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Anchoring of proteins to lactic acid bacteria

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Abstract

The anchoring of proteins to the cell surface of lactic acid bacteria (LAB) using genetic techniques is an exciting and emerging research area that holds great promise for a wide variety of biotechnological applications. This paper reviews five different types of anchoring domains that have been explored for their efficiency in attaching hybrid proteins to the cell membrane or cell wall of LAB. The most exploited anchoring regions are those with the LPXTG box that bind the proteins in a covalent way to the cell wall. In recent years, two new modes of cell wall protein anchoring have been studied and these may provide new approaches in surface display. The important progress that is being made with cell surface display of chimaeric proteins in the areas of vaccine development and enzyme- or whole-cell immobilisation is highlighted.

Introduction

There are at least three reasons to change the outside appearance of a bacterium by expressing foreign proteins at its surface. First, it should help to understand the fundamental mechanisms of protein targeting to the cell envelope. Second, it may enable to control the interactions between the bacterium and its environment. Third, it opens the way to a number of potentially important biotechnological applications. This paper focuses mainly on the third reason.

In the last decade there has been considerable progress towards the development of systems to anchor and display heterologous (poly)peptides at the surface of bacteria, such that they are detectable on the outside of the intact cells. The progress is most advanced in Gram-negative bacteria, notably *Escherichia coli*, for which several efficient fusion protein display systems have been described (Hofnung 1991; Georgiou et al. 1997; Ståhl & Uhlén 1997a). Most of these systems take advantage of the anchoring capacity of outer membrane proteins, employing transmembrane spanning domains (LamB, OmpA, PhoE) or domains that contain a lipomodification signal (Lpp, TraT). Others use the surface exposure capacity of fimbriae, pili or flagellae (FliC, FimH, PapA; Georgiou et al. 1997).

Gram-positive bacteria have also been taken into account for bacterial surface display purposes. The lack of an outer membrane and the presence of multiple peptidoglycan layers in the cell wall of these bacteria resulted in the use of a number of different targeting strategies that link proteins to the membrane or cell wall (Fischetti et al. 1993; Ståhl & Uhlén 1997a). The non-pathogenic staphylococci, *Staphylococcus xylosus* and *carnosus*, and the oral commensal *Streptococcus gordonii* were among the first Gram-positives for which anchored heterologous cell surface proteins were described (Hansson et al. 1992; Pozzi et al. 1992a,b). Recently, other lactic acid bacteria (LAB) like *Lactococcus lactis* and *Lactobacillus* subsp., are being examined for their capacity to target and attach heterologous proteins to the membrane and wall components (Piard et al. 1997b; Pouwels et al. 1998; Poquet et al. 1998; Steidler et al. 1998). The anchoring domains that have been used in these LAB, including *Staph. xylosus*, *Staph. carnosus* and *Strep. gordonii*, to attach heterologous (poly)peptides will be discussed in this short review. The vector systems, expression and translocation signals to produce and transport the hybrid surface proteins are also essential elements in the surface display strategies, but for these specific items the reader is referred to a recent comprehensive

overview (Pozzi & Wells 1997a). The biotechnological applications that form the driving force behind research of surface linked proteins will be briefly addressed. A wide variety of applications are envisaged for LAB carrying surface exposed proteins, among which the immobilisation of enzymes at the bacterial surface, the immobilisation of the production strain at ligand-coated surfaces, generating whole-cell bioadsorbents for environmental purposes, diagnostic tools and display of entire peptide libraries. Hitherto, the most common motivation to attach proteins to the outer compartment of LAB has been the development of live-bacterial-vaccine delivery systems (Fischetti et al. 1996; Medaglini et al. 1998; Pouwels et al. 1998; Pozzi & Wells 1997a; Ståhl et al. 1997b).

Modes of anchoring

The general approach taken to position proteins at the cell surface consists of genetically linking a heterologous polypeptide in such a way to a protein known to be located at the cell surface that the chimaeric protein will be exposed, at least partly, to the outer environment. This approach requires rather detailed knowledge of the topology of the protein containing the anchoring signal, in order to determine an appropriate site for insertion or fusion. Such information may be obtained from protein homology comparisons to make an educated guess or from experimentation. Both strategies have been followed for LAB using cell surface anchoring domains of LAB- or non-LAB proteins.

The application of five different types of anchoring domains has been described or is currently under investigation. Figure 1 summarises these different modes of anchoring and gives also a proposal for nomenclature. Type 1 and type 2 anchors (A1 and A2, respectively) link the hybrid protein to the membrane. At present, the most commonly applied method of anchoring in LAB uses the type 3 anchor (A3), in which the displayed protein is covalently linked to cell-wall components. Attachment domains of type 4 (A4) and type 5 (A5) interact in a yet unknown manner with the cell wall and only very recently received attention. All types of anchors and their use in hybrid proteins are listed in Table 1 and will be discussed below. A1 and A2 domains reside in the N-terminal part of the hybrid proteins. The cell wall anchoring regions A3 to A5 are unique for Gram-positive bacteria and, in the cases described here, require the presence of the attachment domain in the C-terminus of the target protein. The hybrid protein gene is then preceded in the genetic construct by a secretion signal sequence that enables translocation of the expressed product across the cytoplasmic membrane.

Transmembrane anchors. In general, transmembrane spanning domains (TMSs) of any membrane protein may be used as an A1 domain. Protein topology studies that formed the basis for using one or more TMSs as an anchor were performed e.g. for the holin LytP of the lactococcal bacteriophage r1t. Alkaline phophatase (PhoA) fusion and insertions studies of the human immunodeficiency virus (HIV) gp41E epitope (ELDKWAS) revealed suitable sites for anchoring (Leenhouts et al., unpublished). The strategy to insert amino acid sequences in an exterior loop between TMSs may limit the insert size in order not to disturb the membrane protein topology (Hofnung 1991). In addition, this type of anchoring may not result in true surface exposure of the inserted domain since at least 100 amino acids are needed in the extended loop to cross the cell wall (Fischetti et al. 1990). For these reasons a fusion approach is often preferred in which the target protein is simply linked at its N-terminus to one or more TMDs of a cytoplasmic membrane protein. PhoA and β-galactosidase (LacZ) were fused to different N-terminal parts of the *L. lactis* bacteriocin-transport-accessory protein LcnD. Fusions located C-terminally to amino acid residue 44 of LcnD resulted in extracellularly anchored enzymes (Franke et al. 1996). A similar hybrid protein containing the N-terminal 80 amino acids of LcnD and at the C-terminus a 99 amino acid B-cell epitope of the human cytomegalovirus (hCMV) pp65 matrix protein was successfully anchored in this way. The pp65 moiety was accessible to proteinase K from the outside of protoplasts (Franke et al. 1998).

Poquet et al. (1998) identified in a random approach with an export specific reporter enzyme, seven lactococcal gene fragments encoding TMSs that function as membrane anchors. The nuclease reporter protein requires an extracellular location to be active and, therefore, important information on the topology of the fused membrane proteins is obtained.

For none of the above mentioned hybrid proteins has its accessibility from the outside of intact cells been described.

Lipoprotein anchors. A2 or lipoprotein anchoring domains are characterised by covalent binding to the

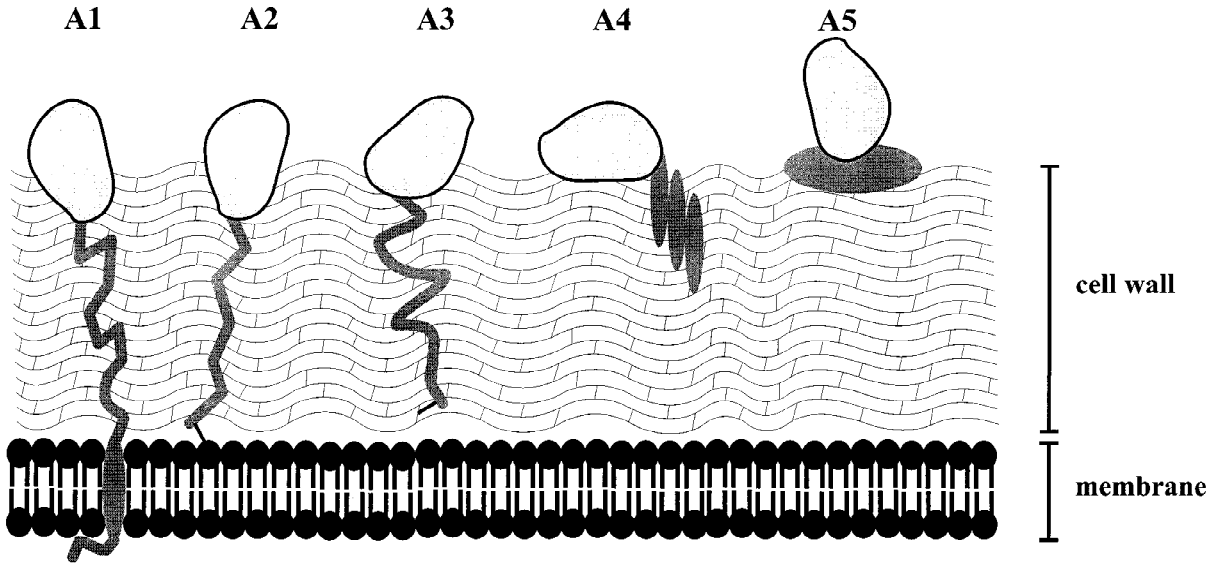

Figure 1. Modes of anchoring of chimaeric proteins to the cell surface of LAB. The light grey areas represent a heterologous (poly)peptide. The grey regions are the different types of anchoring domains. A1: transmembrane anchor region. A2: lipoprotein anchor domain. A3: LPXTG-type cell-wall anchoring domain. A4: AcmA-repeats cell-wall binding domain. A5: surface-layer-protein attachment region. The black lines extending from the A2 and A3 domains represent the covalent bond between the anchors and the lipid bilayer and the peptide crossbridge that connect the peptidoglycan layers, respectively. Components of the cell surface such as (lipo)teichoic acids and oligosaccharides are not shown.

lipid bilayer through N-acyl diglyceride modification of a cysteine residue located immediately C-terminal to the signal sequence cleavage site (Pugsley 1993). Haandrikman et al. (1991) have shown in their study on the proteinase maturation lipoprotein PrtM that this type of modification mechanism operates in *L. lactis*. An A2 signal has been identified on the basis of homology in the *L. lactis* oligopeptide binding protein OppA (Tynkkynen et al., 1993). This putative OppA anchoring signal was used to position the merozoite stage surface antigen MSA2 of *Plasmodium falciparum* on the outside of *L. lactis* (Leenhouts et al. unpublished). Data on the surface display of this OppA::MSA2 fusion protein and of other anchored MSA2 hybrids (Table 1 and below) should be available soon.

In the same random procedure as described above for the transmembrane anchors Poquet et al. (1998) identified four new lactococcal lipoprotein-anchoring signals. In one case, Nlp1, degradation of the fusion protein by proteinase K treatment on intact cells demonstrated its surface exposure.

LPXTG-motif anchor. The type 3 anchor signals (A3) contain highly conserved sequences and start at the C-terminus with a short tail of positively charged residues that remain in the cytoplasm. Upstream of the cytoplasmic domain a stretch of approximately 30 hydrophobic amino acids (optimal length is species dependent; Schneewind et al. 1993) is preceded by the well-conserved pentapeptide LPXTG (Fischetti et al. 1990). The charged tail and hydrophobic domain are thought to function as a temporary stop to position the LPXTG motif for proteolytic cleavage. Correct positioning results in cleavage between the threonine and glycine residues followed by amide-linkage of the threonine residue to the peptide crossbridge in the peptidoglycan of the cell wall, by the action of a postulated sortase (Navarre & Schneewind 1994; Schneewind et al. 1995). The amino acid composition of the peptide crossbridge, which varies among the different LAB species, is flexible with respect to the sorting reaction (Strauss et al. 1998; Ton-That et al. 1998). The sorting signal (LPXTG box, hydrophobic region and charged tail) is preceded by a wall-associated region of about 50 to 125 residues and is characterised by a high percentage of proline/glycine and/or threonine/serine residues (Fischetti et al. 1990). The mechanism by which these A3 domains are cell-surface targeted and subsequently covalently anchored to the cell wall has been elucidated in detail for immunoglobulin binding protein A

Table 1. Anchoring domains that have been used to immobilise hybrid proteins in or on the cell envelope of LAB

Host bacterium	Anchor domain[type]	Origin	Displayed protein	Origin	References
Staph. carnosus and *xylosus*	ProtA[A3]	*Staph. aureus*	CTB	*V. cholerae*	Liljeqvist, 1997
			ABP	*Strep.* subsp.	Andréoni, 1997
			ScFv anti-hIgE	mouse	Gunneriusson, 1996
			gpG (101aa epitope)	hRSV	Nguyen, 1993[a], 1995; Robert, 1996
			M3 (Pf155/RESA)	*P. falciparum*	Samuelson,1995; Hansson,1992
	FnBPB[A3]	*Staph. aureus*	lipase	*Staph. hyicus*	Strauss, 1996[b]
			β-lactamase	*E. coli*	Strauss, 1996[b]
Strep. gordonii	M6[A3]	*Strep. pyogenes*	M6	*Strep. pyogenes*	Pozzi, 1992b; Oggioni, 1996
			E7	HPV	Pozzi, 1992a; Oggioni, 1995; Medaglini, 1997
			gp120 (T-cell epitope)	HIV	Pozzi, 1994
			gp120/E7 (V3 epitope)	HIV/HPV	Di Fabio, 1998; Medaglini, 1998
			Ag5.2	hornet venom	Medaglini, 1995
			HA	measles virus	Medaglini, 1998; Pozzi, 1997
			LTB	*E. coli*	Ricci, 1996; Pozzi, 1997b
			gp120/LTB (V3 epitope)	HIV/*E. coli*	Medaglini, 1998; Pozzi, 1997b
L. lactis	ProtA[A3]	*Staph. aureus*	streptavidin	*Sm. avidinii*	Steidler, 1998
	M6[A3]	*Strep. pyogenes*	nuclease	*Staph. aureus*	Piard, 1997b
	holin[A1]	phage r1t	gp41E (Katinger epitope)	HIV	Leenhouts, unp.
	LcnD[A1]	*L. lactis*	pp65 (99aa epitope)	hCMV	Franke, 1998
	Tmp1-7[A1]	*L. lactis*	nuclease	*Staph. aureus*	Poquet, 1998
	Nlp1-4[A2]	*L. lactis*	nuclease	*Staph. aureus*	Poquet, 1998
	OppA[A2]	*L. lactis*	MSA2	*P. falciparum*	Leenhouts, unp.
	PrtP[A3]	*L. lactis*	TTFC	*C. tetanii*	Norton, 1995; Norton, 1996
			MSA2	*P. falciparum*	Leenhouts, unp.
	AcmA[A4]	*L. lactis*	β-lactamase	*E. coli*	Buist, 1997
			α-amylase	*B. licheniformis*	Buist, 1997
			MSA2	*P. falciparum*	Leenhouts, unp.
Lactobacillus subsp.	M6[A3]	*Strep. pyogenes*	LTB	*E. coli*	Rush, 1997
			gp41E (Katinger epitope)	HIV	Mercenier, 1996
	AcmA[A4]	*L. lactis*	β-lactamase	*E. coli*	Steen, unp.
	PrtP[A3]	*Lb. paracasei*	GusA	*E. coli*	Pouwels, 1996; 1998
			HA (Hackett epitope)	Infuenza virus	Pouwels, 1996
			TTFC	*C. tetanii*	Maassen, 1999
			VP7 and 8	Rotavirus	Leer, 1996; Pouwels, 1998
			Urease A and B	*H. pylori*	Leer, 1996; Pouwels, 1998
	Slp[A5]	*Lb. brevis*	VP1 (11 aa epitope)	Enterovirus	Palva et al., unp.

[A1]: transmembrane anchor; [A2]: lipoprotein membrane anchor; [A3]: LPXTG-type cell-wall anchor; [A4]: AcmA repeats cell-wall anchor; [A5]: surface-layer-protein anchor. [a]: only in *Staph. xylosus*. [b]: only in *Staph. carnosus*.

(ProtA) of *Staphylococcus aureus*. Its description is beyond the scope of this short review and the reader is referred to other reviews and the original literature (Schneewind et al. 1992, 1993, 1995; Ton-That et al. 1997; Navarre et al. 1998, 1999).

The anchoring domain of ProtA has been employed extensively in *Staph. xylosus* and *carnosus* to surface display various antigens for vaccine development purposes (Ståhl et al. 1997b). Electron microscopy, immunofluorescence microscopy and fluorescence-activated cell sorting (FACScan) techniques have been used to determine the proper display of the proteins. Many of the hybrid proteins in these studies contain the albumin binding protein fragment (ABP) from streptococcal protein G, allowing easy isolation of the hybrid proteins and rapid colorimetric analysis of successful display. In a comparative study, mice were immunised with live recombinant bacteria with surface expressed ABP as the model immunogen (Robert et al. 1996; Andréoni et al. 1997). Higher serum antibody titers were obtained with recombinant *Staph. carnosus* than with the corresponding *Staph. xylosus* strain. This result was attributed to the higher number of hybrid proteins present on the surface of the *Staph. carnosus* strain which was calculated to be as much as 10^4 per cell and 3×10^3 for the *Staph. xylosus* strain. Nguyen et al. (1995) engineered the hydrophobicity of a non-secretable portion of the G protein of the respiratory syncytial virus (RSV) by replacing or deleting hydrophobic phenylalanine residues. This resulted in a properly surface displayed protein and showed for the first time that hydrophobicity engineering can be used to optimise surface display. Such a strategy may have important implications in the area of vaccine development if surface display is required of proteins that are normally not secreted. Hydrophobicity engineering may influence the native structure of the protein, which is acceptable as long as protective immunity can be elicited. Correct folding of the displayed proteins is a requirement if they have to exert certain (enzymatic) activities. It was shown that the ProtA anchoring system is efficient in expressing a functional single chain antibody fragment (scFv), the mouse anti-human IgE scFv, on the surface of *Staphylococcus* (Gunneriusson et al. 1996). These recombinant *Staph. carnosus* cells were able to recognise human IgE and this result paves the way for applications such as using recombinant LAB as whole cell diagnostic devices or the surface display of scFv libraries. Cholera toxin fragment B (CTB) of *Vibrio cholerae* was also functionally displayed on *Staph. carnosus* (Liljeqvist et al. 1997). The functionality of the non-toxic CTB requires pentamerization as was demonstrated by binding of the recombinant bacteria to monosialoganglioside GM1, which is present on all epithelial cells of mucosal surfaces.

In another application of the ProtA surface anchor it was fused to the streptavidin monomer of *Streptomyces avidinii* and expressed in *L. lactis* (Steidler et al. 1998). Lysostaphin treatment of producer cells released the fusion protein, indicating that it was linked to the peptidoglycan layer. Immobilisation of the recombinant strain on a biotinylated alkaline phosphatase-coated polystyrene support suggested that the streptavidin moiety of the hybrid proteins is accessible from the outside. These results open possibilities to immobilise LAB on solid surfaces for production purposes.

The C-termini of numerous Gram-positive bacterial surface proteins are highly homologous to the ProtA anchoring signal (Fischetti et al. 1996). Another *Staph. aureus* A3 region, that of the fibronectin binding protein B (FnBPB), was effective in immobilisation of the normally soluble enzymes lipase of *Staphylococcus hyicus* and β-lactamase of *E. coli* on the cell surface of *Staph. carnosus* (Strauss & Götz 1996). A spacer region which exceeded a critical length of approximately 90 amino acids between the LPXTG box and the C-terminus of the enzymes was required to allow efficient folding of the enzymes in their catalytically active form.

An A3 cell wall binding region that has found application in various LAB, like the one of ProtA, is the anchor domain of the fibrillar M6 protein of *Streptococcus pyogenes*. The M6 sorting signal appears to be functional in *Strep. gordonii, L. lactis, Lb. fermentum, Lb. sake,* and *Streptococcus thermophilus* (Pozzi et al. 1992a; Piard et al. 1997a). The M6 anchoring domain was extensively exploited in *Strep. gordonii*. Reported were the cell surface display of the subunit B of heat labile toxin (LTB) of *E. coli*, which has a similar function as CTB, the allergen Ag5.2 of white-faced hornet venom and a variety of relevant immunodominant antigens of human virusses, including the E7 protein of papillomavirus (HPV), parts of gp120 of HIV and the measles virus hemoagglutinin. The hybrid proteins were detected on the cell surface by immunofluorescence microscopy and elicited relevant immune responses in parenteral and local immunisations (Pozzi et al. 1997b). An important step in the development of LAB carriers for vaccine purposes was the successful colonisation of the vaginal mucosa of

Cynomolgus monkeys with recombinant *Strep. gordonii* expressing the HPV E7 protein and part of HIV gp120 on the cell surface, which resulted in antigen-specific vaginal IgA, serum IgG and T-cell responses (Di Fabrio et al. 1998; Medaglini et al. 1998).

A heterologous enzyme, a *Staph. aureus* nuclease, was immobilised on the surface of *L. lactis* using 139 C-terminal residues of M6. The presence of active nuclease in the cell wall fraction suggested correct surface display of the hybrid protein (Piard et al. 1997b). A similar M6 anchoring domain was exploited to surface expose *E. coli* LTB on *Lb. casei*, *Lb. paracasei*, *Lb. acidophilus*, *Lb. plantarum* and *Lb. fermentum* (Rush et al. 1997). The presence of the LTB immunogen on the surface of this set of *Lactobacillus* species allows to investigate the mucosal immune responses to the antigen expressed in colonising and non-colonising strains. Mercenier et al. (1996) expressed M6 hybrid proteins containing parts of HIV gp41 or gp120 on the cell surface of *Lb. paracasei*, as was demonstrated by immunofluorescence techniques. Local administration in mice of recombinant colonising *Lb. paracasei* strains carrying the M6-anchored HIV parts induced significant systemic and mucosal responses.

LAB A3 domains that are being examined for surface display purposes are those of the proteinases (PrtP) of *L. lactis* and *Lb. paracasei* (Kok et al. 1988; Vos et al. 1989; Holck & Næs 1992). The tetanus toxin fragment C (TTFC) of *Clostridium tetanii* was fused to the lactococcal PrtP anchor but, although the hybrid protein was produced in *L. lactis*, it appeared that TTFC could not be detected on the surface of intact cells by immunogold or immunofluorescent labelling techniques. Lysozyme/lysostaphin treatment of the producer demonstrated that all TTFC was present in the cell membrane and not in the cell wall. Nevertheless, membrane located TTFC proved to be approximately 13 to 20-fold more immunogenic than intracellular soluble TTFC. Both forms were capable of evoking protective immune responses in mice subcutaneously immunised with the recombinant strains (Norton et al. 1995; Norton et al. 1996). Whether the inefficient targeting to the cell wall is caused by the lactococcal PrtP anchoring domain or is a TTFC-specific effect, are questions that need to be addressed. At present, a fusion with MSA2 is under investigation (Leenhouts et al., unpublished). The PrtP sorting signal of *Lb. paracasei* seems to work efficiently in a number of different *Lactobacillus* species to surface display various antigens, including TTFC of *C. tetanii*, rotavirus proteins VP7 and 8, urease A and B of *Helicobacter pylori*, and an influenza virus hemoagglutinin epitope fused to β-glucoronidase (GusA) of *E. coli* as a carrier protein (Leer et al. 1996; Pouwels et al. 1996, 1998). FACScan analyses indicated that *Lb. casei* producing surface-anchored TTFC exposed approximately 4×10^3 antigenic molecules per cell to the environment. High levels of serum IgG specific for TTFC were induced following parenteral immunisation of mice with this recombinant strain (Maassen et al. 1999), but low antibody levels were observed after oral administration, which was attributed to the poor viability of the *Lb. casei* strain in the gut of mice (Pouwels et al. 1998).

AcmA-repeats anchor. Nearly all bacterial cell wall hydrolases have a modular design, in which an active site domain degrades the peptidoglycan and a cell wall binding domain immobilises the enzyme in or on the peptidoglycan layer. The cell wall binding domain is often comprised of repeated amino acid sequences. The C-terminal region of the lactococcal cell wall hydrolase AcmA contains three repeated sequences of 44 amino acids separated by stretches of 21 to 31 amino acids rich in serine, threonine and asparagine residues (Buist et al. 1995). This repeat region is defined here as an A4 domain. There are no reports providing insight in the mechanism of binding of these repeats or details on the interaction of the A4 attachment region with the cell wall. Repeats homologous to the ones in AcmA are present in many cell wall- or membrane-associated proteins in Gram-positive and Gram-negative bacteria, but do not necessarily reside in the C-terminus of the protein (Buist 1997). Proteins of Gram-positives with an A4-like domain are usually detected in the wall fraction as well as in the supernatant. This observation suggests an interaction of the A4 attachment region with the cell wall that is less strong than that of the LPXTG type (A3) and is, most likely, of a non-covalent nature.

Although the cell wall binding domain of AcmA contains 3 repeats, 1 repeat proved to be sufficient for cell wall binding (Buist 1997). Interestingly, AcmA present in the supernatant of an *L. lactis* producer was able to bind from the outside to an *L. lactis* strain devoid of AcmA and to a range of Gram-positive bacteria, including *Lactobacillus*, *Clostridium*, *Listeria* and *Bacillus* (Buist and Steen, unpublished results). The A4 domain of AcmA could also be used to immobilise the normally soluble enzymes α-amylase of *E. coli* and β-lactamase of *Bacillus licheniformes* in

an active form on the cell surface of *L. lactis*. Like AcmA, these chimaeric enzymes could bind to *L. lactis* cells when added from the outside (Buist 1997). An attractive possibility of this type of binding is to couple hybrid proteins to non-recombinant Gram-positive bacteria. The possibility to use the AcmA A4 attachment region to display antigens, e.g. like MSA2, on the cell surface of *L. lactis* is currently under investigation (Leenhouts et al., unpublished).

Surface-layer-protein anchor. Some LAB strains contain, like various other Gram-positive bacteria, a layer of proteins exterior to the cell wall. These so-called surface layer (S-layer) proteins form porous lattices of identical subunits completely covering the cell surface and may constitute up to 20% of the total cell protein content. These properties make S-layer proteins (SLPs) an attractive target for protein anchoring studies. At present, the genes of *Lb. brevis*, *Lb. acidophilus*, *Lb. crispatus* and *Lb. helveticus* SLPs have been genetically characterised (Vidgrén et al. 1992; Boot et al. 1993; Toba et al. 1995; Callegari et al. 1998). Comparison of the SLP amino acid sequences of the latter three bacteria revealed a conserved C-terminal one-third of the proteins (>80% identity), that is thought to interact with the cell wall (Pouwels et al. 1998). Expression of SLPs in heterologous hosts (strains of *Lb. casei* and *L. lactis* which lack S-layer proteins) resulted in secretion of the S-layer proteins in the medium (Callegari et al. 1998; Martinez et al., unpublished results). This finding suggests that *Lb. casei* and *L. lactis* lack (a) cell wall component(s) required for proper attachment and this may implicate that cell surface anchoring of SLP hybrid proteins is limited to the host from which the *slp* gene was isolated.

Palva et al. (unpublished) used the *Lb. brevis* SLP to produce chimaeric proteins in *Lb. brevis* with an 11 amino acid insert of an enteroviral capsid protein VP1. Whole cell-ELISA techniques were used to identify two permissive hydrophilic sites in the C-terminal region of this SLP that allow surface exposure of the epitope. These results may suggest that the *Lb. brevis* SLP anchoring domain resides in another part of the protein than it does in the other three SLPs.

Conclusions and perspectives

A number of different anchoring domains have been explored in LAB for the cell surface anchoring of heterologous (poly)peptides and, although still rather premature, a few interesting applications arise at the horizon. From a practical point of view, LAB may have certain advantages over Gram-negative and other Gram-positive bacteria that make them more suitable for bacterial surface-display purposes. LAB have the status of being generally recognised as safe (GRAS) making them certainly more useful in food and medical applications than some other bacterial species. It is exactly in these areas that research on surface display in LAB has concentrated so far. Important progress has been made in the immobilisation of enzymes, the immobilisation of LAB for production purposes and in vaccine development. Although the latter research area has been the major driving force in this field, it is still a matter of debate whether cell-surface display of antigens for vaccine purposes is essential for eliciting effective immune responses. Experimental data for *Staph. xylosus* and *carnosus* indicated that surface exposure is essential (Nguyen et al. 1995). On the other hand, immunisations with *L. lactis* expressing intracellular soluble, membrane-located or cell wall-associated immunogens revealed that all forms were effective in evoking protective immune responses, the particulate form of the antigen being more immunogenic (Norton et al. 1996; Wells et al. 1996; Robinson et al. 1997). Although surface exposure of antigens may, thus, be advantageous in terms of immunogenicity, it may also have drawbacks as, e.g., higher susceptibility to proteolytic degradation in the gastrointestinal tract (GIT) in oral vaccinations. Antigens anchored to the cytoplasmic face of the cell membrane may have the same elevated level of immunogenicity as proteins anchored at the exterior and may be better protected against proteolytic activity in the GIT. This approach has been taken in Gram-negative bacteria (Eko et al. 1999) but, to the best of our knowledge, it has not been investigated in Gram-positives.

The expression of an scFv on the surface of *Staph. carnosus* opens the possibility to express entire (artificial) antibody libraries in LAB. Although construction of such libraries is limited to strains that are highly transformable, strategies like that could help to identify antibody fragments that are reactive to surface components of mucosal cells, such as M-cells that are present in immunoreactive sites. Co-expression of these antibody fragments on the surface of LAB carrying an immunogen may target the bacteria to the desired area and enhance the immune response (Ståhl & Uhlén 1997a). Another interesting application of

recombinant antibody- or artificial binding-protein libraries that warrants further exploration is the selection of ligands that bind specifically to e.g. pollutants, such as heavy metals (Sousa et al. 1996). LAB carrying such surface-located ligands could then be exploited in environmental applications like whole-cell bioadsorbants or biofilters.

The cell wall sorting signals that contain the LPXTG box are clearly the most exploited anchoring signals in LAB. The systems developed in *Staph. carnosus* and *Strep. gordonii* for cell-surface display using this type of anchor belong to the most advanced in LAB. New techniques, such as FACScan, were used for the first time for *Staph. carnosus* and, more recently also for *Lb. casei*, to quantify the number of surface exposed protein molecules on intact cells. A broader use of these techniques will be required to reliably assess each anchoring system in the various bacteria.

In addition to the successful use of heterologous anchor domains from pathogenic bacteria, an important development is the identification and application of LAB anchors. For several purposes the presence of heterologous DNA fragments should be minimised, especially for those in which the GRAS status of the production organism plays a critical role.

The AcmA-repeats and the S-layer proteins represent new types of anchoring devices in LAB and offer alternative approaches for cell surface attachment. Both need further characterisation as they are potentially very versatile. For instance, the AcmA repeat domain binds to the cell wall of a wide range of Gram-positive bacteria, also when it is added from the outside. This property allows to anchor chimaeric proteins, e.g. antigens or active enzymes, to the surface of non-recombinant bacteria, which would prevent the release of recombinant DNA in the environment. Hybrid S-layer proteins may have the limitation that surface anchoring is restricted to the bacterium from which the gene was isolated, but they do have the attractive ability to form crystalline structures *in vitro*. The ability to form lattices was retained for *Lb. acidophilus* and *Lb. crispatus* SLPs that were produced in *E. coli* (Smit et al., unpublished). This property may be exploited in yet other approaches using the isolated protein for, e.g., ultrafiltration membranes, diagnostics, vaccine development and nanotechnology (Sleytr et al. 1997).

As LAB research on membrane proteins, cell wall proteins and surface display advances, the putative biotechnological applications will become increasingly realistic and still new ones may emerge from this exciting and rapidly developing research area.

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References

Andréoni C, Goetsch L, Libon C, Samuelson P, Nguyen TN, Robert A, Uhlén M, Binz H & Ståhl S (1997) Flow cytometric quantification of surface-displayed recombinant receptors on staphylococci. BioTechniques 23: 696–704

Boot HJ, Kolen CPAM, Van Noort JM & Pouwels PH (1993) S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. J. Bacteriol. 175: 6089–6096

Buist G (1997) AcmA of *Lactococcus lactis*, a cell-binding major autolysin. Ph.D. thesis, University of Groningen, Haren, The Netherlands

Buist G, Kok J, Leenhouts KJ, Dabrowska M, Venema G & Haandrikman AJ (1995) Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. 177: 1554–1563

Callegari ML, Riboli B, Sanders JW, Cocconcelli PS, Kok J, Venema G & Morelli L (1998) The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression. Microbiol. 144: 719–726

Di Fabio S, Medaglini D, Rush CM, Corrias F, Panzini GL, Pace M, Verani P, Pozzi G & Titti F (1998) Vaginal immunization of *Cynomolgus* monkeys with *Streptococcus gordonii* expressing HIV-1 and HPV16 antigens. Vaccine 16: 485–492

Eko FO, Witte A, Huter V, Kuen B, Fürst-Ladani S, Haslberger A., Katinger A, Hensel A, Szostak MP, Resch S. Mader S. Raza P, Brand E, Marchart J, Jechlinger W, Haidinger W & Lubitz W (1999) New strategies for combination vaccines based on the extended recombinant bacterial ghost system. Vaccine 17: 1643–1649

Fischetti VA, Medaglini D & Pozzi G (1996) Gram-positive commensal bacteria for mucosal vaccine delivery. Curr. Opin. Biotechnol. 7: 659–666

Fischetti VA, Medaglini D, Oggioni M & Pozzi G (1993) Expression of foreign proteins on Gram-positive commensal bacteria for mucosal vaccine delivery. Curr. Opin. Biotechnol. 4: 603–610

Fischetti VA, Pancholi V & Schneewind O (1990) Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. Mol. Microbiol. 4: 1603–1605

Franke CM (1998) Topology of a type I secretion system for bacteriocins of *Lactococcus lactis*. Ph.D. thesis, University of Groningen, Haren, The Netherlands

Franke CM, Leenhouts KJ, Haandrikman AJ, Kok J, Venema G & Venema K (1996) Topology of LcnD, a protein implicated in the transport of bacteriocins from *Lactococcus lactis*. J. Bacteriol. 178: 1766–1769

Georgiou G, Stathopoulos C, Daugherty PS, Nayak AR, Iverson BL & Curtiss III R (1997) Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. Nature Biotechnol. 15: 29–34

Gunneriusson E, Samuelson P, Uhlén M, Nygren PA & Ståhl S (1996) Surface display of a functional single chain Fv antibody on staphylococci. J. Bacteriol. 178: 1341–1346

Haandrikman AJ, Kok J & Venema G (1991) Lactococcal proteinase maturation protein PrtM is a lipoprotein. J. Bacteriol. 173: 4517–4525

Hansson M, Ståhl S, Nguyen TN, Bächi T, Robert A, Binz H, Sjölander A & Uhlén M (1992) Expression of recombinant proteins on the surface of the coagulase-negative bacterium *Staphylococcus xylosus*. J. Bacteriol. 174: 4239–4245

Hofnung M (1991) Expression of foreign polypeptides at the *Escherichia coli* cell surface. Methods Cell Biol. 34: 77–105

Holck A & Næs H (1992) Cloning, sequencing and expression of the gene encoding the cell-envelope-associated proteinase from *Lactobacillus paracasei* subsp. *Paracasei* NCDO151. J. Gen. Microbiol. 138: 1353–1364

Kok J, Leenhouts KJ, Haandrikman AJ, Ledeboer AM & Venema G (1988) Nucleotide sequence of the cell wall-associated proteinase gene of *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. 54: 231–238

Leer RJ, Antonissen C, Bergmans A, Jore J, Boersma WJA & Pouwels PH (1996) A new series of *Lactobacillus* expression vectors. Abstracts of the Fifth Symposium of Lactic Acid Bacteria: Genetics, Metabolism and Applications. Veldhoven, The Netherlands, Abstract no. E8

Liljeqvist S, Samuelsen P, Hansson M, Nguyen TN, Binz H & Ståhl S (1997) Surface display of the cholera toxin subunit on *Staphylococcus xylosus* and *Staphylococcus carnosus*. Appl. Environ. Microbiol. 63: 2481–2488

Maassen CBM, Laman JD, Heijne Den Bak-Glashouwer MJ, Tielen FJ, Van Holten-Neelen JCPA, Hoogteijling L, Antonissen C, Leer RJ, Pouwels PH, Boersma WJA & Shaw DM (1999) Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. Vaccine 17: 2117–2128

Medaglini D, Oggioni MR & Pozzi G (1998) Vaginal immunization with recombinant gram-positive bacteria. Am. J. Repr. Immunol. 39: 199–208

Medaglini D, Pozzi G, King TP & Fischetti VA (1995) Mucosal and systemic immune responses to a recombinant protein expressed on the surface of the oral commensal bacterium *Streptococcus gordonii* after oral colonization. Proc. Natl. Acad. Sci. USA 92: 6868–6872

Medaglini D, Rush CM, Sestini P & Pozzi G (1997) Commensal bacteria as vectors for mucosal vaccines against sexually transmitted diseases: vaginal colonization with recombinant streptococci induces local and systemic antibodies in mice. Vaccine 15: 1330–1337

Mercenier A, Dutot P, Kleinpeter P, Aguirre M, Paris P, Reymund J & Slos P (1996) Development of lactic acid bacteria as live vectors for oral or local vaccines. Adv. Food Sci. 18: 73–77

Navarre WW & Schneewind O (1994) Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in Gram-positive bacteria. Mol. Microbiol. 14: 115–121

Navarre WW & Schneewind O (1999) Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. 63: 174–229

Navarre WW, Ton-That H, Faull KF & Schneewind O (1998) Anchor structure of staphylococcal surface proteins. II. COOH-terminal structure of muramidase and amidase-solubilized surface protein. J. Biol. Chem. 273: 29135–29142

Nguyen TN, Gourdon MH, Hansson M, Robert A, Samuelson P, Libon C, Andréoni C, Nygren PA, Binz H, Uhlén M, & Ståhl S (1995) Hydrophobicity engineering to facilitate surface display of heterologous gene products on *Staphylococcus xylosus*. J. Biotechnol. 42: 207–219

Nguyen TN, Hansson M, Ståhl S, Bächi T, Robert A, Domzig W, Binz H & Uhlén M (1993) Cell-surface display of heterologous epitopes on *Staphylococcus xylosus* as a potential delivery system for oral vaccination. Gene 128: 89–94

Norton PM, Brown HWG, Wells JM, Macpherson AM, Wilson PW & Le Page RWF (1996) Factors affecting the immunogenicity of tetanus toxin fragment C expressed in *Lactococcus lactis*. FEMS Immunol. Med. Microbiol. 14: 167–177

Norton PM, Le Page RWF & Wells JM (1995) Progress in the development of *Lactococcus lactis* as a recombinant mucosal vaccine delivery system. Folia Microbiol. 40: 225–230

Oggioni MR & Pozzi G (1996) A host-vector system for heterologous gene expression in *Streptococcus gordonii*. Gene 169: 85–90

Oggioni MR, Manganelli R, Contorni M, Tommasimo M & Pozzi G (1995) Immunization of mice by oral colonization with live recombinant commensal streptococci. Vaccine 13: 775–779

Piard JC, Hautefort I, Fischetti VA, Ehrlich SD, Fons M & Gruss A (1997a) Cell wall anchoring of the *Streptococcus pyogenes* M6 protein in various lactic acid bacteria. J. Bacteriol. 179: 3068–3072

Piard JC, Jimenez-Diaz R, Fischetti VA, Ehrlich SD & Gruss A (1997b) The M6 protein of *Streptococcus pyogenes* and its potential as a tool to anchor biologically active molecules at the surface of lactic acid bacteria. In: Horaud et al. (Eds) Streptococci and the Host, (pp. 545–550). Plenum Press, New York, USA

Poquet I, Ehrlich SD & Gruss A (1998) An export-specific reporter designed for gram-positive bacteria: application to *Lactococcus lactis*. J. Bacteriol. 180: 1904–1912

Pouwels PH, Leer RJ & Boersma WJA (1996) The potential of *Lactobacillus* as a carrier for oral immunization: Development and preliminary characterization of vector systems for targeted delivery of antigens. J. Biotechnol. 44: 183–192

Pouwels PH, Leer RJ, Shaw M, Heijne den Bak-Glashouwer MJ, Tielen FD, Smit E, Martinez B, Jore J & Conway PL (1998) Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. Int. J. Food Microbiol. 41: 155–167

Pozzi G & Wells JM (1997a) Gram-Positive Bacteria. Vaccine Vehicles for Mucosal Immunization. Landes Biosciences Georgetown, TX, USA

Pozzi G, Contorni M, Oggioni MR, Manganelli R, Tommasino M, Cavalieri F & Fischetti VA (1992a) Delivery and expression of a heterologous antigen on the surface of streptococci. Infect. Immun. 60: 1902–1907

Pozzi G, Oggioni MR & Medaglini D (1997b) Recombinant *Streptococcus gordonii* as live vehicle for vaccine antigens. In: Pozzi G & Wells JM (Eds) Gram-positives Bacteria. Vaccine Vehicles for Mucosal Immunization (pp. 35–60). Landes Bioscience Georgetown, TX, USA

Pozzi G, Oggioni MR, Manganelli R & Fischetti VA (1992b) Expression of M6 protein gene of *Streptococcus pyogenes* in *Streptococcus gordonii* after chromosomal integration and transcription. Res. Microbiol. 143: 449–457

Pozzi G, Oggioni MR, Manganelli R, Medaglini D, Fischetti VA, Fenoglio D, Valle MT, Kunkl A & Manca F (1994) Human

T-helper cell recognition of an immunodominant epitope of HIV-1 gp120 expressed on the surface of *Streptococcus gordonii*. Vaccine 12: 1071–1077

Pugsley AP (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. 57: 50–108

Ricci S, Rush CM, Medaglini D & Pozzi G (1996) Expression of the *Escherichia coli* heat labile toxin subunit B in *Streptococcus gordonii*. Abstr. XII European Meeting on Bacterial Gene Transfer and Expression. Siena, Italy. p. 88

Robert A, Samuelson P, Andréoni C, Bächi T, Uhlén M, Binz H, Nguyen TN & Ståhl S (1996) Surface display on staphylococci: a comparative study. FEBS Lett. 390: 327–333

Robinson K, Chamberlain LM, Schofield KM, Wells JM & Le Page RWF (1997) Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nature Biotechnol. 15: 653–657

Rush CM, Mercenier A & Pozzi G (1997) Expression of vaccine antigens in *Lactobacillus*. In: Pozzi G & Wells JM (Eds) Gram-positive Bacteria. Vaccine Vehicles for Mucosal Immunization (pp. 107–144). Landes Bioscience Georgetown, TX, USA

Samuelson P, Hansson M, Ahlborg N, Andréoni C, Götz F, Bächi T, Nguyen TN, Binz H, Uhlén M & Ståhl S (1995) Cell surface display of recombinant proteins on *Staphylococcus carnosus*. J. Bacteriol. 177: 1470–1476

Schneewind O, Fowler A & Faull KF (1995) Structure of the cell wall anchor of surface proteins in *Staphylococcus aureus*. Science 268: 103–106

Schneewind O, Mihaylova-Petkov D & Model P (1993) Cell wall sorting signals in surface proteins of Gram-positive bacteria. EMBO J. 12: 4803–4811

Schneewind O, Model P & Fischetti VA (1992) Sorting of protein A to the staphylococcal cell wall. Cell 70: 267–281

Sleytr UB, Bayley H, Sára M, Breitwieser A, Küpcü S, Mader C, Weigert S, Unger FM & et al. (1997) Applications of S-layers. FEMS Microbiol. Rev. 20: 151–175

Sousa C, Cebolla A & De Lorenzo V (1996) Enhanced metalloadsorption of bacterial cells displaying poly-His peptides. Nature Biotechnol. 14: 1017–1020

Ståhl S & Uhlén M (1997a) Bacterial surface display: trends and progress. Trends Biotechnol. 15: 185–192

Ståhl S, Samuelson P, Hansson M, Andréoni C, Goetsch L, Libon C, Liljeqvist S, Gunneriusson E, Binz H, Nguyen TN & Uhlén M (1997b) Development of non-pathogenic staphylococci as vaccine delivery vehicles. In: Pozzi G & Wells JM (Eds) Gram-positive Bacteria. Vaccine Vehicles for Mucosal Immunization (pp. 61–81). Landes Bioscience Georgetown, TX, USA

Steidler L, Viaene J, Fiers W & Remaut E (1998) Functional display of a heterologous protein on the surface of *Lactococcus lactis* by means of the cell wall anchor of *Staphylococcus aureus* protein A. Appl. Environ. Microbiol. 64: 342–345

Strauss A & Götz F (1996) *In vivo* immobilization of enzymatically active polypeptides on the cell surface of *Staphylococcus carnosus*. Mol. Microbiol. 21: 491–500

Strauss A, Thumm G & Götz F (1998) Influence of Lif, the lysostaphin immunity factor, on acceptors of surface proteins and cell wall sorting efficiency in *Staphylococcus carnosus*. J. Bacteriol. 180: 4960–4962

Toba T, Virkola R, Westerlund B, Björkman Y, Sillanpää J, Vartio T, Kalkkinen N & Korhonen TK (1995) A collagen-binding S-layer protein in *Lactobacillus crispatus*. Appl. Environ. Microbiol. 61: 2467–2471

Ton-That H, Faull KF & Schneewind O (1997) Anchor structure of staphylococcal surface proteins. A branched peptide that links the carboxyl terminus of proteins to the cell wall. J. Biol. Chem. 272: 22285–22292

Ton-That H, Labischinski H, Berger-Bächi & Schneewind O (1998) Anchor structure of staphylococcal surface proteins. III. Role of the FemA, FemB, and FemX factors in anchoring surface proteins on the bacterial cell wall. J. Biol. Chem. 273: 29143–29149

Tynkkynen S, Buist G, Kunji E, Kok J, Poolman B, Venema G & Haandrikman AJ (1993) Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. J. Bacteriol. 175: 7523–7532

Vidgrén G, Palva I, Pakkanen R, Lounatmaa K & Palva A (1992) S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. J. Bacteriol. 174: 7419–7427

Vos P, Simons G, Siezen RJ & De Vos WM (1989) Primary structure and organization of the gene for a prokaryotic cell envelope-located serine proteinase. J. Biol. Chem. 264: 13579–13585

Wells JM, Robinson K, Chamberlain LM, Schofield KM & Le Page RWF (1996) Lactic acid bacteria as vaccine delivery vehicles. Antonie van Leeuwenhoek 70: 317–330