

Binding of S1B variants to Stx1B

Microtiter plate was coated overnight at 4 °C with 10 μg/mL of recombinant Stx1B in coating buffer (100 mM Na-bicarbonate/carbonate, pH 9.6). Wells were washed with PBST and blocked with 1 % BSA in PBST for 2 h at RT. Serially diluted S1B-TolA-Avi fusion proteins or ABDwt control in 1 % BSA/PBST were loaded for 1 h. Following washing, bound S1B clones were detected with the addition of HRP-conjugated streptavidin (dilution 1:5000 in 1 % BSA/PBST). The colour was developed with 0.5 mg/mL OPD and 0.01 % H2O2 in 0.1 M citrate buffer (pH 5.0) for 5 min. The reaction was stopped with the addition of 2 M H_2SO_4 and absorbance was read at 492 nm.

Surface plasmon resonance (SPR)

SPR measurements were performed with Biacore T100 (GE Healthcare) at 25 °C. In the first setup we tested binding of S1B variants prepared in fusion with TolA spacer protein and AviTag to Stx1B captured on a Series S sensor chip CM5 (Biacore, GE Healthcare). CM5 sensor chip was activated using Amine coupling Kit (GE Healthcare) according to manufacturer's instructions. For immobilisation of Stx1B standard PBS buffer (137 mM

NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was used as running buffer. Stx1B was diluted into 10 mM sodium acetate buffer, pH 5.0, to a final concentration of 20 μg/mL, and injected over the second flow cell. The final immobilization level was approximately 890 response units (RU). The first flow cell was empty and served as a reference cell to control the level of the non-specific binding. All experiments were performed with running buffer PBS with 300 mM NaCl (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 0.005 % P20). Each compound was injected for 2 min at a flow-rate 20 μL/min, and the dissociation was monitored for 3 min. Regeneration of the sensor surface was achieved with two 30 s pulses of 50 mM NaOH. We used the following concentrations for titration: 0, 15.625, 31.25, 62.5, 125, 250, 500, 1000, 2000 nM and concentration 62.5 nM was repeated at the end of the concentration series.

In reverse setup we immobilised S1B22-TolA-Avi or S1B26-TolA-Avi variants on a sensor chip CM5. CM5 sensor chip activation and immobilization of S1B-TolA-Avi variants was performed as described above, except two injections were needed to rich the final immobilization level of approximately 1100 RU. All experiments were performed with running buffer PBS with 300 mM NaCl (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 0.005 % P20). Each compound was injected for 1 min at a flow-rate of 10 μL/min, and the dissociation was monitored for 30 s. Regeneration of the sensor surface was achieved with 25 mM NaOH for 120 s. We used the following concentrations for titration: 62.5, 125, 250, 500, 1000, 2000, 4000 nM and concentration 500 nM was repeated at the end of the concentration series. We evaluated the obtained data using Biacore T100 Evaluation software. The sensorgrams were reference and blank subtracted and we applied Steady State Affinity model to calculate the affinity constant.

Fluorescence-based thermal shift assay (TSA)

Thermal stability of S1B proteins was tested in PBS and 50 mM Tris, 300 mM NaCl, pH 8.0 (T50N300) buffers with the addition of 5× Sypro Orange dye (Sigma-Aldrich, St. Luis, USA) to the 25 μL total volume. TSA was performed with real-time PCR Detection System CFX touch (BIO-Rad Laboratories, Hercules, USA) according to (12). The data analysis was done by CFX Manager Software and the melting temperatures (Tm) were determined using the first derivative spectra.

Molecular cloning and expression of S1B-cA variants in *L. lactis*

Genes of S1B variants without tolA spacer were amplified with Phusion polymerase (New England Biolabs, Beverly, MA) using pET28-S1Bx-TolA-Avi template and ABD-F primer

and corresponding S1B-R primer (Table 1). Gene for ABDwt was amplified using pET28-ABDwt-TolA-Avi template and ABD-F/ABDwt-R (Table 1) primer pair, while his_6 tag was added with ABDhis_6-F/ABDwt-R primer. All PCR fragments were ligated to pJET 1.2 cloning vector and cloned into plasmid pSDLBA3b, using BamHI and EcoRI restriction enzymes (New England Biolabs). Plasmid DNA was isolated with NucleoSpin plasmid kit (Macherey and Nagel, Düren, Germany), with an additional lysozyme treatment for *L. lactis*. Lactococci were transformed by electroporation using Gene Pulser II apparatus (Bio-Rad, Hercules, CA) according to MoBiTec GmbH instructions (Goettingen, Germany). Plasmids constructed in the study are listed in Table 1. Expression of S1B-cA variants in *L. lactis* NZ9000 was performed in 10 mL cultures. Bacterial suspensions were grown to an A_{600} of 0.8, followed by induction with 25 ng/mL nisin (Fluka) for 3 hours (17, 18, 26, 27). Resulting suspensions were stored at 4 °C for flow cytometric analysis or whole cell ELISA test.

Flow cytometry and whole cell ELISA

For flow cytometric analyses, 10 µL of *L. lactis* suspensions were added to Tris-buffered saline (TBS) and centrifuged. Cells were resuspended in TBS with Alexa 488-labelled recombinant Stx1B (20 µg/mL) prepared using Alexa Fluor 488 Microscale Protein Labelling Kit (Thermo Fischer Scientific) and incubated at RT with constant shaking at 100 rpm. All washings, centrifugations steps and measurements were performed as described in (18, 28).

The whole cell ELISA was carried out according to (29) with a few modifications. *L. lactis* suspension with surface-displayed S1B variant or ABDwt was centrifuged and resuspended in PBS to an A600 of 1.0. 750 µL of the suspensions were washed twice in PBS and incubated with 500 µL of recombinant Stx1B (20 µg/mL in PBS) for 2 hours, followed by additional washing with PBS. Bound toxin subunit was detected with the addition of 200 µL of Anti-Verotoxin I/SLT 1b (antiStx1B) primary antibody (diluted 1:20 in PBS, Abcam, Cambridge, UK) for 1 hour, followed by washing and incubation with 200 µL of (HRP)-conjugated Goat anti-mouse IgG secondary antibody (diluted 1:500 in PBS, Merck Millipore, Darmstadt, Germany). All incubations were performed at RT in tube rotator. After incubation with secondary antibody, cells were first washed with PBS and then with substrate buffer (150 mM Na_2HPO_4, 50 mM citric acid, pH 6.0). Cells were finally resuspended in 1 mL of substrate buffer, and 100 µL of suspensions, or 1:5 dilutions in substrate buffer, were loaded on a microtiter plate. The colour was developed with the addition of 100 µL TMB substrate and the reaction was stopped after 15 min with the addition of 50 µL of 2 M sulphuric acid. Absorbances were read at 450 nm.

Statistical analyses

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

Results

Production of recombinant Stx1B

Recombinant shiga toxin 1B subunit (Stx1B) fusion protein with hexa histidine (his_6) tag was produced as a target for selection of high affinity binders derived from ABD scaffold using ribosome display. The gene for Stx1B was synthesized and cloned to pET28b vector. Different expression conditions (growth at 37°C, 30°C and 25°C, induction at optical densities 0.5, 1, 2 and 3.5-4.0) were tested (data not shown). The highest total amount of Stx1B-his_6 expression was achieved by growing the bacteria at 37 °C to optical density 3.5-4.0, followed by induction with 1 mM IPTG for 3 h at 28 °C. Majority of the fusion protein was produced in the form of inclusion bodies, which were dissolved in 6 M guanidinium HCl and efficiently purified with immobilized metal affinity chromatography (IMAC) (data not shown). We screened different refolding conditions and Stx1B-his_6 was effectively solubilized with rapid dilution method by using 0.5 M arginine and 0.01 % Brij-35-containing 50 mM Tris buffer. Solubilized product was stored in Tris or PBS buffer for characterization.

Characterization of recombinant Stx1B

The molecular weight of recombinant Stx1B-his_6 was determined by analytical gel filtration chromatography (data not shown). The recombinant Stx1B was eluted from calibrated column as one sharp symmetrical peak, with molecular weight that corresponded to pentameric form of recombinant Stx1B.

Functionality of recombinant Stx1B was tested by its ability to bind to immobilised globotriosyl ceramide (Gb_3) receptor *in vitro*, and by its internalization into HeLa cells, which naturally contain Gb_3 on the surface. Strong, specific and concentration depended binding of recombinant Stx1B to immobilized Gb_3 receptor was demonstrated with ELISA (Fig. 1A). In HeLa cells, 1 h after co-incubation with Stx1B the latter was internalized into the cells and was co-localized with the Golgi marker Golgin 97 (Fig. 1B).

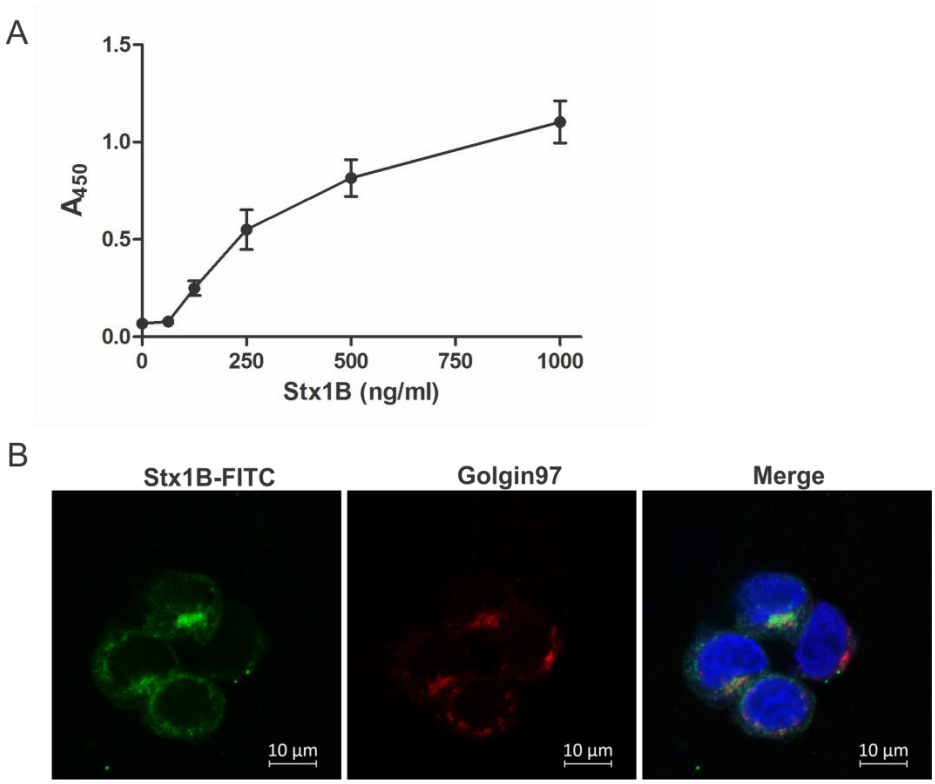


Figure 1 Ability of recombinant Stx1B to bind to Gb_3 receptor *in vitro*. A: Binding of serially diluted Stx1B to Gb_3 as determined by ELISA using THE^{TM} His tag antibody. A_{450}: Absorbance at 450 nm. Error bars denote standard deviations. B: Internalization of FITC-labelled Stx1B into HeLa cells. Golgi was detected with mouse anti-human Golgin97 primary antibody and Alexa Fluor 555-conjugated donkey anti-mouse secondary antibody (red). DAPI staining was used to label nuclei (blue).

Ribosome display selection of Stx1B binders

To select unique binders of Stx1B we used high-complex combinatorial ABD library (11-13). Five cycles of ribosome display selection was performed, yielding collection of 17 unique Stx1B-binders designated as S1B binders (Fig. 2A). Identified S1B binders were produced in *E. coli* as fusion proteins with TolA spacer containing AviTag on C-terminal and his_6 tag on N-terminal (S1B-TolA-Avi). Expression level of corresponding fusion protein was tested by SDS PAGE and western blot analysis (data not shown) and binding affinity was verified by ELISA (Fig. 2B). ELISA-positive cell lysates with appropriate expression level of S1B binders were selected for further analysis.

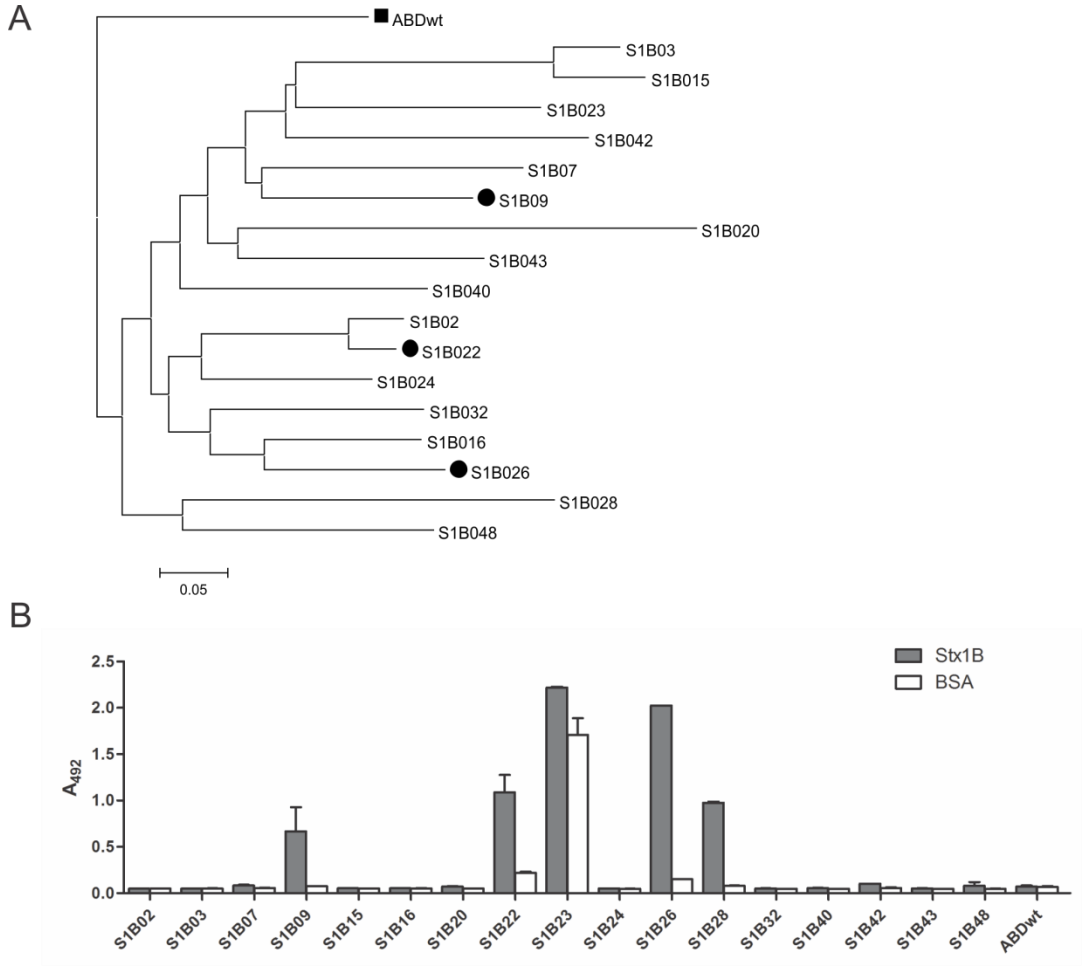
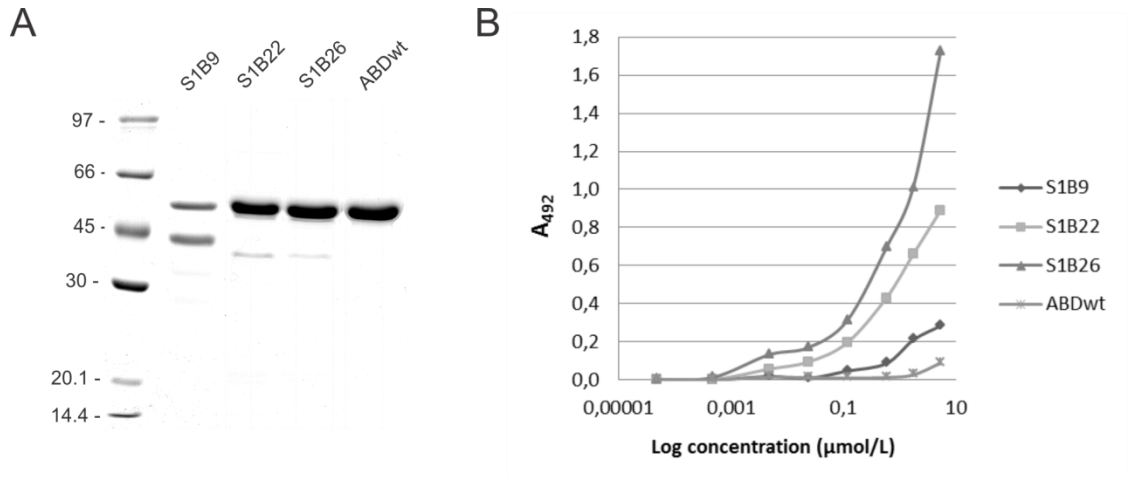


Figure 2. Sequence similarity analysis (A) and binding affinity (B) of 17 S1B binders selected after five rounds of ribosome display. A: The sequence of the parental ABD wild-type domain was used as a root of the tree and is highlighted as square, while S1B variants selected for more detailed analysis are highlighted as circles. B: S1B binders-containing cell lysates were incubated with immobilized Stx1B (grey bars) or BSA (white bars) and detected with HRP-conjugated streptavidin. Error bars denote standard deviations.

Production and characterization of selected S1B binders

Binders S1B9, S1B22 and S1B26 with TolA-Avi fusion were selected for more detailed analysis and were produced in *E. coli* in large scale. Binders were purified with IMAC or HisTrap FPLC and verified on SDS-PAGE electrophoresis (Fig. 3A). Stx1B was immobilized and incubated with serial dilutions of selected S1B binders, detected with HRP-streptavidin. Gradual decrease of the ELISA signal confirmed binding of S1B binders to recombinant Stx1B (Fig. 3B). This was further substantiated by sandwich ELISA in which selected S1B binders were immobilized on microtiter plate and binding of Stx1B was detected with antiStx1B antibody followed by a secondary anti-mouse-HRP conjugate (data not shown). Sequence similarity comparison of selected binders S1B9, S1B22 and S1B26 with parental nonmutated ABDwt is presented in Fig. 3C.

Based on ELISA tests and SDS PAGE analysis clones S1B22 and S1B26 were selected for further characterization of binding with surface plasmon resonance (SPR), and of stability with thermal shift assay (TSA).



C

	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
ABDwt	Y	Y	K	N	L	I	N	N	A	K	T	V	E	G	V	K	A	L	I	D	E	I	L	A	A	L	P
S1B9	M	Y	K	N	V	I	N	M	A	A	T	V	R	H	V	K	W	R	I	D	R	I	L	A	P	L	P
S1B22	W	Y	K	N	V	I	N	I	A	W	P	V	P	P	V	K	R	L	I	D	A	I	L	A	R	L	P
S1B26	V	Y	K	N	T	I	N	W	A	I	S	V	M	P	V	K	L	R	I	D	A	I	L	A	A	L	P

Figure 3. (A) SDS PAGE analysis of selected binders S1B9, S1B22 and S1B26 after purification from E.coli cell lysates, stained with Coomassie brilliant blue. (B) ELISA-determined binding of serially diluted biotinylated selected binders to immobilized recombinant Stx1B. The binding was detected by HRP-conjugated streptavidin. (C) Sequence similarity comparison of selected binders with parental non-mutated ABDwt. Randomized sequences between residues 20 and 46 were compared. Randomized positions are indicated in grey.

Characterization of affinity of selected S1B binders

The first setup of SPR affinity measurements included immobilization of Stx1B on the sensor chip surface and injecting S1B proteins over the surface. Binding was confirmed with different dilutions of both binders, S1B22 and S1B26. Nevertheless, we could not determine the binding constant (KD) for tested interactions with none of the available kinetic modules in BiacoreT100 software, probably due to complex interaction mode. Figure 4 shows a representative response after S1B22, S1B26 and ABDwt-TolA-Avi (ABDwt) at 1 µM concentration were injected over the immobilized Stx1B. Much higher sensor response was observed with S1B22 and S1B26 in comparison with ABDwt.

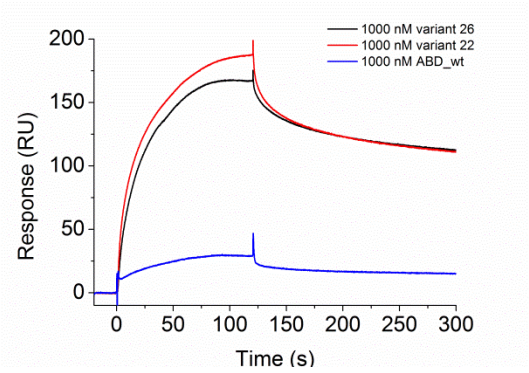


Figure 4. Binding of S1B22 and S1B26 in fusion with TolA-Avi to immobilized recombinant Stx1B.

In reversed SPR setup clones S1B22 (Fig. 5A) and S1B26 (Fig. 5B) were attached to the sensor chip surface and twofold dilutions of recombinant Stx1B, starting with 4 µM concentrations, were injected over the chip surface. To calculate the affinity constant we applied Steady State Affinity model (Fig. 5A, 5B) and an average of three experiments was considered. The binding affinity of recombinant Stx1B to S1B22 was 0.70 ± 0.03 µM, and to S1B26 1.00 ± 0.09 µM.

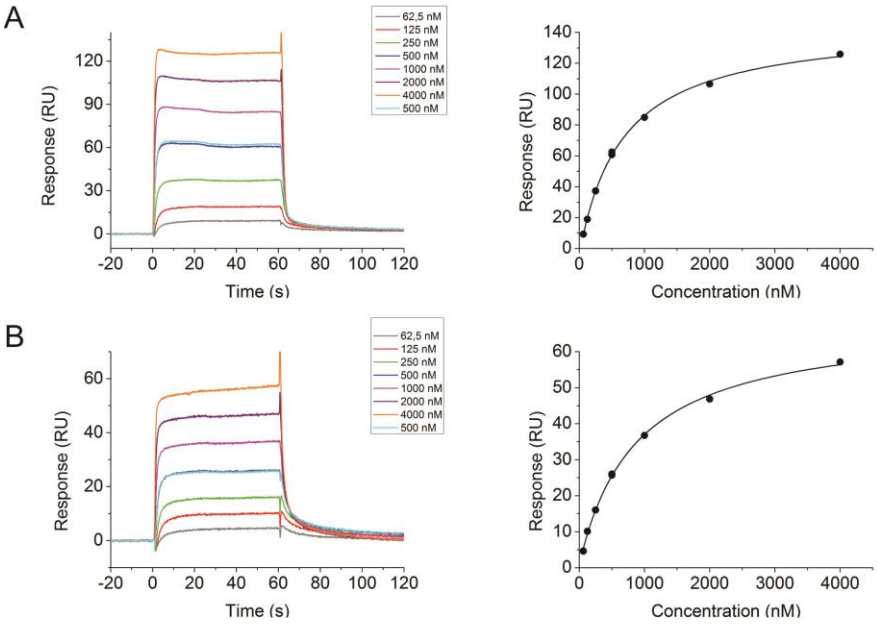


Figure 5. SPR analysis of binding of recombinant Stx1B to CM5-chip-immobilized S1B22-TolA-Avi (A) and S1B26-TolA-Avi (B). Recombinant Stx1B in seven different concentrations was injected over the chip surface (left figures). To calculate the affinity constant we applied Steady State Affinity model (right figures).

Characterization of stability of selected S1B binders

Thermal stabilities of binders S1B22 and S1B26 were investigated by the fluorescence-based thermal shift assay. Melting temperature (Tm) determined for binder S1B26 was 53 °C in

PBS buffer and 50.75 °C in Tris-NaCl buffer. Tm for binder S1B22 could not be determined in any of the tested buffers. Tm value for parental wild-type ABD was previously reported to be 58 °C (12, 13).

Expression and surface display of selected S1B clones on *L. lactis* NZ9000

Genes for S1B9, S1B22, S1B26, ABDwt and ABDwtHis without tolA spacer or tags were amplified with PCR and cloned into the plasmid pSDLBA3b (17), which was previously designed for surface display of target proteins in fusion with Usp45 secretion signal and surface anchoring C-terminal domain of AcmA (cA). Fusion proteins were expressed in *L. lactis* by induction with nisin and visualized in the cell lysates by SDS PAGE and Coomassie brilliant blue staining (Fig. 6A).

Surface display of ABDs was confirmed for ABDwt and ABDwtHis using flow cytometry. *L. lactis* cells expressing ABDwt on their surface efficiently bound FITC-conjugated HSA (Fig. 6B), which also indicates functionality of the displayed binder. Display of ABDwtHis on the surface of *L. lactis* cells was also confirmed with FITC-conjugated antiHis antibody (data not shown).

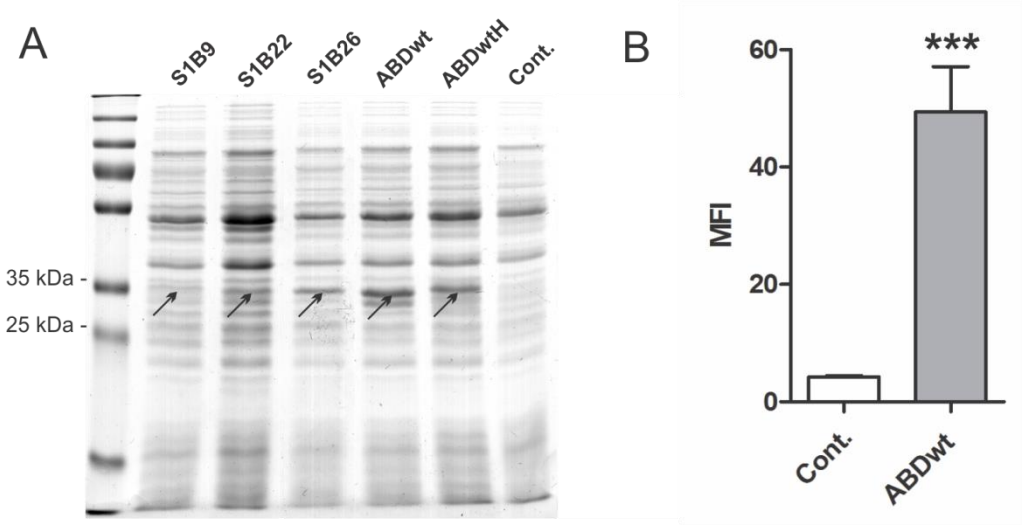


Figure 6. (A) SDS PAGE analysis of lysates of *L. lactis* cells expressing S1B9, S1B22, S1B26, ABDwt and ABDwtHis (ABDwtH), all in fusion with Usp45 secretion signal and the LysM-containing cA domain, stained with Coomassie brilliant blue. ABD fusion proteins are high-lighted with arrows. (B) Flow cytometryc analysis of ABDwt surface display, detection with FITC-conjugated human serum albumin. MFI value of ABDwt was compared to the control using Student's t test. *** $p<0.001$. Cont.: control containing empty plasmid pNZ8148.

Evaluation of binding of Stx1B by recombinant *L. lactis* with surface displayed S1B binders

Binding of Stx1B by recombinant *L. lactis* with surface displayed S1B binders was evaluated by flow cytometry (Fig. 7A) and whole cell ELISA (Fig. 7B). With flow cytometry we

observed statistically significant binding of fluorescently-labelled Stx1B by *L. lactis* cells displaying S1B9 or S1B26 on their surface in comparison with control *L. lactis* cells expressing ABDwt. With whole cell ELISA we confirmed binding of Stx1B by *L. lactis* cells displaying S1B9, S1B22 or S1B26. The comparative strength of binding of Stx1B differed between the two techniques: S1B9 exhibited the strongest binding with flow cytometry, while S1B26 exhibited the strongest binding with whole-cell ELISA.

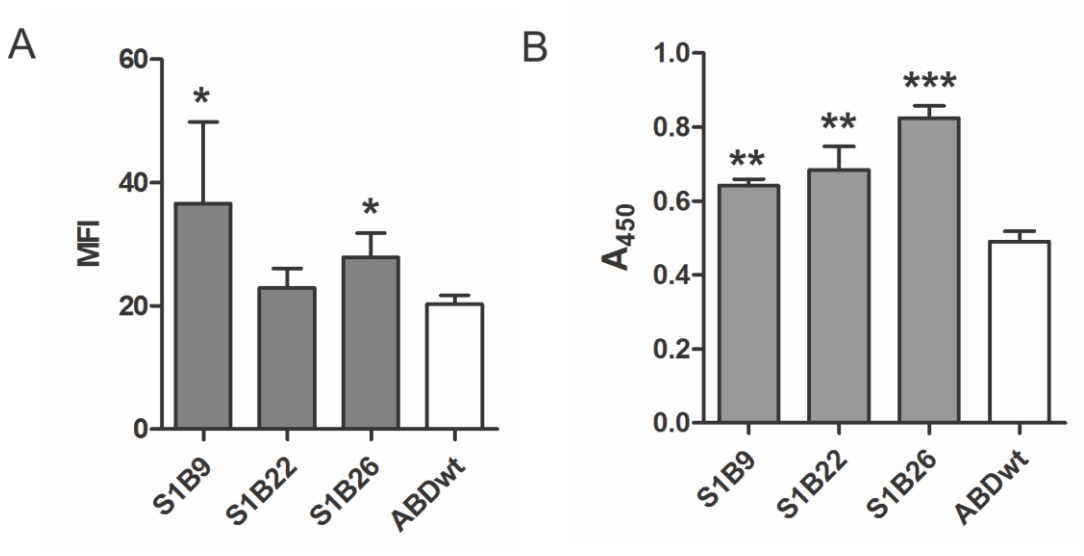


Figure 7. Flow cytometric (A) and whole-cell ELISA (B) analyses of binding of recombinant Stx1B by *L. lactis* cells displaying S1B variants or ABDwt on their surface. (A) Alexa488-conjugated Stx1B was used for detection. MFI: Mean fluorescence intensity. (B) Mouse antiStx1B antibody and HRP-conjugated anti mouse antibody were used for detection of Stx1B. A450: Absorbance at 450 nm. Vertical bars denote standard deviation. MFI or A450 values of S1B binders were compared to ABDwt control using Student's t test. *: $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

Shiga toxin receptor analogs are an attractive treatment option for infections with shiga toxin-producing bacteria (8). They cause no stress on bacteria and do not induce shiga toxin release. They bind the toxin in situ in the gastrointestinal tract, thereby preventing its activity. Engineered bacteria could substitute the polymer analogs and recombinant *E. coli* producing a modified lipopolysaccharide mimicking the shiga toxin receptor has already been reported (20, 21). Probiotic food-grade LAB were previously shown to be able to inhibit shiga toxin-producing bacteria by the production of lactic acid (16) and this could be exploited in creating synergistic therapeutic effect. In the present study we engineered food grade LAB *L. lactis* by displaying protein binders of recombinant shiga toxin 1 B subunit (Stx1B) on its surface.

To select high affinity binders of Stx1B we developed a novel procedure for its recombinant expression in *E. coli* using pET system that encompassed a decrease in growth temperature

from 37 °C to 28 °C after the induction of expression. Stx1B in fusion with hexahistidine tag was expressed in the form of inclusion bodies and was purified with immobilized metal affinity chromatography in the presence of denaturant. Denatured Stx1B was efficiently refolded with rapid dilution method using 0.5 M arginine and 0.01 % Brij-35 detergent-containing 50 mM Tris solubilisation buffer, conditions similar to those reported by Oneda and Inouye (24) for another protein.

Recombinant Stx1B formed pentamers and could bind to Gb_3 receptor in vitro in concentration dependent manner (Fig. 1A). Fluorescently-labelled Stx1B also bound to the receptor on HeLa cells, was internalized into cells and was transferred to Golgi apparatus 1 h after addition to HeLa cells (Fig.1B), which is in accordance with previous study (30). Based on these results it could be concluded that recombinant Stx1B was fully functional and suitable as a target for selection of novel high affinity binders.

Smaller antibody variants, single chain variable fragments (ScFv), against Stx1B were previously selected by screening a phage display library constructed from a naïve human repertoire (31). However, we have observed in our past research that, similar to antibodies themselves, ScFvs are not suitable for surface display on lactococcal cells (unpublished data), especially in comparison to non-Ig scaffolds, such as DARPins and Affibodies, possibly due to larger size and more complex folding. To obtain novel protein binders of shiga toxin, a highly complex combinatorial library derived from streptococcal albumin-binding domain scaffold was therefore applied, which has been successfully used previously for selection of high-affinity binders of IFN-γ (11), for development of IL-23 receptor antagonists (12) and for generation of unique binders of human prostate cancer oncomarker (13). 17 unique sequence variants of Stx1B binders (Fig. 2A), called S1B binders, were obtained by five cycles of ribosome display.

All selected S1B variants were expressed in *E. coli* in fusion with TolA spacer protein with an AviTag installed on C-terminal as reported previously (11-13). Among 17 variants only four cell lysates that contained S1B binders S1B9, S1B22, S1B26 or S1B28 showed specific binding to immobilized recombinant Stx1B (Fig. 2B) using ELISA. S1B28 variant showed lower level of expression, possibly due to unintended mutation in non-randomized part of sequence, and was therefore not included in further characterization.

S1B-TolA-Avi fusion variants S1B9, S1B22, S1B26 and ABDwt were purified from the *E. coli* lysates. Low level of expression of S1B9 variant and some proteolytic degradation was observed by SDS PAGE (Fig. 3A). Nevertheless, binding of all three serially diluted binders (S1B9, S1B22 and S1B26) to immobilized Stx1B was observed (Fig. 3B). Variant S1B26

showed the strongest binding, while binding of S1B9 variant was the weakest, which may be the consequence of lower concentration and degradation mentioned above. Variants S1B22 and S1B26 were therefore chosen for further biophysical characterization using SPR.

When Stx1B was immobilized on the sensor surface and ABD variants were injected over the sensor the response for all tested concentration was recorded. However, binding affinities could not be determined with any of the predetermined kinetic modes. This was probably due to a complex interaction mode between S1B variants and Stx1B, which is in pentameric form, and probably binds more than one S1B molecule. The binding mode between Stx1B and S1B binders could be investigated in the future by structural analysis, such as molecular docking or by solving the structure of the complex. On the contrary, by reverting the SPR setup, immobilizing S1B variants and flowing Stx1B over the sensor, the affinity constants of S1B22 and S1B26 were determined to be in the micromolar range (0.70 μM for S1B22 and 1.00 μM for S1B26). This is lower affinity than previously reported for ScFvs against Stx1B. However, the affinity of ABDs could be further improved by affinity maturation approach, where a new combinatorial library would be constructed from the best binder sequence using error prone PCR and the new library would be subjected to second selection against the same target under conditions of increasing stringency (32).

To display S1B binders on the surface of *L. lactis*, the genes for S1B9, S1B22 and S1B26 without TolA spacer or AviTag were fused to the gene for peptidoglycan-binding C terminus of AcmA protein and to the Usp45 secretion signal, as reported previously (17, 18, 26). The same fusion was constructed with ABDwt, which served as a Stx1B-non-binding control and as a positive control for lactococcal surface display of ABD through its ability to bind human serum albumin. Fusion proteins were expressed in *L. lactis* by using nisin-controlled expression system (NICE), as confirmed by SDS PAGE analysis (Fig. 6). Although S1B9 variant was eliminated from biophysical characterization, it was expressed in *L. lactis* cells and no truncation was observed. This might be due to a change in expression host or removal of the TolA spacer. Surface display of all four tested ABDs (three S1B variants and ABDwt) was confirmed using flow cytometry or whole cell ELISA (Fig. 7). With flow cytometry, binding of fluorescently-labelled Stx1B was observed with surface displayed S1B9 and S1B26, whereby S1B9 variant showed stronger binding. On the contrary, with whole-cell ELISA significant binding was observed with all three S1B variants, the strongest with S1B26 and the weakest with S1B9. Differences could be attributed to sterical hindrances or unfavorable conformations, achieved during different testing conditions that could result from different experimental methods. Nevertheless, on the basis of these results, S1B26 could be

selected as the most promising Stx1B binder for the display on the surface of *L. lactis*, even though S1B22 exhibited lower binding constant in SPR, possibly due to the fusion with TolA-Avi in isolated S1Bs.

In the present study we demonstrated for the first time an efficient and functional display of ABD proteins on the surface of LAB *L. lactis*. This represents an additional class of alternative binding proteins successfully displayed on the surface of *L. lactis*, next to Affibodies (17) and DARPins (18). LAB *L. lactis* capable of binding Stx1B by displaying on its surface ABD-derived Stx1B binding proteins were engineered and could be used for neutralization of shiga toxin in the human intestine in the early stages of infections with shiga toxin producing bacteria. Additionally, novel Stx1B binders on the basis of ABD scaffold could be applied in diagnostics or basic research as a small molecular weight alternative to antibodies.

Acknowledgments

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DRUGO POGLAVJE

Inženiring nosilnega proteina BmpA za površinsko predstavitev IgG-vezavnih domen na bakteriji *Lactococcus lactis*

Engineering BmpA as a carrier for surface display of IgG-binding domain on *Lactococcus lactis*

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POVZETEK

Bazični membranski protein A (BmpA) je potencialni nosilni protein za površinsko predstavitev IgG-vezavnih domen na bakteriji *Lactococcus lactis*. V raziskavi smo dokazali, da lahko 1.3-krat poviša adhezijo bakterij na črevesni celični model in izboljšali BmpA-odvisno površinsko predstavitev z inženiringom BmpA molekule. Izkazalo se je, da je večji del molekule BmpA sicer pomemben za površinsko predstavitev, vendar pa je omejeno krajšanje molekule (različica Bmp1) znatno povišalo zmožnost površinske predstavitve. Bližina N- in C-konca pri Bmp1 modelu ter neučinkovitost vmesnika kažeta na to, da oddaljenost potniškega proteina od membrane ni najpomembnejša pri površinski predstavitvi.

Ključne besede:

bazični membranski protein A, nosilni protein, *Lactococcus lactis*, površinska predstavitev

Izjava

Podpisana Petra Zadravec izjavljam, da članka Engineering BmpA as a carrier for surface display of IgG-binding domain on *Lactococcus lactis* nihče od soavtorjev ni uporabil kot kriterij za zagovor doktorata.

Petra Zadravec

Engineering BmpA as a carrier for surface display of IgG-binding domain on *Lactococcus lactis*

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Running title: Engineering BmpA for surface display

Edited by Jacques Fastrez

Abstract

Basic membrane protein A (BmpA) is a potential carrier protein for surface display of the IgG-binding domain on *L. lactis*. We have shown that it can increase the adhesion of bacteria to intestinal cell model by 1.3-fold and have improved BmpA-based surface display by engineering the BmpA molecule. The bulk of BmpA molecule was shown to be important in surface display; however limited shortening (variant Bmp1) resulted in large increase in surface display ability. The closeness of the N- and C-terminals in Bmp1 model and inefficiency of the spacer suggest that the distance of the passenger from the membrane is not of prime importance in surface display.

Keywords:

Basic membrane protein A / carrier protein / *Lactococcus lactis* / surface display

Introduction

Bacteria with recombinant proteins displayed on their surface have numerous biotechnological applications. They can act as bioadsorbents, biosensors, biocatalysts or oral vaccines. Additionally, bacterial surface display can be exploited in antibody production, screening of peptide libraries and detection of mutations (Georgiou *et al.*, 1997; Lee *et al.*, 2003; Stahl and Uhlen, 1997). For surface display, the protein to be displayed (passenger protein) is fused to an anchoring motif (carrier protein) (Georgiou *et al.*, 1997; Lee *et al.*, 2003; Stahl and Uhlen, 1997). Surface display systems have been developed for Gram-negative bacteria (Georgiou *et al.*, 1997), for Gram-positive bacteria (Stahl and Uhlen, 1997) and for yeasts (Ueda and Tanaka, 2000).

Lactic acid bacteria (LAB) are important hosts in biotechnology due to their safety, industrial applicability and beneficial influence on health. Several strains of different species have been used in therapy as probiotics (de Vrese and Schrezenmeir, 2008). Surface display on LAB has been used mainly for antigens, to prepare mucosal vaccines (Bahey-El-Din, 2012; Berlec *et al.*, 2013; Wells, 2011). Additionally, surface display has been used to display binding molecules directed against pro-inflammatory molecules such as TNFα in inflammatory bowel disease (Ravnikar *et al.*, 2010), for the assembly of macromolecular enzyme complexes (Wieczorek and Martin, 2010), and for immobilization of bacterial cells (Liu *et al.*, 2011). Five different types of surface anchoring domain have been described for LAB, including transmembrane domains, lipoprotein anchor domains, LPXTG-type domains, lysin motif (LysM) domains and surface layer proteins (Leenhouts *et al.*, 1999). In the prototype LAB,

Lactococcus lactis, the most frequently applied surface anchoring domains are the LPXTG sequence of M6 protein of *Streptococcus pyrogenes* (Dieye *et al.*, 2001; Ribeiro *et al.*, 2002; Wieczorek and Martin, 2010), and the C-terminal part of endogenous AcmA that contains the peptidoglycan binding LysM repeats (Lim *et al.*, 2010; Okano *et al.*, 2008; Ramasamy *et al.*, 2006; Ravnikar *et al.*, 2010). However, new surface display approaches are still being sought. In our previous study of the lactococcal surface proteome, we identified the basic membrane protein A (BmpA) as a promising carrier for surface display in *Lactococcus lactis* (Berlec *et al.*, 2011).

BmpA is a lipoprotein that contains the characteristic lipobox in the N-terminal signal peptide that is cleaved by signal peptidase II in the process of covalent anchoring of the protein to the membrane lipid bilayer (Desvaux *et al.*, 2006). BmpA is a solute-binding subunit of the classical ABC transporter (Eitinger *et al.*, 2011) that is responsible for the import of purines (Martinussen *et al.*, 2010). The sequence of lactococcal BmpA molecule shows 31% identity to that of BmpA from *Borrelia burgdorferi*, which is an immunodominant protein (Roessler *et al.*, 1997) with laminin-binding ability (Verma *et al.*, 2009).

In this study we optimized BmpA-based surface display of IgG binding B domain of staphylococcal protein A (Ravnikar *et al.*, 2010). B domain can be detected directly with specific antibodies against protein A, or indirectly, with any antibody whose Fc region can be bound by B domain. It can be used to demonstrate both the location and functionality of the passenger protein, and can serve as affibody prototype (Nygren, 2008). We minimized the relatively bulky BmpA molecule that could limit the translocation of larger fusion proteins. Truncated variant of BmpA without spacer, termed Bmp1, exhibited markedly increased ability for surface display.

Materials and methods

BmpA modeling

The secondary structure of BmpA was predicted with Phyre server (http://www.sbg.bio.ic.ac.uk/~phyre) (Kelley and Sternberg, 2009) and the tertiary structure with I-Tasser (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (Zhang, 2008). Images were prepared with PyMOL (http://www.pymol.org).

Bacterial strains, media and culture conditions

The bacterial strains used in this study are listed in Table S1. *L. lactis* NZ9000 was grown in M-17 medium (Merck) supplemented with 0.5 % glucose (GM-17) and 10 µg/mL of chloramphenicol at 30 °C, without aeration. *E. coli* strain DH5α was grown at 37 °C with aeration in LB medium supplemented with 100 µg/mL ampicillin.

Cloning procedures, mutagenesis and expression of BmpA-variants in *L. lactis*

Cloning procedures, mutagenesis and expression of BmpA-variants are described in Supplementary materials.

SDS PAGE and Western blots

SDS PAGE was performed as described in Supplementary materials.

Adhesion to Caco-2 cells

The Caco-2 human colon adenocarcinoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) with l-glutamine, 10% fetal calf serum (HyClone) and 50 mg/mL of gentamycin sulphate (Sigma-Aldrich) at 37 °C in 10% CO_2. To assay adhesion, cells were seeded at a concentration of 10^4 cells/well in 24-well tissue culture plates and cultured for ca. 3 weeks to form a monolayer.

Cultures of *L. lactis* expressing BmpA, or containing an empty plasmid (control), were washed once with PBS, diluted to OD_{600}=0.50 and enumerated by plate counting on GM-17 plates. 1 ml of cell suspension was added to each well with a Caco-2 monolayer previously washed twice with PBS, and incubated for 30 min. Unattached bacteria were removed by triple washing with PBS. Caco-2 cells were lyzed with 0.05 % Triton X-100 (Sigma-Aldrich) for 5 min, and adherent lactococci enumerated by plate counting on GM-17 plates. Lactococcal cultures were prepared in duplicate and each incubated with Caco-2 monolayer in 10 repeats. Proportions of adherent bacteria were calculated and averaged.

Flow cytometry and fluorescence microscopy

10 µL of cell culture (2×10^9 cells/mL) were added to 500 µL of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and centrifuged for 3 min at 5000 × g at 4 °C. Pellets were resuspended in 500 µL of TBS containing FITC conjugated anti-protein A antibody (Abcam) or Alexa Fluor 488 conjugated rabbit anti-mouse antibody (Abcam) diluted 1:500 and incubated for 2 hours at RT with constant shaking at 100 rpm. Cells were then washed three times with 200 µL 0.1 % TBST and resuspended in 500 µL of TBS. Stained cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson) using 488 nm excitation and 530 nm emission in the FL1

channel. Geometric mean fluorescence intensity (MFI) of at least 20000 bacterial cells was measured. The average of three independent experiments was considered.

The staining protocol for fluorescence microscopy was similar, except that the starting volume of cell cultures was 20 µL and the final volume of TBS for re-suspension 300 µL. Fluorescence microscopy was performed using a Carl Zeiss LSM 710 confocal microscope. Alexa Fluor 488 was excited with an argon laser (488 nm), and emission was filtered using a narrow-band 505–530 nm filter.

Purification of Bmp1 cleavage fragments

Bmp1 with hexa-histidine fusion was expressed in 100 mL culture as described above. The soluble fraction of the cell lysate was loaded on 0.8 ml of BD Talon metal affinity resin (BD Biosciences) according to the manufacturer's instructions, using batch/gravity-flow column purification and imidazole elution.

N-terminal sequencing

Purified Bmp1 was loaded on SDS PAGE and blotted to PVDF membrane. The membrane was stained briefly with Coomassie Brilliant Blue and dried; appropriate bands were excised. The N-terminal amino acid sequences were determined by automated Edman degradation using an Applied Biosystems 492 Protein sequencer.

Results

Adhesion of BmpA-expressing lactococci to Caco-2 monolayer

Expression and surface display of BmpA significantly ($p<0.005$) increased the ability of lactococci to adhere to Caco-2 cell monolayer, a model of intestinal epithelium. After stringent washing, the number of adhered bacteria was increased by 1.3-fold relative to control (Figure 1).

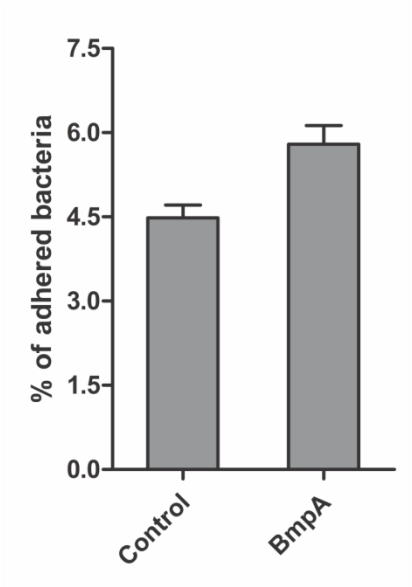

Fig. 1 Percentage of bacteria adhered to Caco-2 monolayer after incubation and washing. BmpA: *L. lactis* expressing BmpA. Control: *L. lactis* carrying empty plasmid. Vertical bars denote standard error.

Design of BmpA variants

Truncated variants of BmpA were designed on the basis of secondary structure prediction (Figure S1). The N-terminus was preserved in all variants. C-termini were defined in loops between alpha helices and beta sheets, where possible, following Ser or Gly residues (Figure S1). A spacer $(SGGGG)_3$ was introduced between BmpA variants and passenger protein (B domain) to increase their mutual distance. All variants are shown schematically on Figure 2.

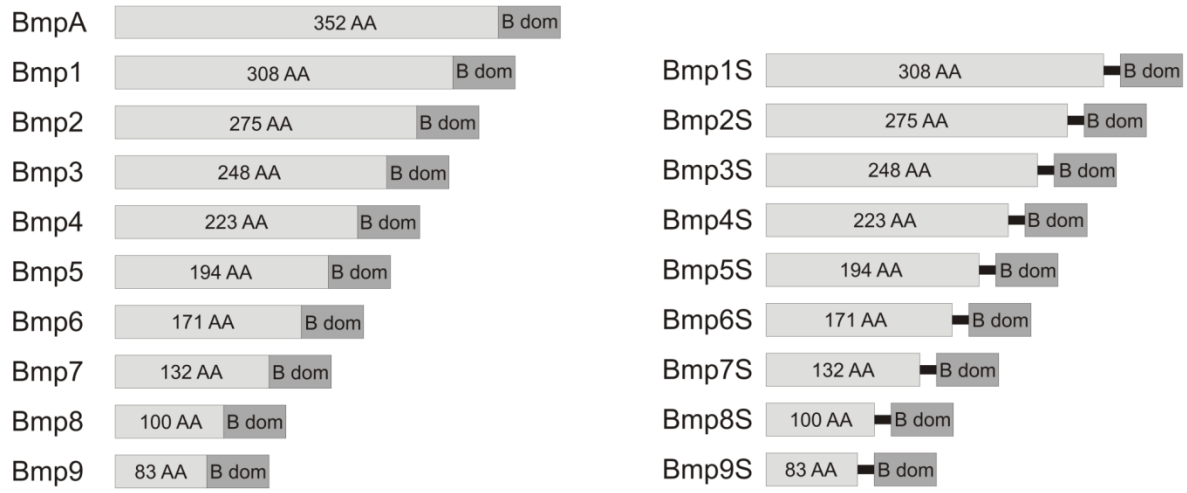

Fig. 2 Schematic representation of BmpA variants, showing their length, position of passenger (B dom) and position of $(SGGGG)_3$ spacer (thick black line).

Expression of BmpA variants

BmpA variants fused with B domain were expressed using nisin-controlled expression (Mierau and Kleerebezem, 2005). Lysates of cells expressing BmpA were compared with those of negative control cells containing empty plasmid, and positive control cells containing pSDLBA3b. All BmpA variants in fusion with B domain were detected on Western blots or on both Western blots and Coomassie-stained gels (Figure S2); however the expression levels of variants were significantly different. Molecular weights of proteins corresponded to those calculated (Table S2). Specific proteolytic cleavage products were observed with Bmp1-4 (Figure S2). B domain-containing cleavage products had molecular weights ca. 15 kDa lower than those of parent proteins.

Evaluation of BmpA variants as potential carrier proteins

The ability of BmpA variants to display B domain on the surface of lactococci was analyzed by flow cytometry and fluorescence microscopy. Mean fluorescence intensity (MFI) was measured for all variants and was normalized relative to MFI of negative control (Figure 3). Similar results were obtained with both antibodies used (anti-protein A, specific for B domain (Figure 3A); anti-mouse, unspecific for B domain and bound via Fc region (Figure 3B)). Of the BmpA variants, Bmp1 increased surface display to the greatest extent. Compared with BmpA, the display was increased by ca. 3.3-fold or 5.6-fold, as determined with the use of specific or unspecific antibodies, respectively. Also, variants Bmp2 and Bmp3, with or without spacer, as well as Bmp1 with spacer, demonstrated a greater ability than BmpA to display B domain on the lactococcal surface; however the display was lower than that with Bmp1. Spacer $(SGGGG)_3$ did not increase surface display of BmpA variants since, in general, variants without spacer performed better than their counterparts with spacer (Figure 3). Bmp1-mediated surface display of B domain was lower than previously described LysM repeats (AcmA)-mediated surface display (Lim *et al.*, 2010; Okano *et al.*, 2008; Ramasamy *et al.*, 2006; Ravnikar *et al.*, 2010) by ca. 3.6-fold or 4.4-fold (as determined with the use of specific or unspecific antibodies, respectively).

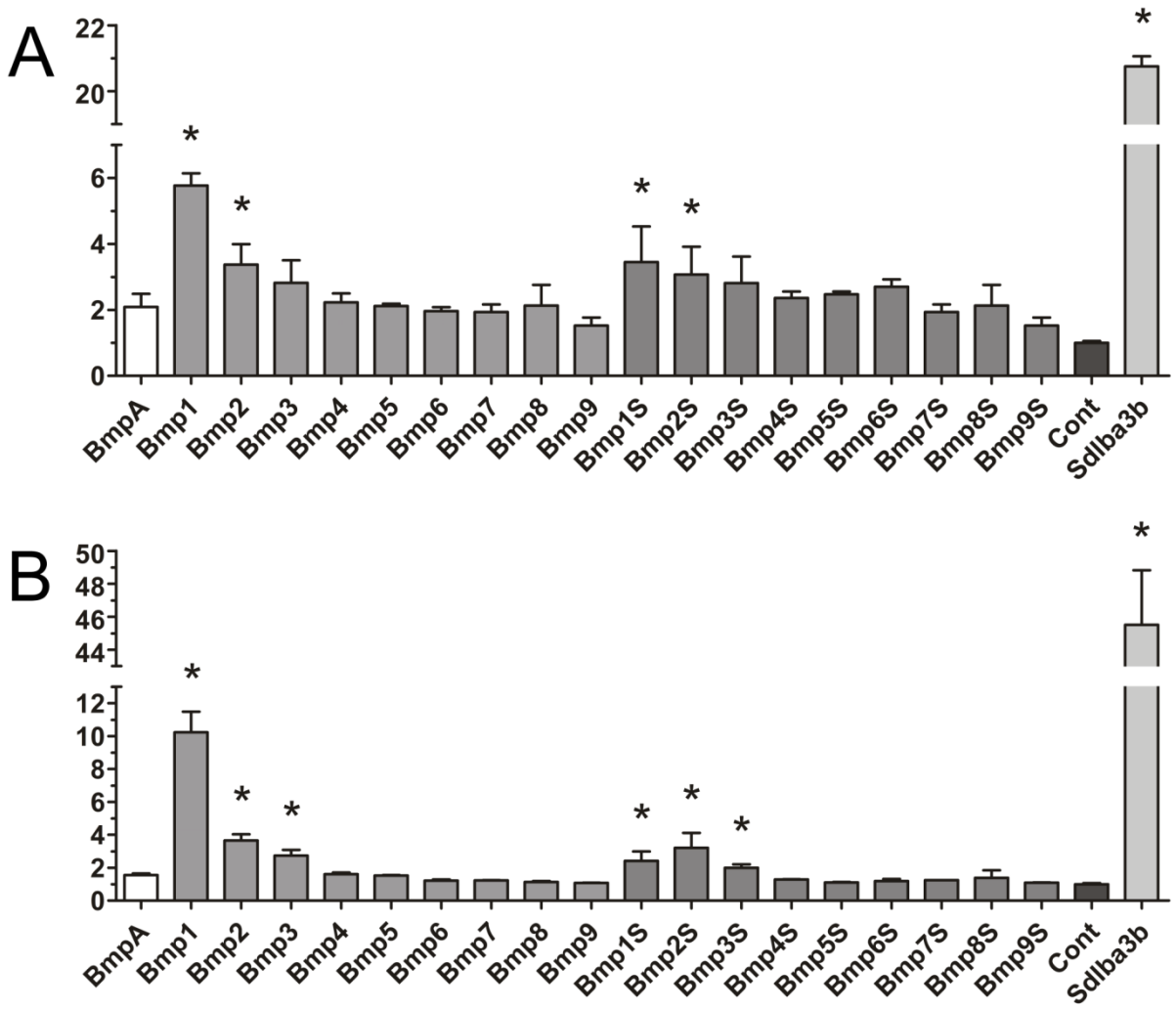

Fig. 3 Flow-cytometric analyses of *L. lactis* cells expressing variants of BmpA (denoted as in Figure 1), and peptidoglycan-binding domain of AcmA (Sdlba3b), all in fusion with B domain and detected with specific antibody against protein A (a) or unspecific antibody which binds to B domain via Fc region (b). Mean fluorescence intensity (MFI) was normalized relative to control (Cont). Vertical bars denote standard deviation. Variants with significantly greater MFI ($p<0.05$) than BmpA are marked with an asterisk.

BmpA-, Bmp1- and AcmA-mediated surface display of B domain was also evaluated with fluorescence microscopy and compared to control cells. The results corresponded well with those of flow cytometry. Cells with Bmp1-displayed B domain had much stronger fluorescence than cells with BmpA-displayed B domain; the fluorescence, however, was even stronger with AcmA-mediated display (Figure 4).

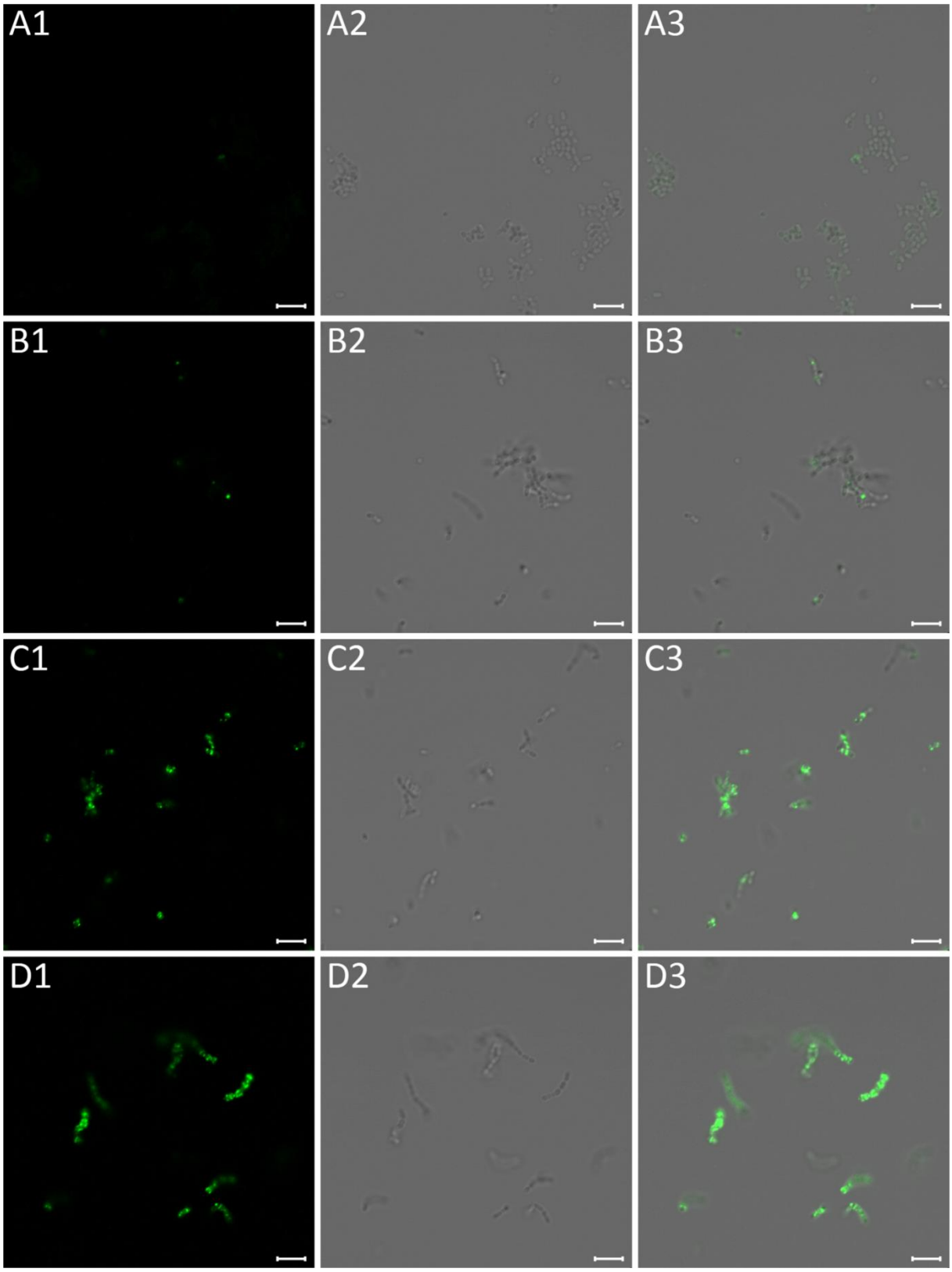

Fig. 4 Control cells (a), and cells expressing BmpA- (b), Bmp1- (c) and peptidoglycan-binding domain of AcmA in fusion with B domain (d), detected with nonspecific Alexa Fluor 488-conjugated antibody. (1) Fluorescent image. (2) Bright field image. (3) Merge. Bar = 5 μm.

Modeling of BmpA and Bmp1 structure

BmpA (Figure 5a) and Bmp1 (Figure 5b) tertiary structures were modeled and superimposed to compare the positions of the C-termini, and the sites of fusion with passenger protein (B domain). BmpA is a solute-binding subunit of an ABC transport protein, predicted to be composed of two similar α/β domains with an intermediate substrate binding cleft. The location of C-termini of BmpA and Bmp1 are predicted on separate domains, but on equivalent positions on a particular domain, as seen in Figure 5.

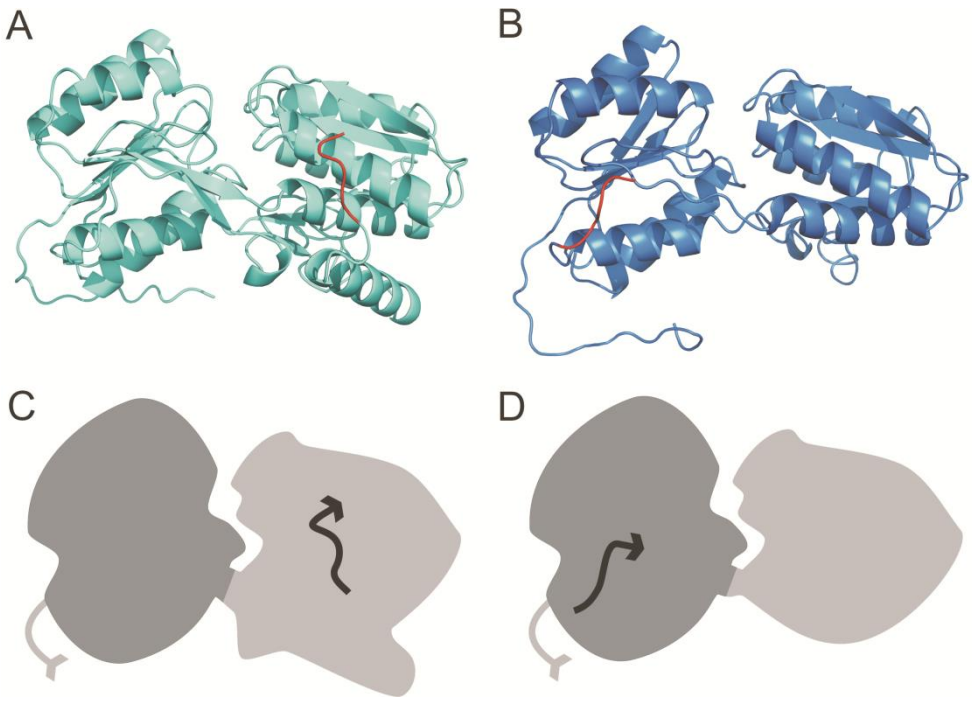

Fig. 5 I-Tasser generated models of (a) BmpA and (b) Bmp1; the five C-terminal amino acids are colored in red. Schematic representation of the two domains (light and dark grey) of BmpA (c) and Bmp1 (d); the N-terminus is shown with a grey arrow and the C-terminus with a black arrow.

Susceptibility of Bmp1 to proteolytic cleavage

In contrast to BmpA, its variants Bmp1-4 were observed to be prone to relatively specific proteolytic degradation (Figure S2), resulting in a residue approximately 15 kDa smaller. This was detected by specific antibody and therefore contained the B domain, indicating removal of the N-terminal part of the protein. Since this could affect the surface display ability of Bmp1-4, we determined the N-terminal sequence of the Bmp1 cleavage product**.** The resulting DSVIK sequence suggested cleavage between Val^{130} and Asp^{131}, indicating the involvement of a putative Asp-N endopeptidase. However, mutating Asp^{131} of Bmp1 to Glu^{131}

(Bmp1Glu) and Asn^{131} (Bmp1Asn) did not prevent the cleavage but slightly reduced the surface display ability (Figure S3).

Discussion

Our previous study established BmpA as a candidate carrier protein for surface display on *L. lactis* (Berlec *et al.*, 2011). In comparison to non-covalent surface attachment mediated by LysM domains, BmpA enables covalent, lipoprotein signal-based surface binding, which may be advantageous for applications that require stronger binding (Berlec *et al.*, 2011). In the present study, we aimed at improving its B domain surface display ability by testing BmpA intestinal adhesion properties and by engineering and minimizing BmpA molecule.

Since BmpA from *B. burgdorferi* has a laminin adhesion property (Verma *et al.*, 2009), we tested whether overexpression of lactococcal BmpA could influence the adhesion of the bacteria to intestinal epithelium in comparison to basal expression of BmpA. A modest increase in binding to the epithelial model, Caco-2 monolayer, was indeed observed with BmpA overexpression.

The BmpA molecule was truncated at its C-terminus at sites corresponding to predicted loops between α-helices and β-sheets. Nine variants were produced in an attempt to reduce the size of the carrier protein, thereby contributing to smaller size of the fusion protein and potentially improving the translocation efficiency and surface display. Additionally, $(SGGGG)_3$ spacer was introduced to all variants between the carrier and passenger to increase their distance apart, to avoid steric hindrance and to move the passenger away from the bacterial surface.

All BmpA variants were expressed, as observed from the results of Coomassie-staining and Western blot; however the truncation of the BmpA molecule affected the protein expression level. The total expression level of the fusion protein (determined by Western blot) was not studied into greater details, because it does not necessarily correspond to the level of surface displayed protein. Not all expressed protein is translocated to the bacterial surface, and not all translocated protein has favourable spatial orientation of the passenger protein. Flow cytometry was used to determine the amount of surface-bound antibody, which corresponds to the amount of surface displayed B domain. Flow cytometry may therefore be regarded as the most relevant measure of the surface display efficiency, while total fusion protein expression may be regarded only as one of the indicators of the latter.

The BmpA variant termed Bmp1, which lacked 44 amino acids at its C-terminal, increased surface display ability over that of BmpA for several fold; however the surface display ability was still inferior to that of peptidoglycan-binding domain of AcmA. Bmp2 and Bmp3 also

increased surface display ability over that of BmpA, but to a lesser extent than Bmp1. Further stepwise shortening of the molecule resulted in stepwise decrease or abolition of the surface display ability, indicating the importance of the bulk of the molecule in surface display. This indicates that the lipoprotein signal peptide is by itself not sufficient for considerable translocation or surface exposure of the passenger protein. Comparison of variants with and without spacer showed that introduction of the spacer had little or no effect on the surface display ability. It is possible that flexibility, introduced by the spacer, hinders efficient interaction with antibodies. This also indicates that direct fusion of carrier and passenger is not a limiting factor in surface display with BmpA or its variants. To summarize, Bmp1 without spacer qualifies as a new carrier in lactococcal surface display of functional proteins. Large improvement of Bmp1- over BmpA-mediated surface display was also confirmed with fluorescent microscopy.

Comparison of BmpA with Bmp1 was therefore studied further by modeling both structures. The models suggest that both proteins are composed of two α/β domains with an intermediate substrate binding cleft, probably for purines. Their C-termini (the sites of passenger protein fusion) are located on opposite domains; the C-terminus of Bmp1 is therefore predicted to be closer to the N-terminus than the C-terminus of BmpA. This is contrary to our initial assumption that increase in the distance of the passenger protein from the membrane (N-terminus) is the most important factor in increasing surface display ability. Our assumption is further refuted by the fact that introduction of the $(SGGGG)_3$ spacer does not increase the ability for surface display. The model does not offer more specific reasons for increased surface display with Bmp1. Removal of part of the second domain in Bmp1 may have resulted in removal of steric hindrance. It is also possible that the passenger protein modifies the position of the N-terminus, particularly with Bmp1, yielding an orientation more favorable for surface display; however modelling of the fusion protein provided no consisting results and is thus not shown.

Another difference between BmpA and Bmp1 is the greater susceptibility of the latter to relatively specific proteolytic digestion, as seen by the appearance of a band of lower molecular weight (ca. 15 kDa) on SDS-PAGE gel. A similar result was observed with variants Bmp2-4 and can probably be attributed to the exposure of amino acid residues normally buried in BmpA. Asp^{131} was established at the cleavage site in Bmp1. An increase in the surface display could be envisaged if the degradation of the carrier protein were prevented. However, changing the Asp to chemically related Asn and Glu did not prevent degradation; it even resulted in a small decrease of the surface display. This indicates lower specificity of the

protease(s) involved, and suggests a broader mutagenesis approach in order to prevent the cleavage.

To summarize, 18 variants of BmpA were investigated for their B domain surface display ability. Substantial minimization of BmpA molecule abolished its surface display ability; however removal of smaller part of the molecule (variant Bmp1) resulted in significantly increased surface display ability of B domain in comparison to BmpA. Bmp1 has therefore been selected as an optimized carrier protein for surface display of B domain in *L. lactis*. Though the display with Bmp1 is inferior to that achieved with peptidoglycan-binding domain of AcmA, it enables stronger covalent surface attachment (in comparison to non-covalent binding of AcmA) and therefore represents an attractive alternative to established surface display approaches. Additionally, BmpA-mediated increase in epithelial cell adhesion could lead to increased intestinal retention of the bacteria. This will be further assessed with Bmp1 in an animal model, with a final goal of establishing novel LAB-based intestinal delivery system.

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Dodatek k drugemu poglavju

Supplementary tables

Table S1: Strains, plasmids and primers.

Strain, plasmid or primer	Relevant features or sequence (5'-3')	Reference
Strain		
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
Plasmid		
pNZ8148	pSH71 derivative, P_{nisA}, Cm^r, nisin-controlled expression	(de Ruyter et al., 1996; Kuipers et al., 1993; Kuipers et al., 1998; Mierau and Kleerebezem, 2005)
pSDLBA3b	pNZ8148 containing gene fusion of Usp45 signal peptide, B domain and AcmA (residues 224-437)	(Ravnikar *et al*., 2010)
pNZ::llmg_1064-his6	pNZ8148 containing $BmpA^{1-352}$ gene with C-terminal hexahistidine tag	(Berlec *et al*., 2011)
pNZ::llmg_1064-B	pNZ8148 containing $BmpA^{1-352}$ gene with C-terminal B domain gene	(Berlec *et al*., 2011)
pNZ::Bmp1-B	pNZ8148 containing $BmpA^{1-308}$ gene with C-terminal B domain gene	This work
pNZ::Bmp2-B	pNZ8148 containing $BmpA^{1-275}$ gene with C-terminal B domain gene	This work
pNZ::Bmp3-B	pNZ8148 containing $BmpA^{1-248}$ gene with C-terminal B domain gene	This work
pNZ::Bmp4-B	pNZ8148 containing $BmpA^{1-223}$ gene with C-terminal B domain gene	This work
pNZ::Bmp5-B	pNZ8148 containing $BmpA^{1-194}$ gene with C-terminal B domain gene	This work
pNZ::Bmp6-B	pNZ8148 containing $BmpA^{1-171}$ gene with C-terminal B domain gene	This work
pNZ::Bmp7-B	pNZ8148 containing $BmpA^{1-132}$ gene with C-terminal B domain gene	This work
pNZ::Bmp8-B	pNZ8148 containing $BmpA^{1-100}$ gene with C-terminal B domain gene	This work
pNZ::Bmp9-B	pNZ8148 containing $BmpA^{1-83}$ gene with C-terminal B domain gene	This work
pNZ::Bmp1-S-B	pNZ8148 containing $BmpA^{1-308}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp2-S-B	pNZ8148 containing $BmpA^{1-275}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp3-S-B	pNZ8148 containing $BmpA^{1-248}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp4-S-B	pNZ8148 containing $BmpA^{1-223}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp5-S-B	pNZ8148 containing $BmpA^{1-194}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp6-S-B	pNZ8148 containing $BmpA^{1-171}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp7-S-B	pNZ8148 containing $BmpA^{1-132}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp8-S-B	pNZ8148 containing $BmpA^{1-100}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp9-S-B	pNZ8148 containing $BmpA^{1-83}$ gene with $(SGGGG)_3$ spacer and C-terminal B domain gene	This work
pNZ::Bmp1-his6	pNZ8148 containing $BmpA^{1-308}$ gene with C-terminal hexahistidine tag	This work
pNZ::Bmp1E-B	pNZ8148 containing $BmpA(D131E)^{1-308}$ gene with C-terminal B domain gene	This work
pNZ::Bmp1N-B	pNZ8148 containing $BmpA(D131N)^{1-308}$ gene with C-terminal B domain gene	This work
Primer		
1064-F	ACCATGGCTAAAAAACGCGTAATCGCAG	(Berlec *et al*., 2011)
Bdom-R-XbaI	ACGTTCTAGATTATTTTGGTGCTTGTGCATC	(Berlec *et al*., 2011)
1064-R1	TGGATCCTCCACCAGGGAATTTACCGTC	This work
1064-R2	AGGATCCACCGTCTTTAGATTTATATTTTC	This work
1064-R3	AGGATCCGCTTGAGTTCAAGGCTTTAG	This work
1064-R4	AGGATCCACCAGAACCATACATAGCTG	This work
1064-R5	TGGATCCTGATTTTACAGACTTGGCAC	This work
1064-R6	AGGATCCACCGATGAAACCAATTTTGTTC	This work
1064-R7	AGGATCCAGAGTCAACGATAACAAAGTTAG	This work
1064-R8	AGGATCCACCTTGTTGCTCCGCAGAG	This work
1064-R9	AGGATCCAGATTGGAAGTAAGTGTATCC	This work
SP-Bdom-F	AGGATCCGGAGGAGGTGGTTCAGGAGGTGGTGGATCTGGTGGAGGTGGTTCCG CTGATAACAAATTCAACAAAGAAC	This work
Bmp1-Glu-F	CTAACTTTGTTATCGTTGAATCTGTCATTAAAGACC	This work
Bmp1-Glu-R	GGTCTTTAATGACAGATTCAACGATAACAAAGTTAG	This work
Bmp1-Asn-F	CTAACTTTGTTATCGTTAACTCTGTCATTAAAGACC	This work
Bmp1-Asn-R	GGTCTTTAATGACAGAGTTAACGATAACAAAGTTAG	This work

Table S2: Calculated molecular weights of BmpA variants.

BmpA variant (B domain fusion)	BmpA	Bmp1	Bmp2	Bmp3	Bmp4	Bmp5	Bmp6	Bmp7	Bmp8	Bmp9
Molecular weight without spacer (kDa)	43.7	39.1	35.6	32.5	30.1	27.1	24.7	20.7	17.3	15.5
Molecular weight with spacer (kDa)		40.0	36.6	33.5	31.0	28.1	25.6	21.6	18.2	16.4

Supplementary figures:

BmpA (352 AA)*

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIGGMQSDVITRFEKGYV
AGAKSVKSDIKVDIQYAGSFSDAAKGKTIAAAMYGSGDDVVYQCAGGVGTGVFSEAKALNSS
KNEADKVWVIGVDQDQEYLGKYKSKDGKDSNFVLVSTIKEVGTVVKDIADKTKDGKFPGGTI
VTYNLKNGGVDLGLDNATSEIKDAVAKAKTDIIDGKITVPSKGSADNKFNKEQQNAFYEILH
LPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPK

Bmp1 (308 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIGGMQSDVITRFEKGYV
AGAKSVKSDIKVDIQYAGSFSDAAKGKTIAAAMYGSGDDVVYQCAGGVGTGVFSEAKALNSS
KNEADKVWVIGVDQDQEYLGKYKSKDGKDSNFVLVSTIKEVGTVVKDIADKTKDGKFPGG

Bmp2 (275 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIGGMQSDVITRFEKGYV
AGAKSVKSDIKVDIQYAGSFSDAAKGKTIAAAMYGSGDDVVYQCAGGVGTGVFSEAKALNSS
KNEADKVWVIGVDQDQEYLGKYKSKDG

Bmp3 (248 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIGGMQSDVITRFEKGYV
AGAKSVKSDIKVDIQYAGSFSDAAKGKTIAAAMYGSGDDVVYQCAGGVGTGVFSEAKALNSS

Bmp4 (223 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIGGMQSDVITRFEKGYV
AGAKSVKSDIKVDIQYAGSFSDAAKGKTIAAAMYGSG

Bmp5 (194 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIGGMQSDVITRFEKGYV
AGAKSVKS

Bmp6 (171 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDSVIKDQKNVTSATFADNESAYLAGVAAAKATKTNKIGFIG

Bmp7 (132 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQGYKLLFGIGFSLQDATSAAAKNNPK
SNFVIVDS

Bmp8 (100 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQSNSASDYTTNYNSAEQQG

Bmp9 (83 AA)

MAKKRVIAVSAIALASVAVLAGCRSHDASGTSGKVKTDLKAAIVTDANGVNDRSFNQSAWEG
LQSWGKENNLKKGTGYTYFQS

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*The BmpA sequence differs from that in GeneBank in that it includes an Ala at position 2 due to cloning requirements. Modified numbering is therefore used throughout the manuscript.

Figure S1: Amino acid (AA) sequences of BmpA and its variants with predicted secondary structure (using Phyre server). Dark orange: α-helix. Light orange: β-strand.

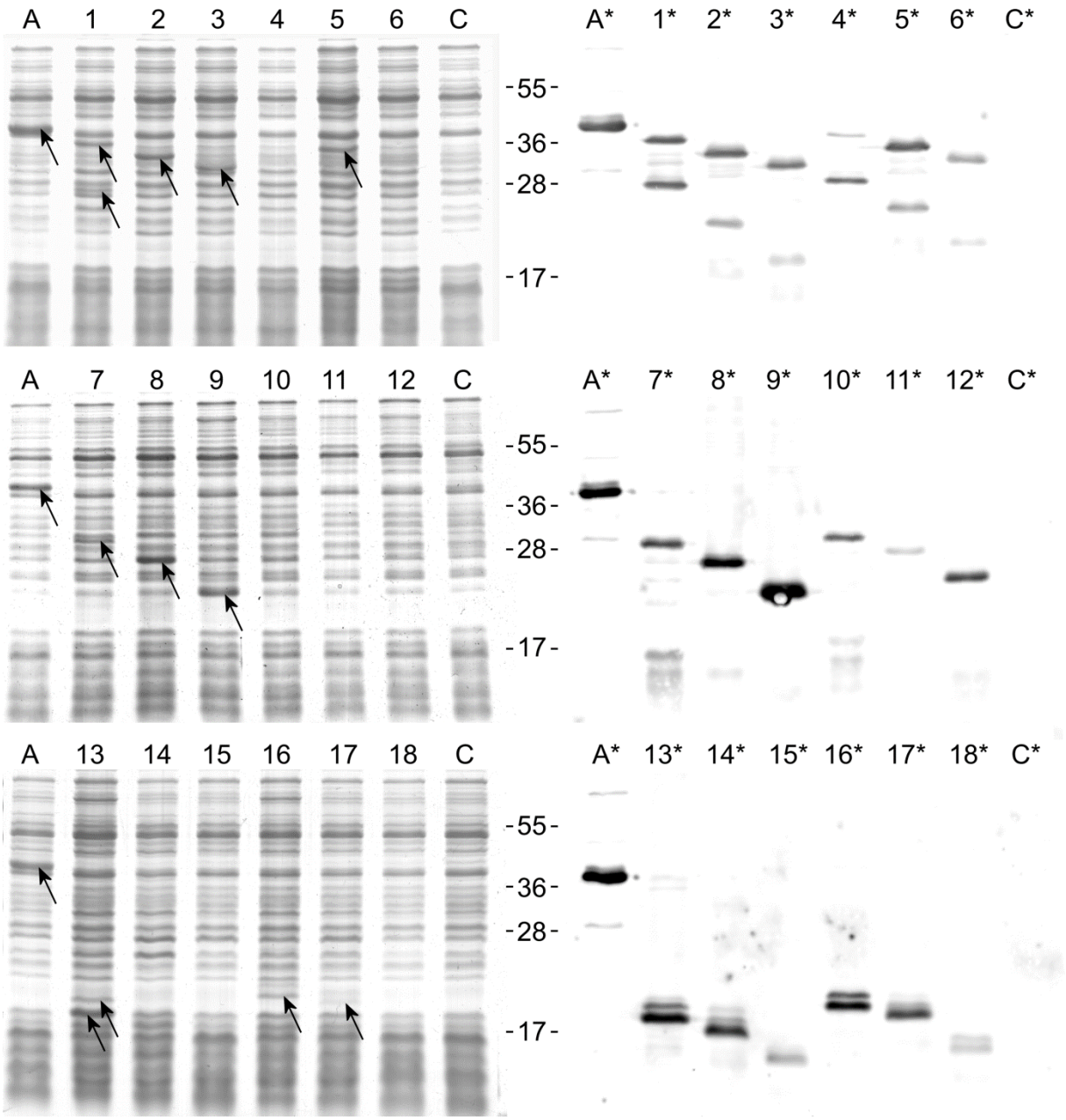

Figure S2: SDS-PAGE of lysates of *L. lactis* cells expressing BmpA variants in fusion with B domain, stained with Coomassie brilliant blue, or detected with anti-protein A antibody following Western blot (denoted with asterisk). A: BmpA. C: control, containing empty plasmid. 1-3: Bmp1-Bmp3. 4-6: Bmp1S-Bmp3S. 7-9: Bmp4-Bmp6. 10-12: Bmp4S-6S. 13-15: Bmp7-Bmp9. 16-18: Bmp7S-Bmp9S. Designation of variants corresponds to that in Figure 1. Proteins or degradation products that can be distinguished in Coomassie-stained gels are highlighted with arrows.

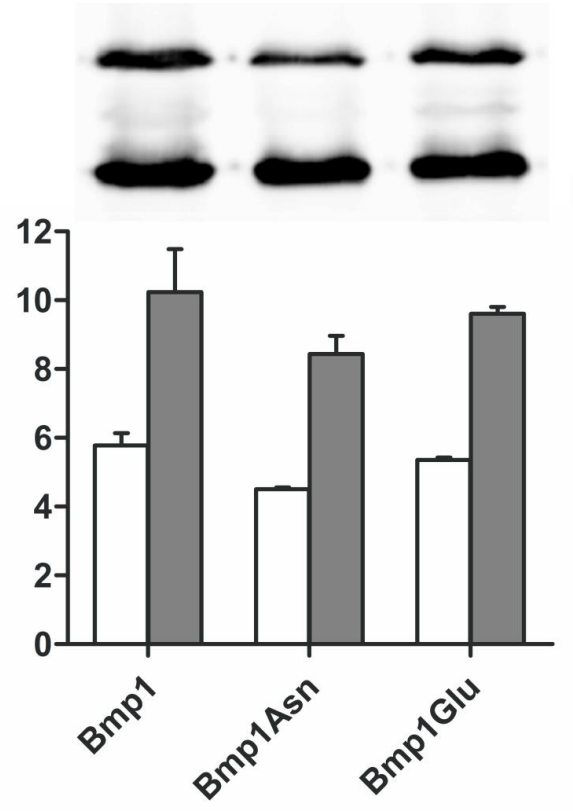

Figure S3: Flow-cytometric and western blot analyses of *L. lactis* cells expressing variants of Bmp1, Bmp1Asn and Bmp1Glu. Open bars: specific antibody. Grey bars: unspecific antibody. Mean fluorescence intensity (MFI) was normalized relative to control (not shown). Vertical bars denote standard deviation. Western blot bands above the graph correspond to lysates of cells denoted on the graph. Upper bands correspond to whole proteins, lower bands to cleavage products.

Supplementary methods:

Cloning procedures and mutagenesis

DNA fragments were amplified with Taq polymerase (Fermentas) and routinely ligated to pGEM-T Easy (Promega) for sequencing and further cloning. Restriction enzymes were from Fermentas or New England Biolabs. Plasmid DNA was isolated with Wizard SV Minipreps (Promega), with an additional lysozyme treatment step for *L. lactis*. The latter was transformed with electroporation, using Gene Pulser II apparatus (Biorad) (Holo and Nes, 1989). Nucleotide sequencing was performed by Eurofins MWG Operon. Primers (Thermo Scientific) and plasmids are listed in Table S1.

Truncated variants of BmpA gene were amplified using forward primer 1064-F and reverse primers 1064-R1 to 1064-R9. The products were used to replace BmpA in pNZ::llmg_1064-B via *Nco*I/*Bam*HI sites. The gene for the $(SGGGG)_3$ spacer was fused to the N-terminal of B domain of staphylococcal protein A gene with primers SP-Bdom-F and Bdom-R-XbaI and

used to replace B domain via *Bam*HI/*Xba*I sites in pNZ::Bmp1-B. Bmp1 gene in the resulting plasmid pNZ::Bmp1-S-B was replaced by the remaining shortened variants of BmpA (Bmp2 to Bmp9). Bmp1 gene was also used to replace BmpA in pNZ::llmg_1064-his_6, yielding pNZ::Bmp1-his_6.

Bmp1 gene mutants D131E and D131N were prepared with QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions, using Bmp1-Glu-F/R and Bmp1-Asn-F/R primer sets.

Expression of BmpA-variants in L. lactis

Overnight cultures of *L. lactis* NZ9000 harboring the appropriate plasmids were diluted (1:100) in 10 mL of fresh GM-17 medium. Cultures were grown to optical density A_{600} = 0.80, induced with 25 ng/mL nisin (Fluka) and grown for a further 3 hours. An aliquot (1 mL) was stored at 4 °C for flow cytometric analysis. The remaining culture was pelleted, resuspended in 500 µl of 0.1 M potassium phosphate buffer (pH 7.00), and frozen for SDS PAGE and Western blot analysis.

SDS PAGE and Western blots

SDS PAGE was performed with a mini-Protean II apparatus (Bio-Rad). Page Ruler Plus (Fermentas) pre-stained standard was used for molecular weight comparison. Samples were thawed on ice, briefly sonicated and denatured by heating at 100 °C in the presence of DTT before loading. Proteins were stained with Coomassie Brilliant Blue or transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore). The membrane was blocked in 5 % skimmed milk and incubated overnight at 4°C with FITC conjugated anti-protein A antibody (Abcam; 1:2000 dilution). After washing with 0.05 % TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween 20, pH 7.5), fluorescence was detected with Typhoon 9410 (GE Healthcare) imager using blue excitation (488 nm).

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TRETJE POGLAVJE

Izboljšanje LysM-posredovane površinske predstavitve načrtovanih proteinov z ankirinskimi ponovitvami (DARPinov) pri rekombinantih in nerekombinantih bakterijah *Lactococcus lactis* in *Lactobacillus sp.*

Improvement of LysM-mediated surface display of DARPins in recombinant and non-recombinant *Lactococcus lactis* and *Lactobacillus* species

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POVZETEK

Mlečnokislinske bakterije (MKB) so zaradi varnosti in probiotičnih lastnosti zanimivi gostiteljski organizmi za površinsko predstavitev heterolognih proteinov. Za terapevtske in prehrambne namene je zaradi regulatornih zahtev zaželena uporaba površinska predstavitve na nerekombinantih mikroorganizmih. V raziskavi smo na površini bakterije *Lactococcus lactis* predstavili dva načrtovana proteina z ankirinskimi ponovitvami (DARPina), ki imata sposobnost vezave Fc regije človeških IgG, s fuzijo z Usp45 signalnim peptidom za izločanje in peptidoglikan-vezavnim C-koncem proteina AcmA, ki vsebuje ponovitve lizinskega motiva (LysM). Za testiranje heterologne vezave izločenih fuzijskih proteinov, ki so se nahajali v gojišču, na 10 sevov iz rodu *Lactobacillus*, smo uporabili pretočno citometrijo, encimsko-imunski test (ELISA) s celimi celicami in fluorescenčno mikroskopijo. Fuzijski proteini so se vezali na površino vseh laktobacilov, vendar je bila vezava na večino bakterij le 2- do 5-krat močnejša v primerjavi s kontrolo. *Lactobacillus salivarius* ATCC 11741 je izkazal izjemno močno vezavo (32- do 55-krat višjo od kontrole), zaradi česar je lahko zanimiv gostiteljski organizem za nerekombinantno površinsko predstavitev. Genomska primerjava sevov kaže na to, da so eksopolisaharidi seva *Lb. salivarius* možen razlog za to razliko. Hkrati smo pokazali koncentracijsko odvisno, 15-kratno povišanje nerekombinantne površinske predstavitve ob gojenju bakterij v subletalnih koncentracijah antibiotikov kloramfenikol in eritromicin. V raziskavi smo optimizirali nerekombinantno površinsko predstavitev na MKB, ki temelji na LysM ponovitvah, z izborom seva *Lactobacillus salivarius* ATCC 11741 kot optimalnega gostiteljskega organizma in z uvedbo antibiotikov kot dodatkov za povišanje površinske predstavitve na bakteriji *L. lactis*. Poleg tega učinkovita predstavitev DARPinov na površini nerekombinantnih bakterij odpira številne nove možnosti uporabe v terapiji.

Ključne besede:

LysM, površinska predstavitev, DARPini, *Lactobacillus salivarius, Lactococcus lactis*

Izjava

Podpisana Petra Zadravec izjavljam, da članka Improvement of LysM-mediated surface display of DARPins in recombinant and non-recombinant *Lactococcus lactis* and *Lactobacillus* species nihče od soavtorjev ni uporabil kot kriterij za zagovor doktorata.

Petra Zadravec

Improvement of LysM-mediated surface display of DARPins in recombinant and non-recombinant *Lactococcus lactis* and *Lactobacillus* species

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Running title: Surface display of DARPins in Lactic Acid Bacteria

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Abstract

Safety and probiotic properties make Lactic Acid Bacteria (LAB) attractive hosts for surface display of heterologous proteins. Their display on non-recombinant microorganisms is preferred for therapeutic and food applications due to regulatory requirements. We have displayed two designed ankyrin repeat proteins (DARPins), each possessing affinity for the Fc region of human IgG on the surface of *Lactococcus lactis*, by fusing them to the Usp45 secretion signal and to the peptidoglycan binding C-terminal of AcmA containing lysine motif (LysM) repeats. Growth medium containing secreted fusion protein was used to test its heterologous binding to ten strains of species of genus *Lactobacillus*, using flow cytometry, whole-cell ELISA and fluorescence microscopy. Fusion protein bound to the surface of all lactobacilli; however binding to the majority of the bacteria was only 2-5-fold stronger than control. *Lactobacillus salivarius* ATCC11741 demonstrated exceptionally strong binding, 32-55-fold higher than control, and could therefore be an attractive host for non-recombinant surface display. Genomic comparison of the species indicated the exopolysaccharides of *Lb. salivarius* as a possible reason for the difference. Additionally, a 15-fold concentration-dependent increase in non-recombinant surface display in *L. lactis* was demonstrated by growing the bacteria in sub lethal concentrations of antibiotics chloramphenicol and erythromycin. Non-recombinant surface display on LAB, based on the LysM repeats, was optimized by selecting *Lactobacillus salivarius* ATCC11741 as the optimal host, and by introducing antibiotics as additives for increasing surface display in *L. lactis*. Additionally, effective display of DARPins on the surface of non-recombinant LAB has opened up several new therapeutic possibilities.

Keywords

LysM, surface display, DARPins, *Lactobacillus salivarius*, *Lactococcus lactis*

Introduction

The ability to display heterologous proteins on bacterial surfaces is becoming increasingly important in various fields of biotechnology (1-3). Bacteria displaying heterologous proteins can be used as bioadsorbents, biosensors, biocatalysts and oral vaccines, and in antibody production, screening of peptide libraries and detection of mutations (1-3). Lactic Acid Bacteria (LAB) are particularly attractive hosts due to their long-term usage in food, industrial applicability and general acceptance of their probiotic properties (4). Several approaches to the display of heterologous proteins on the surface of LAB have been established, including

LPXTG-type domains (5), lysine motif (LysM) domains (6), surface layer proteins (7) and lipoprotein anchors (8). Display of heterologous proteins on the surface of LAB has been exploited for the preparation of mucosal vaccines (9, 10), for the delivery of binding molecules to the gastrointestinal tract (11), for the assembly of macromolecular enzyme complexes (12), and for bacterial immobilization (13).

The non- recombinant approach to surface display is preferred for therapeutic and food applications. This can be achieved by non-covalent binding of recombinant proteins to the surface of non-recombinant bacteria. The feasibility of such an approach has already been demonstrated by using the C-terminal part of lactococcal AcmA protein (cA) as the cell wall anchor in lactobacilli (14) and in non-viable gram-positive enhancer matrix (GEM) particles (15). AcmA is an autolysin (N-acetylmuramidase) with a vital role in cell division in *Lactococcus lactis.* It comprises an enzymatic domain at the N-terminus and a peptidoglycan-binding domain at the C-terminus that comprises 3 LysM repeats (14, 16, 17). cA has been used as a fusion partner for non-covalent attachment of heterologous proteins to the surface of LAB, *e.g.* malaria antigen to *Lactobacillus sake* and *Lactobacillus casei* (14), α-amylase to *Lactobacillus plantarum* and *Lactobacillus casei* (18), chicken anaemia virus coat proteins to *Lactobacillus acidophilus* (19) and TNFα-specific affibody to *Lactococcus lactis* (11).

Other LysM repeat-containing proteins have also been applied as anchors for surface display on LAB with similar effectiveness. These include bacteriophage endolysin Lyb5 (20) and muropeptidase MurO from *Lactobacillus plantarum* (21). Differences in the ability to bind LysM repeat-containing proteins have been observed between *Lactobacillus* species, which reflect the differences in lactobacilli surfaces (22). *Lb. casei* bound LysM-containing proteins at the cell poles, *Lb. sake* over the entire surface and *Lb. helveticus* did not bind at all (14). This suggests that a more detailed and quantitative comparison of various *Lactobacillus* species could yield species or strains that would be particularly suitable for LysM repeat-based surface display.

Binding of LysM-containing proteins to the bacterial surface can be hindered by surface structures that spatially obstruct the accessibility of peptidoglycan, such as lipoteichoic acid (LTA) or surface layer proteins (14). Bacterial surface structures and surface proteome composition can be perturbed by changing the growth conditions or exposing bacteria to exogenous substances, which could consequently lead to differences in LysM-mediated binding. Antibiotics can cause significant alterations in gene transcription at sub-inhibitory concentrations, and stress response in sub-lethal concentrations (23). Erythromycin (24) and chloramphenicol (25) altered the transcription of more than 600 genes in *Enterococcus*

faecalis. Treatment with bile, which also has antimicrobial activity, altered the transcription of more than 300 genes, and caused changes in several cell-envelope related functions in *Lactobacillus rhamnosus* (26).

We have previously demonstrated LysM-mediated surface display of affibodies (11). Here we introduce designed ankyrin repeat proteins (DARPins) that bind the Fc domain of human IgGs (27) as model proteins for testing LysM-mediated surface binding. DARPins are small, non-antibody protein scaffolds that can be selected from combinatorial libraries to bind numerous proteins (28). The first successful display of DARPins on the surface of LAB broadened the spectrum of successfully displayed binding proteins and opened up several new therapeutic and other possibilities.

The goal of the present study was to improve heterologous LysM-mediated non-recombinant surface display in LAB. In order to achieve this, 10 strains of lactobacilli species were screened to select an optimal *Lactobacillus* carrier for heterologous non-recombinant surface display. Additionally, erythromycin and chloramphenicol, two antibiotics commonly used in LAB research, were tested for their impact on LysM-mediated surface display.

Materials and methods

Bacterial strains, media and culture conditions

The bacterial strains used in this study are shown in Table 1. *L. lactis* NZ9000 was grown at 30 °C in M-17 medium (Merck) supplemented with 0.5 % glucose (GM-17) without aeration. To maintain selection pressure on transformation, 10 μg/mL of chloramphenicol was added. To evoke surface changes in untransformed strains, 1, 2 and 3 μg/mL of chloramphenicol and 0.05, 0.075 and 0.1 μg/mL of erythromycin were added. *Lactobacillus* strains were grown in De Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany) at 37 °C without aeration. *E. coli* strain DH5α was grown at 37 °C with aeration in LB medium supplemented with 100 μg/mL ampicillin or 10 μg/mL of kanamycin.

Molecular cloning

DARPins I_07 and I_19 (27) were used as model binding proteins for surface display on *L. lactis*. Amino acid sequences of DARPins were back-translated and codon-optimized for expression in *L. lactis*, yielding genes DARPin I_07 and DARPin I_19 (Table 1). Genes were purchased from GeneArt Gene Synthesis (Regensburg, Germany) and cloned to plasmid pSDLBA3b (Table 1) using BamHI/EcoRI restriction enzymes (New England Biolabs, Beverly, USA), yielding plasmids pSD_I07 and pSD_I19. 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. All plasmids were sequenced by GATC (Constance, Germany).

TABLE 1 Strains, plasmids, and genes used in this study

Strain, plasmid, or gene	Relevant features or sequence (5' – 3')[a]	Reference or source
Strains		
E. coli DH5α	endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR F- Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–	Invitrogen
L. lactis NZ9000	MG1363 nisRK ΔpepN	NIZO
Lb. acidophilus ATCC 4356	Wild type	ATCC
Lb. delbrueckii subsp. *bulgaricus* ATCC 1184	Wild type	ATCC
Lb. casei ATCC 393	Wild type	ATCC
Lb. gasseri ATCC 33323	Wild type	ATCC
Lb. gasseri K7	Wild type	44
Lb. paracasei ATCC 25302	Wild type	ATCC
Lb. plantarum ATCC 8014	Wild type	ATCC
Lb. reuteri ATCC 55730	Wild type	ATCC
Lb. rhamnosus ATCC 53103	Wild type	ATCC
Lb. salivarius ATCC 11741	Wild type	ATCC
Plasmids		
pNZ8148	pSH71 derivative, PnisA, CmR, nisin-controlled expression	NIZO
pSDLBA3b	pNZ8148 containing gene fusion of Usp45 signal peptide, B domain and cA	11
pMA-T-I07	pMA-T containing DARPin I_07 gene, AmpR	Gene Art
pMK-RQ-I_19	pMK-RQ containing DARPin I_19 gene, KanR	Gene Art
pSD_I07	pNZ8148 containing gene fusion of Usp45 signal peptide, DARPin I07 and cA	This work
pSD_I19	pNZ8148 containing gene fusion of Usp45 signal peptide, DARPin I19 and cA	This work
Genes		
DARPin I_07	GGATCCGATCTTGGTAAAAAACTTCTTGAAGCAGCACGAGCTGGACAAGACG ACGAAGTACGTATCCTTATGGCTAACGGTGCCGATGTTAACGCATCAGATA AATCTGGTTATACTCCATTACATTTGGCCGCACATATTGGTCATTTAGAAAT TGTTGAGGTATTATTGAAGCATGGTGCTGATGTTAATGCACATGATTCATGG GGAGACACTCCTCTTCATTTAGCCGCCACTTTTGGTCACTTAGAAATCGTAG AAGTACTTTTAAAACATGGTGCTGACGTTAATGCACAAGACAAATTTGGAA AAACAGCATTTGACATTTCAATTGATAACGGTAATGAAGACTTGGCAGAGA TCTTACAAGAATTC	This work
DARPin I_19	GGATCCGATTTAGGAAAGAAGTTATTAGAAGCAGCACGAGCAGGACAAGACG ATGAGGTAAGAATCTTAATGGCTAATGGTGCTGACGTAAACGCTGATGATA ACAAAGGAGATACTCCACTTCATTTAGCTGCATCATTCGGTCACTTAGAAAT TGTCGAAGTACTTCTTAAAAACGGAGCTGATGTAAACGCAGACGATTACTTT GGAGATACACCATTACACTTAGCAGCTTGGTCTGGTCATCTTGAAATTGTTG AAGTTTTATTAAAATATGGTGCAGATGTAAATGCACAAGATCAACGTGGAT TTACACCTTTACATCTTGCTGCTATCGCTGGACACCTTGAAATTGTAGAAGT TTTACTTAAATATGGAGCTGACGTTAACGCACAAGATAAGTTTGGAAAAAC TGCTTTCGATATTAGTATTGATAATGGTAACGAAGACCTTGCAGAAATCTTA CAAGAATTC	This work

[a] The introduced restriction sites are underlined in sequences.

Expression of DARPin-cA fusion proteins in *L. lactis*

Overnight cultures of *L. lactis* NZ9000 harbouring plasmids pSD_I07 and pSD_I19 were diluted (1:100) in 10 mL (or 100 mL) of fresh GM-17 medium and grown to optical density A_{600} = 0.80. Fusion protein expression was induced with 25 ng/mL nisin (Fluka AG, Buchs, Switzerland). After three hours of 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. The cell pellet was resuspended in 500 µL of 0.1 M potassium phosphate buffer (PBS, pH 7.4) and

stored at -20 °C for SDS PAGE analysis, or resuspended in PBS to optical density A_{600} = 1.0 and stored at 4 °C for whole-cell ELISA. The supernatant was decanted, filtered with a 0.22 μm pore size filter (Minisart, Millipore, Billerica, USA), aliquoted, and stored at -20 °C for testing DARPin-cA fusion protein binding to non-recombinant bacteria.

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). The membrane was blocked in 5 % skimmed milk and incubated overnight at 4°C with FITC conjugated human IgG (Jackson ImmunoResearch, West Grove, USA; 1:1000 dilution). After washing with 0.05 % TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween 20, pH 7.5), fluorescence was detected with ChemiDoc MP Imaging System (BioRad) using blue excitation (488 nm).

Whole-cell ELISA

The whole-cell enzyme-linked immunosorbent assay (ELISA) was performed as described (29). For testing DARPin surface display, 750 μL of *L. lactis* or 1 mL of *Lactobacillus sp.* cell suspension in PBS with optical density A_{600} = 1.0 was centrifuged (5000 × g, 5 min, 4 °C) and washed twice with 500 μL PBS. Cells were then resuspended in 200 μL of fluorescein isothiocyanate (FITC)-conjugated human IgG (Jackson ImmunoResearch, West Grove, USA; diluted 1: 500 in PBS), and incubated 1 h at room temperature (RT) with gentle shaking. Cells were then washed twice with PBS and resuspended in 200 μL of peroxidase-conjugated mouse anti-human IgG (Jackson ImmunoResearch, West Grove, USA; diluted 1:2500 in PBS). After 1 h of incubation at RT with gentle shaking, cells were washed first with PBS, and then with substrate buffer (150 mM Na_2HPO_4, 50 mM citric acid, pH 6.0). Cells were then resuspended in 1 mL of substrate buffer, and 100 μL of appropriate dilutions in substrate buffer (1:5 and 1:25) were loaded on a microtiter plate. 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, Steinheim, Germany) was added and the reaction stopped after 15 min by the addition of 50 μL of 2 M sulphuric acid. Absorbances were read at 450 nm using an Infinite M1000 (Tecan, Salzburg, Austria).

A similar protocol was used, with a few modifications, for quantifying lipoteichoic acid (LTA) on the surface of *Lactobacillus* species. Monoclonal antibody to LTA (clone 55, Hycult biotech, Uden, The Netherlands; diluted 1:250 in PBS) was used as primary antibody. Peroxidase-conjugated goat anti-mouse IgG (Merck Millipore, Billerica, USA; diluted 1:500 in PBS) was used as secondary antibody. Colour was developed for 20 min before stopping the reaction.

Flow cytometry and fluorescence microscopy

For flow cytometry 10 µL of cell culture in stationary phase was added to 500 µL of Tris-buffered saline (TBS; 50mM 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 and 1 µL of FITC-conjugated human IgG antibody (Jackson ImmunoResearch, West Grove, USA) was added. 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 analysed 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 cell in the appropriate gate was measured. The average of at least two independent experiments was considered.

The protocol for preparing specimens for fluorescence microscopy was similar, except that the starting volume of the cell cultures was 20 µL and the final resuspension volume 300 µL. Stained cells were fixed to the microscopic slide using the StatSpine Cytofuge 2 (Iris Sample Processing, Westwood, USA) for 10 min at maximum speed. A LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) was used for observing prepared specimens. Alexa Fluor 488 was excited with an argon laser (488 nm), and the emission was filtered with a narrow-band 505-530 nm filter. All images were taken under the same settings and were analysed using Carl Zeiss ZEN lite 2012 software.

Binding of DARPIN-cA fusion proteins to non-recombinant bacteria

Cell cultures of *L. lactis* NZ9000 and *Lactobacillus* sp. were grown to optical density 2.0 – 3.0 (late exponential phase). For flow cytometry and fluorescence microscopy, 20 µL of the cell culture was added to 500 µL of TBS and centrifuged for 5 min at 5000 × g at 4 °C, followed by resuspension of the pellet in 500 µL of filtered DARPin I07-cA medium. Suspensions were incubated for 2 h at RT in a tube rotator. After incubation, cells were washed once with 500 µL TBS and stained as described above for flow cytometry and

fluorescence microscopy. Control samples were stained without prior binding of DARPin I07-cA fusion to the bacterial surface.

For whole-cell ELISA, 1 mL of *Lactobacillus sp.* cell suspension in PBS with optical density A_{600} = 1.0 was added to 20 mL of filtered DARPin I07-cA medium and incubated for 2 h at RT in a tube rocker. Control bacteria were incubated with filtered growth medium of empty plasmid (pNZ8148)-containing bacteria and treated in the same fashion. After incubation, cells were centrifuged for 10 min at 5000 × g at 4 °C and resuspended in 500 μL of PBS, washed and stained as described above for whole-cell ELISA.

Genomic comparison of *Lactobacillus* species

Integrated microbial genomes (IMG) software (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) was used to compare the eight out of ten *Lactobacillus* species that were used in the study and whose genomes have been sequenced. Additionally, the well-studied probiotic *Lb. salivarius* UCC 118, and *Lb. plantarum* ATCC 14917 (close relative of *Lb. plantarum* ATCC 8014 (30)) were included in the comparison. Clusters of orthologous groups (COGs) from the category cell wall/membrane/envelope biogenesis were compared. COGs that were present either in *Lb. salivarius* alone, or absent only from *Lb. salivarius* were identified and studied further in the genome of *Lb. salivarius* ATCC 11741 to elucidate their possible functional role. BLAST analysis of *Lb. salivarius* ATCC 11741 genome was performed to determine the presence of LTA biosynthesis genes and LTA D-alanylation genes. Genes from *Lb. acidophilus* NCFM, in which LTA biosynthesis pathway was annotated (31), were used as query sequences for BLAST search.

Statistics

Statistical analyses were performed with GraphPad Prism 5.0 software. Student's t test was used to compare the significance of the differences between samples.

Results

Expression and surface display of DARPIN-cA fusion proteins on *L. lactis* NZ9000

Genes for two DARPins (I_07 and I_19), both expressing affinity for the Fc region of human IgG, were codon-optimized for expression in *L. lactis* and synthesized. Both genes were cloned, under the control of NisA promoter, to our previously reported plasmid for surface-display (11) that enables fusion to the Usp45 secretion signal and to the LysM repeats-containing C-terminal domain of AcmA protein (cA). Expression of fusion proteins was induced by adding nisin to the growth medium and was confirmed in the cell lysate by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 1A). After Western blot, functional DARPin-containing fusion proteins were detected indirectly via their ability to bind the Fc region of human IgG (Fig. 1B). Stronger binding was observed with DARPin I07.

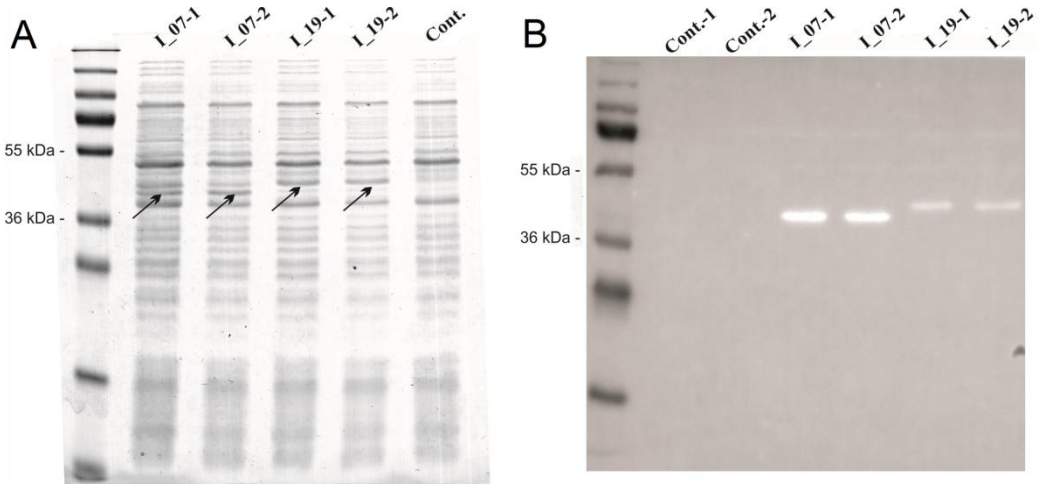

FIG 1 SDS-PAGE (A) and Western blot (B) of lysates of *L. lactis* cells expressing DARPins in fusion with Usp45 secretion signal and LysM-containing cA domain, stained with Coomassie Brilliant Blue. I_07: DARPin I_07-cA. I_19: DARPin I_19-cA fusion. Cont.: control containing empty plasmid pNZ8148. DARPin-cA fusion proteins are indicated with arrows

Surface display of both DARPins was also evaluated indirectly via their ability to bind the Fc region of human IgGs and confirmed with flow cytometry (Fig. 2A), whole-cell ELISA (Fig. 2B) and fluorescence microscopy (Fig. 2C). The amount of IgG bound by bacteria with surface-displayed I_07 DARPin was at least two fold higher than those with I_19 DARPin-displaying bacteria, as determined by both flow cytometry and whole-cell ELISA.

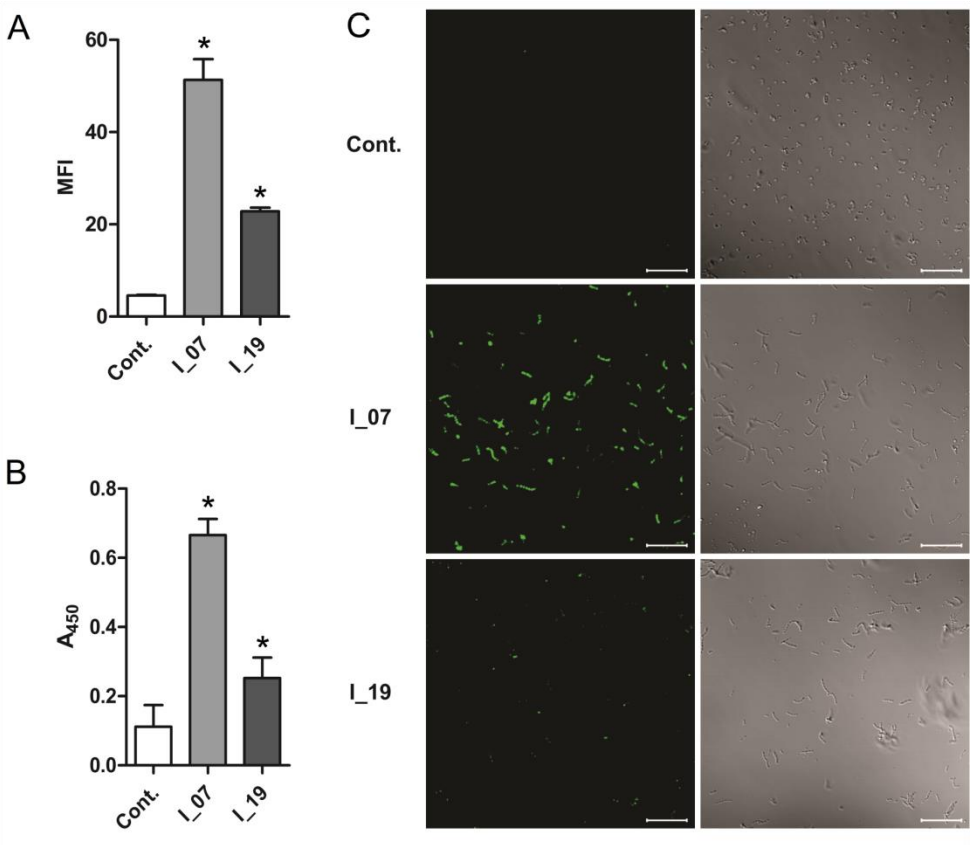

FIG 2 Flow cytometry (A), whole cell ELISA (B) and fluorescence microscopy (C) of *L. lactis* cells expressing DARPins I_07 and I_19, detected with FITC-conjugated human IgG. Cont.: control containing empty plasmid pNZ8148. MFI: Mean fluorescence intensity. A450: Absorbance at 450 nm. Error bars denote standard deviation. *, significant difference (t test; $P<0.05$) in comparison to control. (C) Left: Fluorescence image. Right: Bright field image. Bars = 20 μm.

Selection of the optimal *Lactobacillus* host for LysM-mediated non-recombinant surface display of DARPin-cA fusion proteins

Sterile growth medium containing DARPin I07-cA fusion was incubated with ten strains of genus *Lactobacillus* (Table 1). Binding of DARPin I07-cA fusion to the bacterial surface was detected indirectly via the ability of DARPin I07 to bind Fc region of human IgG. DARPin-bound immunoglobulins were quantitated with flow cytometry and whole-cell ELISA. The mean fluorescence intensity (MFI) value, obtained with flow cytometry for each *Lactobacillus* species, was normalized relative to the control sample, in which bacteria were incubated with immunoglobulins without prior binding of DARPin I07-cA fusion to the bacterial surface (Fig. 3A). The A_{450} values, obtained with whole-cell ELISA, were normalized relative to the control samples, in which bacteria were incubated with growth medium of empty-plasmid-containing bacterial culture (Fig. 3B). Fluorescence microscopy (Supplementary material; Fig. S1) was used to observe the difference between DARPin I07-cA- bound and control bacteria on a single cell level.

Binding of DARPin I07-cA fusion to the surface of lactobacilli was confirmed for all bacterial species with whole-cell ELISA, and for seven species out of ten with flow cytometry. Majority of species exhibited weak to moderate binding (approximately 2-5-fold higher than control), depending on the method of detection used. Stronger binding of *Lb. paracasei* ATCC 25302 (more than 16-fold higher than control) was determined with whole-cell ELISA, but could not be confirmed with flow cytometry or fluorescence microscopy. The strongest binding was observed with *Lb. salivarius* ATCC 11741 and was confirmed with all three methods: flow cytometry (more than 55-fold higher MFI), whole-cell ELISA (more than 32-fold higher A_{450}) and fluorescence microscopy (much higher fluorescence than with any other *Lactobacillus* species).

To establish whether the increased content of LTA on the surface of lactobacilli correlates with lower LysM-mediated surface binding we quantified LTA on the surface of lactobacilli using whole-cell ELISA with LTA-specific monoclonal antibody (Fig. 3C). Even though not statistically significant, the results suggest that higher amounts of LTA correlate with lower LysM-mediated surface binding, as observed with *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. plantarum* and *Lb. reuteri*.

The exceptional surface properties of *Lb. salivarius*

Genome sequences were compared to identify possible traits that separate *Lb. salivarius* from the rest of the *Lactobacillus* species tested. We narrowed the comparison to genes involved, or predicted to be involved, in synthesis of the cell wall, cell membrane or exopolysaccharides (EPS). 4 COGs were identified that are present in *Lb. salivarius* only, and 1 that is present in all *Lactobacillus* species tested, except in *Lb. salivarius* (Table 2). The unique *Lb. salivarius* COGs contain 6 genes, among which 5 are included in the EPS biosynthesis cluster (Table 2), which suggests a role for EPS in LysM-mediated surface display.

To establish possible influence of LTA (or its absence) on the surface properties of *Lb. salivarius*, the presence of LTA biosynthesis and LTA D-alanylation genes were confirmed with high probability in the genome of *Lb. salivarius* with BLAST analysis (E-values of < $1{,}0\times10^{-21}$), suggesting that *Lb. salivarius* is capable of producing LTA (Table 3).

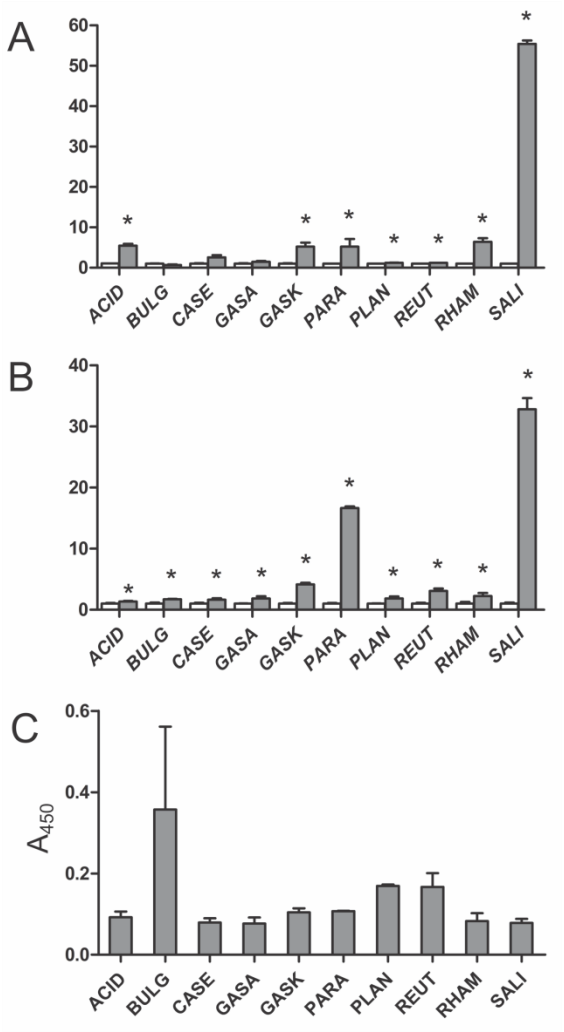

FIG 3 Flow cytometric (A) and whole-cell ELISA (B) analysis of *Lactobacillus sp.* cells after incubation with DARPin I07-cA fusion-containing growth medium and detection with FITC-conjugated human IgG (gray bars). MFI and A_{450} values were normalized to those for the control samples (white bars). (C) Quantification of LTA on the surface of *Lactobacillus sp.* by whole-cell ELISA, using an LTA-specific monoclonal antibody. ACID: *Lb. acidophilus* ATCC 4356, BULG: *Lb. delbrueckii* subsp. *bulgaricus* ATCC 1184, CASE: *Lb. casei* ATCC 393, GASA: *Lb. gasseri* ATCC 33323, GASK: *Lb. gasseri* K7, PARA: *Lb. paracasei* ATCC 25302, PLAN: *Lb. plantarum* ATCC 8014, REUT: *Lb. reuteri* ATCC 55730, RHAM: *Lb. rhamnosus* ATCC 53103, SALI: *Lb. salivarius ATCC 11741*. *, significant increase in IgG binding (t test; $P<0.05$) in comparison to control.

TABLE 2 IMG analysis of COGs unique to *Lb. salivarius*

Locus tag	Gene product description	COG no.	COG description
HMPREF0545_0918	UDP-glucose 6-dehydrogenase	COG1004	Predicted UDP-glucose 6-dehydrogenase
HMPREF0545_0471[a]	UDP-glucose 6-dehydrogenase	COG1004	Predicted UDP-glucose 6-dehydrogenase
HMPREF0545_0474[a]	Glycosyltransferase	COG3754	Lipopolysaccharide biosynthesis protein
HMPREF0545_0475[a]	Glycosyltransferase	COG3754	Lipopolysaccharide biosynthesis protein
HMPREF0545_0486[a]	Pyridoxal-phosphate-dependent aminotransferase	COG0399	Predicted pyridoxal phosphate-dependent enzyme apparently involved in regulation of cell wall biogenesis
HMPREF0545_0487[a]	Possible capsular polysaccharide biosynthesis protein	COG1086	Predicted nucleoside-diphosphate sugar epimerases
NP[b]	NP[b]	COG1732	Periplasmic glycine betaine/choline-binding (lipo)protein of an ABC-type transport system (osmoprotectant binding protein)

[a] Part of the EPS biosynthesis cluster.
[b] NP, not present in *Lactobacillus salivarius* ATCC 11741.

TABLE 3 BLAST analysis of *Lb. salivarius* ATCC 11741 genes

Gene category and locus tag	Gene description	Query sequence[a]	E-value
Genes involved in LTA byosynthesis			
HMPREF0545_1244	Glycosyltransferase	LBA0444	$3{,}0\times10^{-144}$
HMPREF0545_1243	Glycosyltransferase	LBA0445	$2{,}0\times10^{-140}$
HMPREF0545_1242	Integral membrane protein	LBA0446	$4{,}0\times10^{-65}$
HMPREF0545_1240	Phosphatidylglycerol-membrane-oligosaccharide glycerophosphotransferase	LBA0447	0
Genes involved in LTA D-alanylation			
HMPREF0545_0394	D-alanine-D-alanyl carrier protein ligase (DltA)	LBA1926	0
HMPREF0545_0393	DltB	LBA1925	$1{,}0\times10^{-166}$
HMPREF0545_0392	D-alanine-poly(phosphoribitol) ligase subunit (DltC)	LBA1924	$1{,}0\times10^{-21}$
HMPREF0545_0391	DltD precursor	LBA1923	$1{,}0\times10^{-125}$

[a] Query sequences are from *Lactobacillus acidophilus* NCFM.

Antibiotic-induced increase in LysM-mediated non-recombinant surface display on *L. lactis*

Non-recombinant *L. lactis* cells were grown to late exponential phase in the presence of several concentrations of erythromycin or chloramphenicol, followed by incubation with DARPin I07-cA fusion. Surface binding was measured with flow cytometry. The presence of erythromycin or chloramphenicol in the growth medium caused significant, antibiotic concentration-dependent, increases in LysM-mediated binding to the bacterial surface (Fig. 4). The largest increases in surface binding were observed with 0.1 μg/ml erythromycin (Fig. 4A), and 3 μg/ml chloramphenicol (Fig. 4B).

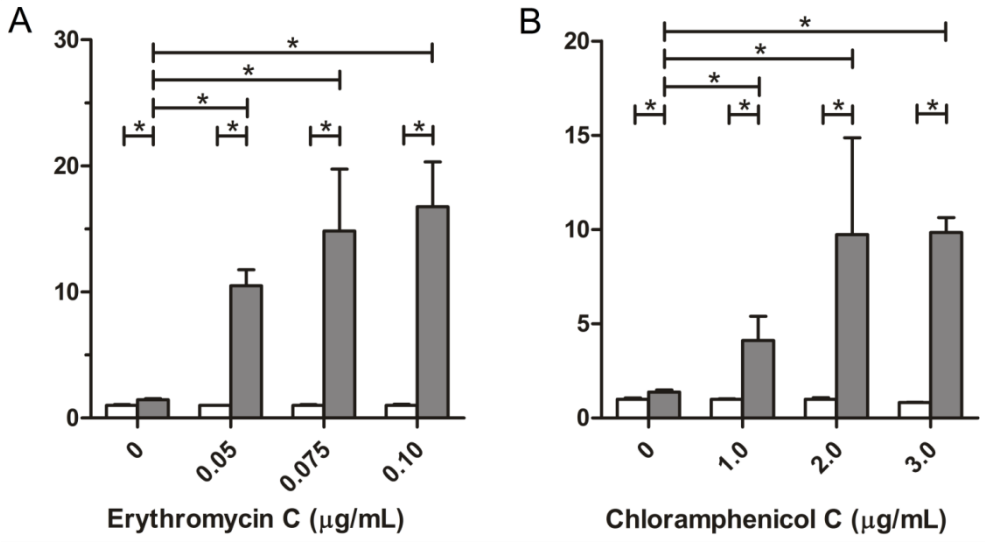

FIG 4 Flow cytometric analysis of LysM-mediated non-recombinant surface display on *L. lactis* after treatment of bacteria with different concentrations of antibiotics erythromycin (A) and chloramphenicol (B). Binding of DARPin I07-cA fusion to the bacterial surface was detected with FITC-conjugated human IgG (gray bars). MFI values were normalized to those for the control samples (white bars) in which bacteria were incubated with immunoglobulins without prior binding of DARPin I07-cA fusion to the bacterial surface. Error bars denote standard deviations. *, significant difference (t test; $P<0.05$) between the pair of samples indicated.

Discussion

LysM-mediated binding to the surface of LAB was assessed by introducing two DARPins (I_07 and I_19) as model binding proteins, both expressing affinity for the Fc region of human immunoglobulins. DARPins that have already been developed against several other targets are potentially applicable in LAB-mediated therapy, such as lactococcal phage TP901-1 (32), HIV gp120 (33) and human IgE (34). We have genetically fused DARPins I_07 and I_19 to the C-terminal of AcmA protein and to the Usp45 secretion signal and expressed the resulting fusion proteins in *L. lactis* using nisin-controlled expression (NICE). Both fusion proteins (I_07-cA and I_19-cA) were over-expressed in the cytoplasm (Fig. 1A) and were able to bind Fc of human IgG (Fig. 1B); however stronger binding was observed with DARPin I_07. The secretion of both fusion proteins into the growth medium, subsequent surface binding and human IgG Fc-binding ability were confirmed with flow cytometry, whole-cell ELISA and fluorescence microscopy. I_07-cA again bound more strongly to IgG Fc than I_19-cA, as determined with all three methods (Fig. 2), and was therefore used for further testing of surface display. The stronger I_07-cA binding contrasts with that reported (27), in which I_19 was found to bind more strongly. The expression levels of the two DARPin fusion proteins were the same and we attribute the stronger IgG binding with I_07-cA to the more complex folding of the larger I_19 (containing 5 ankyrin repeats) in comparison to I_07 (containing 4 ankyrin repeats). The display of human IgG-binding DARPins on the surface of *L. lactis* adds another class of LAB-displayed binders to the existing pool that includes affibodies, nanobodies and single-chain variable fragments (11, 35, 36).

The LysM repeat-containing fusion protein is secreted to the growth medium with the Usp45 secretion signal. Because not all fusion protein binds to the producer cells, the spent growth medium can be applied for heterologous binding of LysM repeat-containing fusion protein to unmodified bacterial cells, as already demonstrated (14, 20). Heterologous binding can be considered to be non-recombinant, which is a great advantage in view of the significant regulatory obstacles and low public acceptance of genetically modified organisms (GMOs) (37). Heterologous binding was very effective on naked peptidoglycan particles, while binding to lactobacilli varied, and was species-dependent (14, 15). The differences between lactobacilli were quantified by flow cytometry and whole-cell ELISA, using DARPin-cA fusion protein as a probe for surface binding mediated by the LysM repeats (Fig. 3). The results of flow cytometry were substantiated by those obtained using fluorescence microscopy

(Fig. S1). DARPin-cA fusion protein bound to the surface of all lactobacilli species tested; however for the majority of species weak to moderate binding was observed. Stronger binding to the surface of *Lb. paracasei* ATCC 25302 was observed with whole-cell ELISA, but not with flow cytometry or fluorescence microscopy, suggesting intraspecies variability which might be growth-phase dependent and would require further study. The strongest binding was observed with *Lb. salivarius* ATCC 11741 and was confirmed with all three methods. *Lb. salivarius* ATCC 11741 exhibited so far unique properties for LysM mediated surface display, largely surpassing all other lactobacilli strains (more than 15-fold or 8.5-fold stronger binding than the average in other lactobacilli, as determined with flow cytometry and whole-cell ELISA, respectively). *Lb. salivarius* ATCC 11741 therefore has the potential to become a non-recombinant surface display host, particularly as it has already been reported to be a probiotic with antipathogenic and TNFα-stimulatory activity (38). However, its applicability will have to be verified in further studies. Our future work will therefore include testing of *Lb. salivarius* ATCC 11741 as a non-recombinant vector for the intestinal delivery of cytokine- and toxin-binding proteins in mouse disease models.

The observed difference in surface display ability between *Lb. salivarius* ATCC 11741 and the other lactobacilli tested may be explained as the consequence of different surface structures or of their chemical composition. Higher content of cell wall LTA has already been shown to correlate with lower LysM-mediated surface binding. We also observed that species containing more LTA on their surface exhibited lower LysM-mediated display ability (*Lb. delbrueckii* subsp. *bulgaricus* ATCC 1184, *Lb. plantarum* ATCC 8014 and *Lb. reuteri* ATCC 55730). However, this is not the case for all species and, in particular, could not explain the much larger LysM-mediated display ability observed with *Lb. salivarius*. We therefore performed a genomic comparison of *Lb. salivarius* with the rest of the species for which genomic information is available. There are no apparent differences in the presence of LTA biosynthetic genes between *Lb. salivarius* and the rest of the lactobacilli, although this comparison is hampered by differences in LTA biosynthesis and the lack of complete understanding of LTA biosynthesis in lactobacilli. The presence of LTA biosynthesis cluster, homologous to that of *Lb. acidophilus* NCFM (31), and of LTA D-alanylation cluster in *Lb. salivarius* has been confirmed. More general comparison of genes involved in the synthesis of cell wall, membrane or envelope across the tested species revealed five *Lb. salivarius*-specific genes (present in unique clusters of orthologous groups – COGs) that are located in one of the two *Lb. salivarius* EPS biosynthetic clusters (39). *Lb. salivarius* ATCC 11741 has already been described as a more intensive producer of EPS than other lactobacilli strains (40). The

most prominent difference observed was the presence of UDP-glucose-6-dehydrogenase (UGDH) gene (HMPREF0545_0471) in the *Lb. salivarius* ATCC11741 genome. UGDH can convert UDP-glucose to UDP-glucoronate which is rarely observed in lactobacilli EPS (41), but is a relatively common constituent of the capsule of pathogenic Gram-positive bacteria (42, 43). The interdependence of UDP-glucoronate monomer in the EPS structure and LysM-mediated binding remains to be confirmed, and more detailed characterization is therefore needed to confirm the possible involvement of UGDH or EPS structure.

The amount of LysM repeat-containing fusion protein on the surface was higher on a recombinant expression host, than on non-recombinant lactococci that bound the fusion protein from the spent growth medium of recombinant producer. This can be attributed to partial removal of the LysM repeat-containing fusion protein from the growth medium by the producer cells which, to a certain extent, limits the applicability of the procedure. We also observed that recombinant expression and display of the fusion protein was significantly increased if the producer cells contained a gene that conferred resistance to a second antibiotic (the first being part of the NICE system; data not shown). We therefore turned our attention to the influence of non-lethal concentrations of antibiotics on non-recombinant lactococci. Surprisingly, a concentration dependent, up to 10-fold increase in LysM-mediated surface binding was observed when the bacterial culture was grown in the presence of chloramphenicol or erythromycin. This is unexpected, because both antibiotics are ribosome inhibitors and do not interfere with cell-wall synthesis. However, stress (including antibiotic stress) can cause massive perturbations in patterns of bacterial gene expression, including genes in the cell surface homeostasis pathways (24, 25). In our experiments, treatment of cultures with antibiotics consistently resulted in greater LysM-mediated surface display, although the amount of the increase was less reproducible. Presumably there are factors that play an important role in antibiotic-induced surface changes that were not completely controlled in our experiments. Nevertheless, addition of antibiotics or, alternatively, more acceptable stress compounds, could provide an important means of increasing LysM-mediated display in non-recombinant bacteria.

The display of proteins on the surface of LAB usually requires their genetic modification, which limits or prevents its medical and food application due to poor public acceptance and unresolved legal issues. Non-covalent binding of heterologous proteins from a recombinant host to the surface of non-recombinant bacteria could constitute a regulatory bypass. In the present research we improved non-recombinant surface display in LAB. Ten species of lactobacilli have been screened and *Lb. salivarius* ATCC 11741 shown to have the greatest

LysM-mediated surface display ability, making it an attractive candidate for non-recombinant LysM-mediated surface display. Additionally, sub-lethal concentrations of the antibiotics erythromycin and chloramphenicol have been shown to increase LysM-mediated surface display in *L. lactis* in a concentration dependent manner. Addition of such stress factors could be used as a complementary strategy in improving LysM-mediated surface display in LAB. Finally, surface display of DARPins on various LAB species has been established, which offers an opportunity for new therapeutic applications.

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Dodatek k tretjemu poglavju

Supplemental figure 1

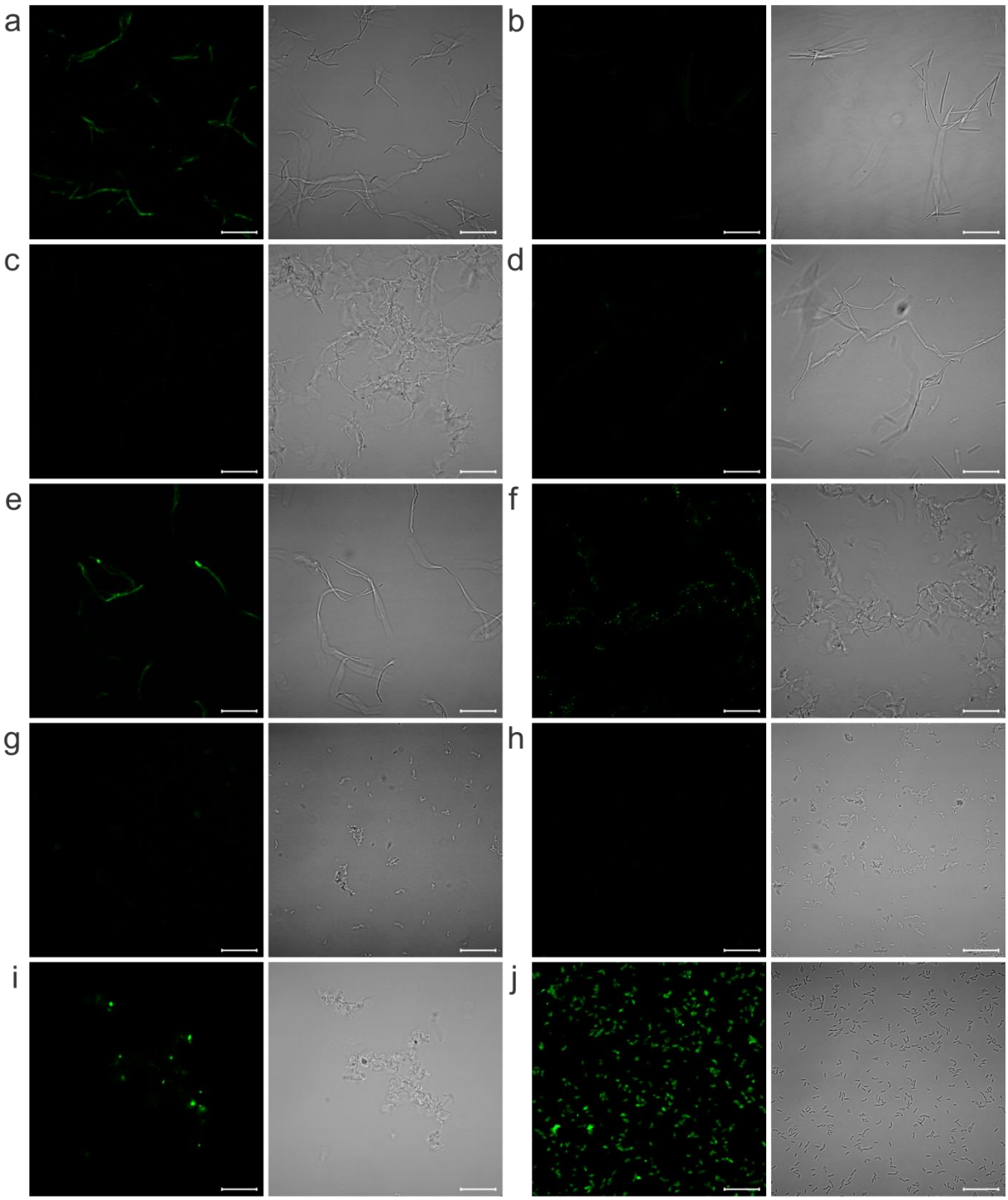

FIG S1 Fluorescence microscopy of *Lactobacillus sp.* cells after incubation with DARPin I07-cA medium and detection with FITC-conjugated human IgG. A: *Lb. acidophilus*; b: *Lb. delbrueckii* ssp. *bulgaricus;* c: *Lb. casei;* d: *Lb. gasseri* ATCC33323; e: *Lb. gasseri* K7; f: *Lb. paracasei;* g: *Lb. reuteri;* h: *Lb. plantarum;* i: *Lb. rhamnosus;* j: *Lb. salivarius.* Left: Fluorescence image. Right: Bright field image. Bar = 20 µm.

ČETRO POGLAVJE

Heterologna površinska predstavitev na mlečnokislinskih bakterijah: ne GSO alternativa?

Heterologous surface display on lactic acid bacteria: non-GMO alternative?

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POVZETEK

Mlečnokislinske bakterije (MKB) so prehransko sprejemljivi gostiteljski organizmi za površinsko predstavitev z velikim potencialom v živilstvu in terapiji. Z alternativnimi pristopi k površinski predstavitvi na MKB bi se izognili uporabi rekombinantne DNA tehnologije in regulatornim zahtevam, ki so povezane z gensko spremenjenimi organizmi (GSO). Nekovalentno površinsko predstavitev proteinov lahko dosežemo s fuzijo z različnimi domenami, ki se vežejo na celično steno in med katerimi je še posebej dobro proučena domena z lizinskimi motivi (LysM). Heterologna površinska predstavitev na površini nespremenjenih bakterij je mogoča z izolacijo fuzijskih proteinov iz rekombinantnih bakterij ali iz njihovih gojišč. Tovrsten način predstavitve je bil dokazan na neživih celicah brez proteinske vsebine, imenovanih bakterijam podobni delci, in na različnih sevih iz rodu *Lactobacillus*. Med njimi je bil sev *Lactobacillus salivarius* ATCC 11741 nedavno prikazan kot posebej primeren za LysM-posredovano predstavitev. Prispevek obravnava možne regulatorne implikacije heterologne površinske predstavitve, še posebej te, ki zadevajo Evropsko Unijo.

Ključne besede:

površinska predstavitev, domene, ki se vežejo na celično steno, LysM, bakterijam podobni delci, GEM, gensko spremenjeni organizmi, regulatorna agencija, EFSA, EMA

Izjava

Podpisana Petra Zadravec izjavljam, da članka Heterologous surface display on lactic acid bacteria: non-GMO alternative? nihče od soavtorjev ni uporabil kot kriterij za zagovor doktorata.

Petra Zadravec

Heterologous surface display on lactic acid bacteria: non-GMO alternative?

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Keywords

surface display, cell-wall binding domains, LysM, bacteria-like particles, Gram-positive enhancer matrix, genetically-modified organism, regulatory agency, EFSA, EMA

Abstract

Lactic acid bacteria (LAB) are food-grade hosts for surface display with potential applications in food and therapy. Alternative approaches to surface display on LAB would avoid the use of recombinant DNA technology and genetically-modified organism (GMO)-related regulatory requirements. Non-covalent surface display of proteins can be achieved by fusing them to various cell-wall binding domains, of which the Lysine motif domain (LysM) is particularly well studied. Fusion proteins have been isolated from recombinant bacteria or from their growth medium and displayed on unmodified bacteria, enabling heterologous surface display. This was demonstrated on non-viable cells devoid of protein content, termed bacteria-like particles, and on various species of genus *Lactobacillus*. Of the latter, *Lactobacillus salivarius* ATCC 11741 was recently shown to be particularly amenable for LysM-mediated display.

Possible regulatory implications of heterologous surface display are discussed, particularly those relevant for the European Union.

Heterologous surface display on LAB

Microbial surface display is an emerging technology with numerous potential applications in various fields of biotechnology. Engineering microorganisms to display proteins of choice on their surface renders them useful as whole-cell biocatalysts, vaccines, biosensors, delivery vectors, bioadsorbents, etc.[1] Microbial surface display can also be exploited for screening peptide libraries, detecting mutations and selecting binders.[1] Lactic acid bacteria (LAB) are attractive platforms for the surface display of heterologous proteins due to their "generally recognized as safe" status, industrial applicability and assumed beneficial health effects as probiotics.[2] Surface-engineered LAB could be used in food or in therapy; however recombinant LAB are genetically modified organisms (GMO) and therefore face low public acceptance and severe regulatory scrutiny, particularly in the European Union. Alternative non-GMO approaches to surface display are therefore being sought.

"Genetic modification" of bacteria for surface display could be avoided by non-covalent anchoring of recombinant heterologous proteins to the surface of wild-type (non-genetically modified) LAB, or to non-viable bacteria-like particles (BLPs). The latter are basically just peptidoglycan sacculi without proteinaceous content of the cytoplasm or of the cell wall (Figure 1). Since the recombinant protein is produced in a host different from the one on which it is to be displayed, the approach could be called heterologous surface display. Such an approach involves no change in the bacterial genetic information, but includes recombinant proteins. To enable heterologous surface display, the recombinant proteins have to be fused to a domain capable of binding to the cell wall. Covalent binding would require enzymatic intervention or addition of cross-linking reagents. Non-covalent binding requires no additives and is therefore preferred. Several non-covalent surface-binding domains that can bind to bacterial peptidoglycan have been described in the Pfam database (Table 1). These domains differ in length (20-200 amino acid residues), comprise various numbers of repeats (1 to 64) and can be positioned at the centre, N- or C-terminus of the recombinant protein. The exact binding sites on the cell surface for most of the presently considered non-covalent anchoring domains have not so far been determined. A few, however, have been characterized. For example, LysM domains recognize N-acetylglucosamine moieties of peptidoglycan[3], bacterial SH3 domains (Sh3b; common for genus *Staphylococcus*) recognize pentaglycine cross-

bridges, cell wall binding repeats (CW or ChBD) bind to choline of teichoic acids, and Cpl-7 binding domains bind to peptidoglycan in choline-independent fashion.[4, 5]

The majority of advances in non-covalent heterologous surface display have been achieved by involving the Lysine motif (LysM) domain from lactococcal AcmA protein as a surface anchor. This domain binds non-covalently to peptidoglycan in a species non-specific manner.[6] It consists of 1 to 12 LysM repeats, each of which contains 44-65 amino acid residues. The LysM domain is usually located on the C- or the N-terminus of proteins but can also be present in the middle. LysM domains, comprising more than 27,000 different sequences, from more than 4,500 species of prokaryotes, eukaryotes and viruses are described in the Pfam database.[7] LysM domains are the most studied non-covalent surface-binding domains and are used in several different industrial and medical applications, including surface display, cell immobilization, detection of bacteria and fungi and separation/purification processes.[8]

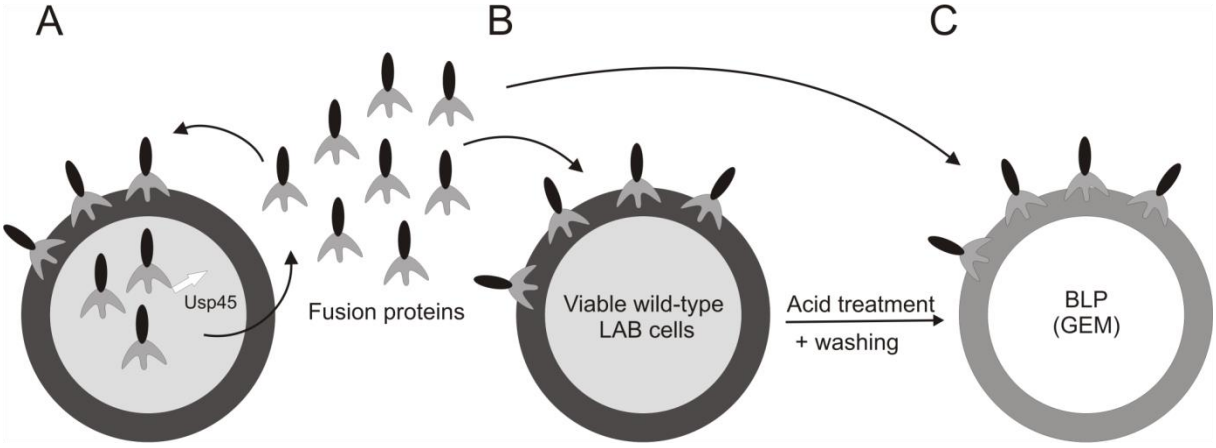

Figure 1 Schematic representation of non-covalent surface display on recombinant lactic acid bacteria (LAB; A), heterologous surface display on un-modified (wild-type) LAB (B) and heterologous surface display on bacteria like particles (BLPs) (C). Approaches (B) and (C) contain no recombinant DNA. Usp45: secretion signal. GEM: Gram-positive enhancer matrix.

Heterologous surface display on living LAB cells

The peptidoglycan-binding domain of lactococcal AcmA protein has been used as an anchor for heterologous surface display of various proteins on several LAB. AcmA is an N-acetylglucosaminidase with a role in cell wall remodelling during growth and division. It contains 3 LysM repeats at its C-terminal part (cA).[9] Steen et al.[6] demonstrated affective binding of *Plasmodium* antigen (MSA2) via cA to *Lactococcus lactis, Lb. sake, Lb. buchneri, Lb. plantarum* and *Lb. casei*. The distribution of MSA2_cA fusion protein on bacterial surfaces was species dependent; *Lb. casei* bound fusion protein only at the cell poles, while

Lb. sake bound over the entire surface. *Lb. helveticus*, on the other hand, bound poorly, which was attributed to the presence of S-layer proteins on the surface of this species.[6] *Lb. acidophilus* has been shown to be very effective vehicle for oral immunization of chickens against chicken anemia virus. The viral coat protein VP1 in fusion with cA has been displayed on the surface of non-recombinant *Lb. acidophilus* cells and used as a vaccine.[10] Other LysM-repeat containing proteins, including endolysin Lyb5[11] and muropeptidase MurO[12], have also been used as anchors for surface display of heterologous protein on non-recombinant LAB. The LysM domain of endolysin Lyb5 from *Lb. fermentum* bacteriophage has been fused with the reporter proteins GFP and β-galactosidase and displayed successfully on the surface of *L. lactis*, *Lb. brevis*, *Lb. plantarum*, *Lb. fermentum*, *Lb. delbrueckii*, and *Lb. helveticus.*[11] The same reporter proteins (GFP and β-galactosidase) have been functionally displayed on the surface of non-recombinant *Lb. plantarum* via two LysM repeat containing LysM domains from putative muropeptidase MurO, the autolysin from *Lb. plantarum.*[12] Surface display of non-recombinant heterologous proteins on LAB has also been established with the C-terminal region of the S-layer protein SlpB of *Lactobacillus crispatus* K2-4-3.[13] The efficacy of SlpB-mediated surface display has been confirmed by displaying GFP and β-galactosidase on the surfaces of *L. lactis*, *Lb. delbrueckii*, *Lb. brevis*, *Lb. helveticus*, *Lb. johnsonii*, *Lb. crispatus*, and *Lb. salivarius*.

Ten different species of lactobacilli have recently been screened for the possibility of their successful use in LysM-mediated non-recombinant heterologous surface display.[14] Human IgG-binding designed ankyrin repeat protein (DARPin), in fusion with cA, was used as a reporter protein. Instead of using the recombinant fusion protein, expressed and purified from *E. coli*, *L. lactis* was used as a production host. Expressed fusion protein was secreted into the growth medium that was, upon cell removal, used further as a source of recombinant fusion protein for un-modified *Lactobacillus* cells. This significantly simplifies the process, since recombinant protein purification is not required. Surface display of DARPin was observed with all species tested, but the protein amount displayed on *Lactobacillus salivarius* ATCC 11741 greatly surpassed that on other species. *Lb. salivarius* ATCC 11741 has therefore been suggested as the optimal host for heterologous surface display on living bacteria.[14]

Further improvement in heterologous display on living cells could also be achieved by using other non-covalent surface-binding domains (Table 1). Significant differences in the affinity of non-covalent surface-binding domains between species have already been observed. It has been shown that they can differentiate between different strains of the same species (e. g., strains from genus *Listeria*[15]) or are genus specific (e.g., genus *Staphylococcus*[16]). Of the cell-

wall binding domains described in the PFAM database, only four (CW_binding_2, PG_binding_2, PG_binding_3 and SPOR) are not present in *Lactobacillus* genus (Table 1). This makes the majority of them good potential candidates for non-covalent surface display of heterologous proteins on *Lactobacillus* strains in general. This is further substantiated by the occurrence of the majority of non-covalent cell wall-binding domains as cell wall anchors of phage endolysins that are directed against *Bacillus*, *Lactobacillus*, *Lactococcus*, *Mycobacterium*, *Staphylococcus*, and *Streptococcus* genera[4] and can be highly specific.

Heterologous surface display on non-living LAB cells

An alternative approach to heterologous surface display on LAB uses non-viable or non-living LAB (Figure 1), designated bacteria-like particles (BLP) or, formerly, Gram-positive enhancer matrix (GEM). This approach has been developed by Bosma et al.[17], based on the findings of Steen et al.[6], who discovered that treatment of live LAB cells with trichloroacetic acid (TCA) dramatically increases the ability for surface display of MSA2_cA fusion proteins on the surface of treated cells. TCA treatment caused the removal of proteins and lipoteichoic acids from peptidoglycan, consequently enabling binding of heterologous fusion proteins over the entire surface.[6] The simple and rapid preparation of BLPs includes pre-treatment of cells with 10% TCA, boiling for 30 min and washing with phosphate buffered saline (PBS).[17] Prepared BLPs could be used immediately or stored at -80 °C. BLPs are usually produced from lactococcal cells, but the method of preparation is applicable to all Gram-positive bacteria. In recent years, lactococcal BLPs have been widely used for the development of mucosal vaccines against viruses (influenza, respiratory syncytial virus and hepatitis B virus), bacteria (*Streptococcus pnemoniae*, *Staphylococcus aureus*, *Yersinia pestis* and *Shigella flexneri*) and parasites (*Plasmodium falciparum* and *Plasmodium berghei*)[8, 18]. Most of these vaccines have employed LysM domains as anchors for pathogen antigens, and have been investigated in pre-clinical studies. Influenza vaccine has been evaluated in a Phase I clinical trial.[18] In addition to lactococcal BLPs, *Lb. salivarius* IBB3154 BLPs have recently been evaluated as vaccine delivery vehicles by displaying two *Campylobacter jejuni* antigens (CjaA and CjaD) in fusion with cA.[19] In our studies, BLPs prepared from *Lb. salivarius* ATCC 11741 resulted in a more than 8-fold increase in LysM-mediated surface display over that in un-modified living cells (unpublished), indicating the potential of *Lb. salivarius* ATCC 11741 as a source of BLPs. The advantages of BLPs for vaccination purposes include their immunostimulatory activity, their possible nasal or oral administration, and their ability to bind multiple antigens.[18]

Table 1 Noncovalent peptidoglycan binding domains from PFAM database

Pfam Name	Description	Pfam Number	Repeats	Position	Length	Ligand	Sequences in *Lactobacillus* genus
Cpl-7	Cpl-7 lysozyme C-terminal domain	PF08230	1-3	Central, C-term.	42	PG in choline independent fashion	6 in 6 species
ChW	Clostridial hydrophobic W[a]	PF07538	1-13	Central, N-, C-term.	36	PG	1 in 1 species
CW_binding_1	Putative cell wall binding repeat[b]	PF01473	1-64	Central, N-, C-term.	19	Choline in teichoic acids	77 in 27 species
CW_binding_2	Putative cell wall binding repeat 2	PF04122	1-4	Central, N-, C-term.	92	PG	/
LysM	LysM domain	PF01476	1-12	Central, N-, C-term.	44	*N*-acetylglucosamine	466 in 110 species
PG_binding_1	Putative peptidoglycan binding domain	PF01471	1-9	Central, N-, C-term.	57	PG	75 in 71 species
PG_binding_2	Putative peptidoglycan binding domain	PF08823	1	Central, C-term.	74	PG	/
PG_binding_3	Predicted peptidoglycan domain	PF09374	1	Central, N-, C-term.	72	PG	/
PG_binding_4	Putative peptidoglycan binding domain	PF12229	1-4	Central	114	Two unlinked PG chains	136 in 117 species
SH3_3	Bacterial SH3 domain (Sh3b)	PF08239	1-13	Central, N-, C-term.	65	Pentaglycine cross-bridge	64 in 62 species
SH3_5	Bacterial SH3 domain (Sh3b)	PF08460	1-9	Central, N-, C-term.	65	Pentaglycine cross-bridge	107 in 51 species
SLAP	Bacterial surface layer protein	PF03217	1-6	Central, N-, C-term.	120	PG	436 in 47 species
SPOR	Sporulation related domain	PF05036	1-3	Central, N-, C-term.	76	PG	/
WxL	WxL domain surface cell wall-binding	PF13731	1	C-term.	215	PG	257 in 31 species

[a]conserved tryptophan
[b]also known as the choline-binding repeats (ChBr) or the choline-binding domain (ChBD)
PG-peptidoglycan

Heterologous surface display: the regulatory perspective

BLPs with displayed protein contain no viable cells, and are, technically speaking, a combination of recombinant protein and cell fragments. From the regulatory perspective this can thus be described as a more complex recombinant protein preparation. Their application is purely medical and involves no potential food usage. We therefore focus our further discussion on the difference between recombinant bacteria that produce surface displayed fusion protein and unmodified (wild-type) bacteria with a surface-displayed fusion protein that originates from recombinant bacteria. The difference could have important regulatory implications since the two types of bacteria differ significantly in the levels of their genetic modification. The former contain modified genetic material in viable bacterial cells capable of reproduction. The latter, on the other hand, contain a combination of unmodified bacteria and metabolic products (including secreted fusion protein) of recombinant bacteria – the mixture is devoid of viable recombinant bacteria or their DNA. This is of particular importance in the European Union, given its strict regulation of genetically modified organisms (GMO). Both types of bacteria can be used for the delivery of therapeutic proteins, such as anti-inflammatory mediators, with the aim of eliciting beneficial effects on health. Their effects on health could be regarded as health claims or as medical claims. Health claims are achieved through the administration of food or food supplements to a general healthy population; the usage is regulated by European food safety authority (EFSA). Medical claims, however, are achieved through the administration of drugs to a specific population of patients and are regulated by the European medicines agency (EMA).

EFSA has prepared a guidance document to assist in the preparation of applications for marketing genetically modified microorganisms (GMM) for food or feed.[20] It requires the submission of an appropriate risk assessment. GMMs are separated into 4 categories that eventually differ in the stringency of their risk assessment. Category 1 contains chemically defined, purified compounds produced by GMMs (without recombinant genetic material). Category 2 contains complex products produced by GMMs (without recombinant genetic material), Category 3 contains inactivated GMMs (with recombinant genetic material) and Category 4 contains viable GMMs with recombinant genetic material. According to this division, the recombinant microorganisms would be classified into Category 4, while microorganisms with adhered recombinant protein would be classified under Category 2 – if lack of recombinant genetic material and viable cells could be reliably established. The administrative burden of preparing a risk assessment for Category 2 would be significantly

lighter than that for category 4. Risk assessments for both microorganisms would require information on the GMM, its production process and on the product preparation process. Environmental risk assessment for Category 2 would be simpler, due to the negligible environmental exposure, and would include only the proof of absence of recombinant DNA or viable recombinant cells. An environmental risk assessment for Category 4 would, on the other hand, have to be much more detailed and would have to characterize GMM-receiving environments, the potential of GMMs to survive in these environments and interact with indigenous microorganisms, plants and animals, the stability of the recombinant DNA, the mechanism of its transfer to environmental organisms, the presence of recipients of recombinant DNA, the consequences of horizontal transfer and the effects of GMMSs on plants and animals. Additionally, Category 4 requires post-market environmental monitoring which has to include the monitoring of predicted adverse effects, as well as the identification of unpredicted adverse effects. Reports on monitoring should be submitted periodically to EFSA.

In the final product the content of recombinant protein on the surface of unmodified bacteria would be very low and would almost certainly fall below the 0.9% labelling threshold.[21] However, the threshold does not apply for "intentional" addition of recombinant protein[21], which would be the case, since the addition of the protein would actually be part of the production process. Unmodified bacteria with added recombinant protein would therefore have to be labelled as GMOs, regardless of the recombinant protein content.

Recombinant bacteria and unmodified bacteria with bound recombinant protein could also be viewed differently from the regulatory perspective of EMA. The marketing of a new human pharmaceutical product in the EU requires an assessment of quality, safety and efficacy and, additionally, an assessment of environmental risks.[22] Recombinant bacteria with therapeutic indications would probably be regarded as an advanced therapy medicinal product.[23] The Committee for Advanced Therapies has made a scientific recommendation on the classification of a few recombinant bacteria and has classified them as gene therapy medicinal products.[24] Assessment of environmental risk differs in stringency if the pharmaceutical product contains or consists of GMO, and is described in a special guideline.[25] The guideline predicts 4 possible routes of contact between the GMO-containing product and the environment: dispersal during normal use, accidental dissemination, disposal of unused product and dispersal through patient excreta. The environmental risk assessment should address the identification of characteristics that could cause adverse effects, their potential consequences, the probability of their occurrence, an estimation of risks posed by GMO

characteristics, risk management strategies and estimation of overall risk of GMO. On the other hand, the guideline specifies that the components of biotechnological medicinal products can be proteins produced by GM cell cultures. However, if recombinant organisms are not present in the final product then the product is not regarded as "containing or consisting of GMO". This implies the possibility that the recombinant protein for bacterial surface attachment would be regarded as a biotechnological/biological product; the unmodified bacteria (non-GMO), however, would be a special regulatory case *per se*, but would require no GMO-related risk assessment.

Recombinant bacteria and unmodified bacteria with bound recombinant protein are not treated differently by the US Food and drug administration (FDA) when human challenge studies are to be performed with the purpose of establishing beneficial health effect. Both types of bacteria would require an Investigational New Drug Application that would include pharmacologic and toxicity studies and three phases of human clinical trials.[26]

Advantages and disadvantages

To conclude, heterologous surface display on un-modified bacteria has the advantage of less complex regulatory requirements. However, the requirements are still not trivial and would have to include the confirmation of lack of recombinant DNA and viable recombinant bacterial cells, apart from other safety data. It is questionable whether receiving the approval would be economical, particularly for the food industry. The advantage of recombinant bacteria is the possibility of constant production (also in *in vivo* conditions) of recombinant protein. The heterologous binding to unmodified bacteria, on the other hand, has to be performed in advance, and surface-bound recombinant protein is diluted upon cell division. BLPs can be loaded with larger amounts of recombinant protein although they lack the advantages of live bacterial delivery, such as potential probiotic synergism. Heterologous binding of recombinant protein to the surface of un-modified bacteria will probably not constitute the ultimate solution in surface display. It could, however, represent an interesting "middle way" between BLPs and recombinant bacteria, especially in combination with recent findings.

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PREGLED IN ZAKLJUČKI

Bakterije, ki tvorijo toksine šiga (*E. coli*, *S. dysenteriae*), pri ljudeh povzročajo drisko in hemoragični kolitis, ki se lahko razvije v smrtno nevaren hemolitični uremični sindrom. Kljub temu da se razvijajo številni novi načini zdravljenja tovrstnih okužb, specifično in učinkovito zdravljenje še vedno ni na voljo. Mlečnokislinske bakterije (MKB) so kot naravni prebivalci gastrointestinalnega trakta primeren gostiteljski organizem za dostavo terapevtskih molekul v prebavni trakt, saj so varne za uporabo, ugodno vplivajo na zdravje ljudi kot probiotiki in imajo razvite različne sisteme za izražanje proteinov in za površinsko predstavitev. Majhni vezavni proteini, ki ne temeljijo na imunoglobulinskem ogrodju (ne-Ig vezavni proteini), npr. načrtovani proteini z ankirinskimi ponovitvami (DARPini) in albumin-vezavna domena (ABD), predstavljajo učinkovito alternativo protitelesom in so primerni za površinsko predstavitev na MKB. V okviru doktorskega dela smo razvili gensko spremenjeno MKB *L. lactis* s sposobnostjo vezave toksina šiga, tako da smo na njeni površini predstavili nove načrtovane ABD s sposobnostjo vezave rekombinantne B podenote toksina šiga (Stx1B).

V prvem delu doktorata smo najprej pripravili funkcionalen rekombinanten protein Stx1B v bakteriji *E. coli*. Razvili smo nov postopek za ekspresijo Stx1B v *E. coli* z uporabo sistema pET, ki je vključeval gojenje bakterij do visoke gostote in nato, po indukciji izražanja, znižanje temperature s 37 °C na 28 °C. Netopen rekombinantni protein smo ponovno zvili z uporabo hitre redčitve v pufru Tris z dodanim 0.5 M argininom in 0.01 % detergentom Brij-35, pri čemer smo prilagodili postopek Oneda in sod. (102). Z določitvijo molekulske mase proteina, ki je ustrezala pentamerni obliki, potrditvijo koncentracijsko odvisne vezave Stx1B na Gb_3 receptor *in vitro* in internalizacijo Stx1B v HeLa celice, smo lahko zaključili, da je pripravljeni protein funkcionalen in primerna tarča za selekcijo vezalcev Stx1B z visoko afiniteto. Z uporabo kompleksne knjižnice, pridobljene iz ogrodja streptokoknega proteina ABD, in petih ciklov selekcije z uporabo predstavitve na ribosomih, smo pridobili 17 edinstvenih različic ABD, ki smo jih poimenovali S1B vezalci. Med njimi smo tri najobetavnejše vezalce S1B9, S1B22 in S1B26 podrobneje ovrednotili z ELISA in površinsko plazmonsko resonanco. Različicama S1B22 in S1B26 smo določili konstano vezave Stx1B v mikromolarnem območju (0.70 μM za S1B22 in 1.00 μM za S1B26), kar kaže na nižjo afiniteto kot pri predhodno objavljenih kratkih enoverižnih variabilnih fragmentih protiteles (ScFv) proti Stx1B, pridobljenih s pomočjo predstavitve naivne človeške knjižnice na fagih (103). Kljub višji afiniteti vezave Stx1B, ScFv zaradi velikosti in kompleksnega načina zvitja niso primerni za površinsko predstavitev na bakteriji *L. lactis*, kar smo ugotovili v naših

predhodnih raziskavah (neobjavljeno). Poleg tega lahko v primeru ABD izboljšamo afiniteto s procesom zorenja, ki vključuje pripravo nove knjižnice različic ABD na osnovi sekvence najboljšega vezalca. Pri tem se s ciljem randomizacije uporabi napakam podvržen PCR, pridobljena knjižnica pa se uporabi pri novi selekciji proti isti tarči pod čedalje ostrejšimi pogoji (104). Vse izbrane S1B različice, vključno z izvorno ABD (ABDwt), smo uspešno predstavili na površini laktokoknih celic v obliki fuzije z Usp45 signalom za izločanje in s peptidoglikan-vezavnim C-koncem proteina AcmA (cA). Vezavo Stx1B smo potrdili z uporabo pretočne citometrije in ELISE s celimi celicami. Na podlagi rezultatov lahko S1B26 izpostavimo kot vezalec Stx1B, ki je najobetavnejši za površinsko predstavitev na bakteriji *L. lactis*. S tem smo delno potrdili prvo hipotezo, ki pravi, da so MKB s površinsko predstavljenimi vezavnimi proteini uporabne za vezavo toksina šiga. Da bi lahko dokončno potrdili zastavljeno hipotezo, bi bilo potrebno v prihodnosti potrditi vezavo celotnega toksina šiga na v raziskavi razvite laktokokne celice. Tovrstno delo bi morali zaradi varnosti in zahtev za delo z nevarnimi biološkimi dejavniki opraviti v sodelovanju z laboratorijem, ki ima dovoljenje za delo s toksinom šiga. V okviru raziskave smo, poleg uspešne vezave Stx1B, kot prvi pokazali tudi uspešno predstavitev ABD proteinov na površini MKB *L. lactis*. Pridobljene rekombinantne MKB *L. lactis* s sposobnostjo vezave Stx1B lahko potencialno uporabimo za nevtralizacijo toksina šiga v črevesju ljudi v prvih stopnjah okužbe z bakterijami, ki tvorijo toksin šiga. Prav tako lahko nove Stx1B vezalce uporabimo za diagnostiko in bazične raziskave okužb, povezanih s toksinom šiga.

V drugem in tretjem delu doktorata smo se posvetili površinski predstavitvi na MKB. Pri bakteriji *L. lactis* smo želeli optimizirati nov način površinske predstavitve s pomočjo bazičnega membranskega proteina A (BmpA), ki se je v naši prejšnji študiji izkazal kot primeren nosilni protein za površinsko predstavitev IgG-vezavne domene (B-domene) na bakteriji *L. lactis* (64), hkrati pa tudi izboljšati uveljavljeni sistem na podlagi nosilnega proteina cA. V študiji z nosilnim proteinom BmpA je bil namen izboljšati zmožnost površinske predstavitve B domene z manjšanjem BmpA molekule in preveriti zmožnost adhezije BmpA na črevesni epiteljski model, saj je znano, da se BmpA iz *B. burgdorferi* veže na laminin (66). Na modelu črevesnih celic smo dokazali, da prekomerno izražanje BmpA na površini bakterije *L. lactis* poviša adhezijo bakterij na črevesne celice. Za optimizacijo površinske predstavitve B domene smo pripravili 18 krajših različic BmpA. Dokazali smo, da krajšanje molekule BmpA (različice Bmp1, Bmp2 in Bmp3) poveča zmožnost površinske predstavitve B domene v primerjavi z BmpA, vendar je to krajšanje omejeno, saj ob nadaljevanju pride do zmanjšane zmožnosti površinske predstavitve ali do popolne

nezmožnosti. Hkrati smo ugotovili, da razdalja med potniškim proteinom in membrano nima večjega pomena pri površinski predstavitvi, saj dodatek vmesnika med nosilni in potniški protein ni bistveno vplival na zmožnost površinske predstavitve. Različico Bmp1 smo zaradi občutnega povišanja površinske predstavitve B domene v primerjavi z BmpA izbrali kot optimiziran nosilni protein za površinsko predstavitev B domene v *L. lactis*. Čeprav je zmožnost površinske predstavitve z Bmp1 v primerjavi s petidoglikan-vezavnim cA nosilnim proteinom nižja, Bmp1 omogoča močnejšo kovalentno vezavo na površino MKB. BmpA in Bmp1 tako predstavljata učinkovito alternativo ostalim nosilnim proteinom pri površinski predstavitvi na MKB.

Za uporabo v prehrani in terapiji je zaželena nerekombinantna površinska predstavitev (brez GSO). Za testiranje heterologne nerekombinantne površinske predstavitve smo v tretjem delu doktorata na površini bakterije *L. lactis* predstavili dva modelna DARPina (I_07 in I_19) s sposobnostjo vezave Fc regije človeških IgG. Oba DARPina smo uspešno predstavili na površini bakterije *L. lactis* v obliki fuzije z Usp45 signalnim zaporedjem in cA nosilnim proteinom ter dokazali vezavo človeških IgG protiteles s pomočjo pretočne citometrije, ELISE s celimi celicami in konfokalne mikroskopije. Fuzijski protein I_07-cA, ki je na površini vezal človeška IgG protitelesa močneje od proteina I_19-cA, smo uporabili pri nadaljnjih testiranjih heterologne površinske predstavitve na nerekombinantnih MKB. Pri izražanju fuzijskih proteinov I_07-cA v bakteriji *L. lactis* pride zaradi signala Usp45 do izločanja fuzijskih proteinov v gojišče, kjer se jih del pripne na površino bakterij, del pa ostane v gojišču. Izrabljeno gojišče s prisotnimi fuzijskimi proteini smo uporabili kot vir le-teh za testiranje heterologne površinske predstavitve na površini bakterij desetih nerekombinantih sevov iz rodu *Lactobacillus*. Opazili smo, da je prišlo do vezave fuzijskih proteinov I_07-cA na površino vseh testiranih laktobacilov. Ob tem je *Lb. salivarius* ATCC 11741 izkazoval daleč najmočnejšo vezavo, zato smo ga izbrali kot optimalen gostiteljski organizem za nerekombinanto površinsko predstavitev na osnovi cA nosilnega proteina. S tem smo tudi potrdili četrto hipotezo, ki pravi, da so izbrani sevi iz rodu *Lactobacillus* primerni za heterologno nerekombinantno površinsko predstavitev proteinov. V nadaljevanju smo želeli ovrednotiti vpliv lipoteihoične kisline (LTA) in antibiotikov kloramfenikol ter eritromicin na uspešnost površinske predstavitve z nosilnim proteinom cA. Domneva se namreč, da LTA, sestavina celične stene, na površini MKB ovira vezavo cA nosilnega proteina na peptidoglikan (63). Z uporabo ELISA smo primerjali količino LTA na površini desetih sevov iz rodu *Lactobacillus*. Opazili smo, da nekateri sevi iz rodu *Lactobacillus*, ki imajo večjo količino LTA na površini, izkazujejo manjšo zmožnost cA-posredovane

površinske predstavitve. Vendar to ni veljajo za vse seve in tudi ni razložilo boljše površinske predstavitve na bakteriji *Lb. salivarius* ATCC 11741. Na osnovi teh rezultatov in zaradi neuspešne priprave seva *L. lactis* z inaktiviranim genom za LTA sintazo nismo mogli z gotovostjo potrditi tretje hipoteze, da LTA na površini mlečnokislinskih bakterij vpliva na uspešnost predstavitve proteinov z nosilnim proteinom cA. Smo pa kot prvi dokazali, da subletalne koncentracije antibiotikov eritromicin in kloramfenikol izboljšajo cA-posredovano površinsko predstavitev na bakteriji *L. lactis* v odvisnosti od koncentracije, kar ni bilo pričakovano, saj gre za antibiotika, ki vplivata na sintezo proteinov in ne na sintezo celične stene. Do izboljšanja površinske predstavitve pride najverjetneje zaradi odziva bakterij na antibiotični stres, kar privede do sprememb pri izražanju genov, vključno s tistimi, ki so vključeni v sintezo celične stene. Dodatek antibiotikov ali alternativnih, sprejemljivejših stresnih faktorjev bi se lahko uporabil kot komplementarni pristop k izboljšanju cA-posredovane površinske predstavitve pri MKB. Nerekombinantne MKB s površinsko predstavljenimi DARPini ali drugimi vezavnimi proteini bi se lahko uporabile za zdravljene infekcij s STEC ali *S. dysenteriae*, pa tudi v različne druge terapevtske namene. V okviru doktorske naloge smo na površini MKB kot prvi uspešno predstavili vezavne proteine DARPine in ABD-je, s čimer smo potrdili drugo zastavljeno hipotezo, da so DARPini in ABD-ji primerni za površinsko predstavitev na MKB.

V zadnjem delu doktorata smo predstavili in ovrednotili heterologno nerekombinantno površinsko predstavitev na MKB ter pogled nanjo s stališča regulative. Poleg predhodno predstavljenega pristopa, kjer na površino viabilnih nerekombinantnih MKB pripnemo rekombinante proteine, lahko kot nosilce za pritrditev peptidoglikan-vezavnih fuzijskih proteinov uporabimo tudi nežive bakterijam podobne delce (BLP, prej poznane kot GEM delci), ki jih pripravimo z enostavno kislinsko obdelavo. BLP imajo v primerjavi z nerekombinantnimi bakterijami višjo sposobnost vezave fuzijskih proteinov, vendar jim manjkajo prednosti živih bakterij, kot npr. potencialno probiotično delovanje. S stališča regulative se BLP obravnavajo kot kompleksni pripravki rekombinantih proteinov, saj gre za kombinacijo rekombinantih proteinov in celičnih fragmentov. Namenjeni so predvsem uporabi v medicini in ne v prehrani. Bakterije s površinsko predstavljenimi rekombinantimi proteini bi se kot produkti lahko uporabljale v prehrani, kar bi v Evropi regulirala Evropska agencija za varnost hrane (EFSA), ali v medicini, kar bi regulirala Evropska agencija za zdravila (EMA). Produkt, ki bi vključeval površinsko predstavitev rekombinantnih proteinov na rekombinantnih bakterijah (GSO), bi EFSA uvrstila v 4. kategorijo, za katero je potrebna priprava podrobne ocene tveganja in dodatni monitoring po sprostitvi izdelka na trg. Produkt,

ki bi vključeval površinsko predstavitev na nerekombinantih bakterijah (ne-GSO pristop), pa bi EFSA uvrstila v 2. kategorijo, za katero je potrebna enostavnejša ocena tveganja, ki bi morala vsebovati dokaz odsotnosti rekombinantne DNK. Tudi EMA za GSO produkte zahteva podrobno oceno tveganja za okolje, medtem ko se nerekombinantne bakterije s površinsko izraženimi rekombinantnimi proteini pri EMA ne obravnavajo kot GSO, ampak kot biotehnološki produkt, za katerega je potrebna enostavnejša ocena tveganja kot za GSO. Ameriška uprava za hrano in zdravila (FDA) enakovredno obravnava rekombinante bakterije in nerekombinantne bakterije s površinsko vezanimi rekombinantnimi proteini. Pri prijavi novega produkta FDA zahteva vse potrebne študije na ljudeh, vključno s farmakološkimi študijami, študijami toksičnosti in vsemi tremi fazami kliničnega testiranja. Heterologna površinska predstavitev na nespremenjenih MKB ima s stališča regulatornih zahtev prednost v primerjavi z rekombinantnimi bakterijami in predstavlja srednjo pot med rekombinantnimi bakterijami in BLP.

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Objavljeni povzetek znanstvenega prispevka na konferenci

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Samostojni znanstveni sestavek ali poglavje v monografski publikaciji

15. ZADRAVEC, Petra, ŠTRUKELJ, Borut, BERLEC, Aleš. Manipulation of intestinal flora as a way to treat Crohn's disease : the role of probiotics, prebiotics and antibiotics. V: KAROUI, Sami (ur.). *Crohn's disease*. Rijeka: InTech, cop. 2011, str. 145-168. [COBISS.SI-ID 25502503]

MONOGRAFIJE IN DRUGA ZAKLJUČENA DELA

Diplomsko delo

16. ZADRAVEC, Petra. *Predstavitev različic antigena hepatitisa A na površini rekombinante mlečnokislinske bakterije Lactococcus lactis : diplomsko delo = Display of antigen varieties of hepatitis A on surface of recombinant lactic acid bacterium Lactococcus lactis : graduation thesis*, (Biotehniška fakulteta, Študij biotehnologije, Diplomska dela, 29). Ljubljana: [P. Zadravec], 2010. XII, 84 f., [18] f. pril., ilustr., preglednice. http://www.digitalna-knjiznica.bf.uni-lj.si/dn_zadravec_petra.pdf. [COBISS.SI-ID 6340985]

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- Raziskovalno delo v biotehnološkem laboratoriju (tehnike kloniranja, delo z gensko spremenjenimi organizmi, izražanje proteinov v različnih ekpresijskih sistemih, izolacija in čiščenje proteinov, detekcija z SDS PAGE, prenos po Western-u, pretočna citometrija, konfokalna mikroskopija, ELISA, analize s površinsko plazmonsko resonanco, …
- Pedagoško delo: izvedba vaj za študente farmacije in biotehnologije
- Pisanje strokovne literature v slovenskem in angleškem jeziku (objavljeni 3 strokovni članki s prvim avtorstvom v revijah s faktorjem vpliva, četrti v pripravi)
- Aktivna udeležba na številnih mednarodnih in domačih konferencah.

Raziskovalno delo v tujini
Inštitut za biotehnologijo, Praga, Češka junij – september 2013

- Selekcija vezavnih proteinov z uporabo tehnologije predstavitve na ribosomih
- Testiranje vezavnih proteinov z uporabo ELISA

BIOCEV, Inštitut za biotehnologijo, Vestec, Češka 3 tedni v 2016

- Termoforeza za testiranje afinitete vezave in določanje stabilnosti proteinov s Prometheus NanoTemper

Delovno mesto: svetovalec
Ministrstvo za okolje in prostor, Oddelek za biotehnologijo 23.8.2010 – 22.8.2011

- Poznavanje zakonodajnega okvirja na področju biološke varnosti v Sloveniji, Evropski uniji in na mednarodnem področju
- Organizacija sestankov znanstvenih odborov
- Priprava in pregled dokumentov
- Opravljane deset mesečnega pripravništva v javni upravi

Izobrazba

Doktor znanosti
(področje biokemija in molekularna biologija) oktober 2011–junij 2016
Univerza v Ljubljani, Medicinska fakulteta, študij Biomedicina

- Biokemija
- Farmacevtska biotehnologija
- Doktorat na področju biotehnologije mlečnokislinskih bakterij: Razvoj gensko spremenjenih mlečnokislinskih bakterij s sposobnostjo vezave toksina šiga

Univerzitetni diplomiran biotehnolog 2005–2010
VII. - univerzitetni programi / 2. bolonjska stopnja
Univerza v Ljubljani, Biotehniška fakulteta, Program Biotehnologija

- Diplomsko delo Predstavitev različic antigena hepatitisa A na površini rekombinantne mlečnokislinske bakterije *Lactococcus lactis*

Gimnazijski maturant
Gimnazija Franca Miklošiča Ljutomer 2001–2005

Dodatna znanja

Tuji jeziki:

- Angleščina: zelo dobro
- Nemščina: osnovno
- Hrvaščina: osnovno

Vozniški izpit: B kategorija

Računalniška znanja:

- MS Word, Excel, Powerpoint, Outlook, CorelDRAW

Dodatna izobraževanja:

D**elavnica na temo pisanja znanstvenih člankov** maj 2015
Izvajalec prof. dr. Roger Pain

Delavnica **On being a scientist: Essential skills of a biomedical investigator** april 2014
Izvajalec prof. dr. Tung-Tien Sun

Izobraževanje za delo z inštrumentom februar 2013
Applied Biosystem StepOnePlus Real-Time PCR System
Izvajalec Omega d.o.o

Didaktično izpopolnjevanje: **Uspešna komunikacija na predavanjih in vajah** september 2012
Izvajalec prof. ddr. Barica Marentič Požarnik v organizaciji UL FFA

Delavnica **Akademsko podjetništvo** februar 2012
Izvajalec Kemijski inštitut v sodelovanju z Institutom Jožef Stefan

Nagrade

Fakultetna Prešernova nagrada

Za diplomsko delo z naslovom Predstavitev različič antigena hepatitisa A na površini rekombinantne mlečnokislinske bakterije *Lactococcus lactis*

Zoisova štipendija

Celotna srednja šola in do zaključka fakultete.

Članstva v društvih

- Slovensko biokemijsko društvo
- Slovensko mikrobiološko društvo
- Športno društvo Kog (namestnik in tajnik društva 2011 – 2015)

Zanimanja

- Športne aktivnosti in izleti v naravo
- Branje
- Kuhanje