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

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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.

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

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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, biadsorbents, 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 (Fig. 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 center, 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, 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 LysM domain 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

### Heterologous Surface Display on Living LAB Cells

The peptidoglycan-binding domain of lactococcal AcmA protein has been used

![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.](image)

### 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 W⁹    | PF07538     | 1–13    | Central, N-, C-term.| 36     | PG                                           | 1 in 1 species                            |
| CW_binding_1      | Putative cell wall binding repeatb | 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      | Predicted peptidoglycan binding domain | PF08823    | 1       | Central, C-term.| 74     | PG                                           | /                                         |
| PG_binding_3      | Predicted peptidoglycan binding 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                           |

⁹ also known as the choline-binding repeat (ChBr) or the choline-binding domain (ChBD).

PG-peptidoglycan.
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). Steen et al. demonstrated aff ective binding of Proteus (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. 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. Other LysM-repeat containing proteins, including endolysin Lyb5 and mureopeptidase MurO, 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. The same reporter proteins (GFP and β-galactosidase) have been functionally displayed on the surface of non-recombinant Lb. plantarum via 2 LysM repeat containing LysM domains from putative muropeptidase MurO, the autolysin from Lb. plantarum. 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 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. 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. 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) or are genus specific (e.g., genus Staphylococcus). Of the cell-wall binding domains described in the PFAM database, only 4 (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 phase endolysins that are directed against Bacillus, Lactobacillus, Lactococcus, Mycobacterium, Staphylococcus, and Streptococcus genera 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 (Fig. 1), designated bacteria-like particles (BLP) or, formally, Gram-positive enhancer matrix (GEM). This approach has been developed by Bosma et al., based on the findings of Steen et al. 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. 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). Prepared BLPs could be used immediately or stored at −80°C. BLPs are usually produced from lacticoccal cells, but the method of preparation is applicable to all Gram-positive bacteria. In recent years, lacticoccal BLPs have been widely used for the development of mucosal vaccines against viruses (influenza, respiratory syncytial virus and hepatitis B virus), bacteria (Streptococcus pneumoniae, Staphylococcus aureus, Yersinia pestis and Shigella flexneri) and parasites (Plasmodium falciparum and Plasmadium berghei). 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. In addition to lacticoccal BLPs, Lb. salivarius IBB3154 BLPs have recently been evaluated as vaccine delivery vehicles by displaying 2 Campylobacter jejuni antigens (CjaA and CjaD) in fusion with cA. 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.
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 2 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. 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 GMMs 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% labeling threshold. However, the threshold does not apply for “intentional” addition of recombinant protein, 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 labeled 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. Recombinant bacteria with therapeutic indications would probably be regarded as an advanced therapy medicinal product. 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. Assessment of environmental risk differs in stringency if the pharmaceutical product contains or consists of GMO, and is described in a special guideline. 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 pharmacological and toxicology studies and 3 phases of human clinical trials.

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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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