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Minireview

Relevant uses of surface proteins – display on self-organized biological structures

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Summary

Proteins are often found attached to surfaces of self-assembling biological units such as whole microbial cells or subcellular structures, e.g. intracellular inclusions. In the last two decades surface proteins were identified that could serve as anchors for the display of foreign protein functions. Extensive protein engineering based on structure–function data enabled efficient display of technically and/or medically relevant protein functions. Small size, diversity of the anchor protein as well as support structure, genetic manipulability and controlled cultivation of phages, bacterial cells and yeasts contributed to the establishment of designed and specifically functionalized tools for applications as sensors, catalysis, biomedicine, vaccine development and library-based screening technologies. Traditionally, phage display is employed for library screening but applications in biomedicine and vaccine development are also perceived. For some diagnostic purposes phages are even too small in size so other carrier materials where needed and gave way for cell and yeast display. Only recently, intracellular inclusions such as magnetosomes, polyhydroxyalkanoate granules and lipid bodies were conceived as stable subcellular structures enabling the display of foreign protein functions and showing potential as specific and tailor-made devices for medical and biotechnological applications.

Introduction

The microbial cell surface localization of molecules engaged in, e.g. cell recognition, signal transduction, surface adherence, immunoreactions or movement and/or colonization is a frequently applied principle in nature. Steric effects often cause poor exposure of surface-displayed heterologous polypeptides resulting in low interaction of the displayed protein with antibodies, small molecules or ligands. Therefore, the fusion of the protein of interest to exposed natural surface proteins or appendages such as flagella or pili is a strategy to overcome these constraints and to allow increased exposure i.e. functionality of the displayed protein function. This review will focus on the different surface display systems investigated so far for phage, bacterial and yeast cell display, endospores, magnetosomes and polyhydroxyalkanoate (PHA) granules.

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Phage display

Phage display can be regarded as one of the first extensively developed and utilized foreign protein display systems harnessing the prolific self-assembling process of the bacteriophage and its defined surface protein architecture (Smith, 1985; Gao *et al*., 2010; Rakonjac *et al*., 2010). Most widely used is *Escherichia coli* phage M13 but also λ and T7 (Benhar, 2001). Affinity screening of phage display libraries is also called 'biopanning' (Parmley and Smith, 1988). In several rounds of panning the engineered phage particles are incubated with the ligand of interest, which is immobilized on a solid support material. Several washes are performed to exclude unspecific binding, followed by elution of the bound particles (Rakonjac *et al*., 2010). Phage display systems are especially applied to combinatorial approaches and are the main tool for the isolation and engineering of recombinant antibodies (Benhar, 2001). Recombinant antibody display started with the fusion of the variable region of a single-chain antibody (scFv) fragment to the amino terminus of the phage minor coat protein III (McCaffterty *et al*., 1990). A following successful application has been the isolation of monoclonal antibodies using phage display and library screening (Winter *et al*., 1994). In recent years techniques were optimized allowing now design and construction of large libraries of screening targets but especially antibodies (Hoogenboom *et al*., 1998). For most phages purification is easy and large-scale production is inexpensive; therefore, phage display

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has also been considered for protein or antibody purification (Clark and March, 2006). Phage display is a very versatile technology, e.g. a treatment against cocaine addiction is based on phage display technology. Nasally delivered whole phage particles displaying a specific cocaine-sequestering antibody can enter the central nervous system and bind to cocaine molecules, thus inhibiting their action on the brain (Dickerson *et al.*, 2005).

One of the main characteristics of bacteriophages is their specificity for the bacterial host. Using this natural affinity, phages have been used for the detection and genotyping of bacteria. Either, the bacteria-bound phages were detected with labelled antibodies, thereby increasing the detection sensitivity (Watson and Eveland, 1965) or the phages were already labelled with the fluorescent dye covalently attached to the coat proteins (Goodridge *et al.*, 1999). Also, phages are specifically employed to deliver reporter genes, encoding fluorescent proteins such as luciferase or the green fluorescent protein (GFP), which are expressed after the infection of the specific host bacteria (Kodikara *et al.*, 1991; Funatsu *et al.*, 2002).

Bacteriophages are also investigated as new vaccines. As bacteriophages are not able to replicate in eukaryotic hosts they are seen as inert antigens and processed by antigen-presenting cells (Gaubin *et al.*, 2003; Gao *et al.*, 2010). As one of the first examples, the protective epitope 173–187 from the glycoprotein G of the human respiratory syncytial virus was fused to the fd phage pIII coat protein. The heterologous protein was displayed at the phage surface and the recombinant phage particles were used to immunize mice. These immunogenic peptide presenting phage particles could be applied as vaccine and conferred a protective immune response. Immunized mice were challenged but the phage vaccine prevented an infection of the immunized animals by the pathogen (Bastien *et al.*, 1997). For vaccination purposes the oral application route seemed more favourable but results varied regarding survival of phage vaccines in gastric fluid, which was also depending on the chosen anchoring motif and on whether purified proteins or whole phage particles were used (Zuercher *et al.*, 2000). Generally, epitope displaying phage particles can induce a specific antibody response when applied orally (Benhar, 2001) and a study involving human volunteers demonstrated the safety of an oral application of phages (Bruttin and Brussow, 2005).

The disadvantage of the smallness of the phage particles is the incompatibility with fluorescence activated cell sorting (FACS) technology (Francisco and Georgiou, 1994). Nevertheless, phage display is a useful tool in biomedical applications and can potentially be used for imaging and diagnosis of tumours and cancerous cell aggregates. Phage display is a potent technique, which permits the screening of vast sequences, can be employed for peptide affinity improvement and generation of unique binding peptides (Deutscher, 2010).

Virus-like particles (VLPs)

Virus-like particles are composed of viral capsid proteins that show the intrinsic ability to spontaneously self-assemble after expression. VLPs can be isolated after recombinant coat protein expression or assembled *in vitro* from purified, recombinantly expressed coat protein subunits. VLPs can be expressed in a wide variety of hosts, ranging from bacterial to mammalian cells (Garcea and Gissmann, 2004). Capsid proteins display basic domains facing the inside of the particle and are therefore predestined to bind nucleic acids via stable, non-specific ionic interactions. Similarly, small molecules with the appropriate ion charge can be internalized by VLPs. Inclusion of nucleic acid or small molecules can be triggered by an 'osmotic shock' reaction, where dilution into low ionic strength solution is causing increased spaces between the coat protein subunits and the interior positive charges are pulling the nucleic acid or other molecules to the inside (Barr *et al.*, 1979). Incorporation can also occur during *in vitro* assembly of coat proteins in the presence of nucleic acids (Braun *et al.*, 1999; Henke *et al.*, 2000). The best characterized models for the generation and application of VLPs are papilloma and polyoma virus (Petry *et al.*, 2003). The polyoma VP1 coat protein was engineered to display the IgG binding domain Z from *Staphylococcus aureus* in its H1 loop (Gleiter and Lilie, 2001; May *et al.*, 2002). The generated chimeric proteins were still able to self-assemble as well as to bind DNA. A plasmid encoding GFP was incorporated into the engineered VLPs and an antibody directed against the cell-surface receptor ErbB2 was bound to the surface displayed Z domain. The VLPs were specifically targeted to ErbB2 receptor displaying cells where after incubation GFP fluorescence could be observed (Gleiter and Lilie, 2003). The main application of VLPs is the development of new, effective, fast and inexpensive vaccines. VLPs are very immunogenic and interact very well with dendritic cells and other antigen presenting cells (Lenz *et al.*, 2001; Rudolf *et al.*, 2001; Warfield *et al.*, 2003). One already approved VLP vaccine is used for immunization against human papilloma virus 16. It is composed of the major structural protein L1 and is able to induce a high titre of neutralizing antibodies and confers protection against human papilloma virus infection (Koutsky *et al.*, 2002). Recently, researchers are focussing on the development of an efficient and rapidly prepared influenza vaccine. Current influenza vaccine development is time-consuming and limited to chicken egg production whereas influenza-VLPs could be rapidly prepared as soon as the targeted influenza virus RNA is isolated (Szécsi *et al.*, 2006). It has been proven

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Table 1. Examples for different surface display systems.

		Function			
Display system	Anchor	Anchor	Fusion partner	Application	References
Phage	pIII	M13 coat protein	scFv	Antibody engineering	McCaffterty *et al.* (1990)
			Epitopes	Vaccines	Bastien *et al.* (1997)
	pVIII	M13 coat protein	Peptides/antibodies	Library screening	Benhar (2001)
Virus-like particles	VP1	Polyoma virus coat	Z domain of protein A	Antibody purification	Gleiter and Lilie (2001)
	L1	Human papilloma virus	–	Vaccine	Koutsky *et al.* (2002)
Gram-negative cells	LamB	Maltoporin	Peptides	Library screening	Benhar (2001)
	OmpA	Outer membrane protein	Peptides	Library screening	Benhar (2001)
	Lpp-OmpA	Lipoprotein/OmpA chimere	Enzymes/antibodies	Biosensors	Chen and Georgiou (2002)
	INP	Ice nucleation protein	Enzymes	Biosensors	Li *et al.* (2003)
	FliC	Flagellae protein (flagellin)	Peptides/antigens	Library screening	Lu *et al.* (1995)
	FimA	Fimbriae protein (fimbrillin)	Epitopes/antigens	Vaccines	Samuelson *et al.* (2002)
Bacterial ghosts		*E. coli* O157:H7 antigens		Vaccines	Mayr *et al.* (2005)
		Vibrio cholerae antigens		Vaccines	Eko *et al.* (2003b)
	L′	DNA-binding protein of MS2	DNA	DNA vaccine	Mayrhofer *et al.* (2005)
Gram-positive cells	Protein A	Cell wall anchor (covalent)	Streptavidin	Biotin-binding	Steidler *et al.* (1998)
			Metal binding peptides	Bioremediation	Samuelson *et al.* (2000)
	LysM	Cell wall anchor (non-covalent)	GFP/laccase	Enzyme display	Shao *et al.* (2009)
	FnBPB	Fibronectin-binding protein	Lipase/lactamase	Bioassays	Strauss and Götz (1996)
	M6	Cell wall binding	Cohesins	Enzyme display	Wieczorek and Martin (2010)
S-layers	SbpA	*Lysinibacillus sphaericus*	Bet v1	Vaccines	Ilk *et al.* (2011)
	RsaA	*Caulobacter crescentus*	Protein G	Antibody binding	Nomellini *et al.* (2007)
Yeast	Agα1	α-agglutinin	Z domain of protein A	Immunoassays	Shimojo *et al.* (2004)
	Aga1/Aga2	a-agglutinin	Wasp venom antigens	Vaccines	Borodina *et al.* (2010)
			Cohesins	Biofuel production	Wen *et al.* (2010)
	Flo1	Flocculin	Lipase	Enzyme display	Matsumoto *et al.* (2002)
Endospores	CotB, C, D	Coat proteins	Toxins/antigens	Vaccines	Isticato *et al.* (2001)
					Mauriello *et al.* (2004)
					Duc *et al.* (2007)
Magnetosomes	Mms13	Transmembrane protein	Protein A/G	IgG purification	Matsunaga *et al.* (2006)
PHA beads	PhaF	Phasin	Enzymes	Protein purification	Moldes *et al.* (2004)
	PhaP	Phasin	Enzymes	Protein purification	Banki and Wood (2005)
	PhaZ	Depolymerase	Fluorophores	Protein purification	Lee *et al.* (2005)
	PhaR	Regulator	GFP	Protein purification	Zhang *et al.* (2010)
	PhaC	Synthase	Protein A/enzymes	Bioassays	Grage *et al.* (2009)
			Antigens	Vaccines	Parlane *et al.* (2009)
Lipid bodies	PhaP1	Phasin	Enzymes	Enzyme display	Hänisch *et al.* (2006b)
	LD domain	Eukaryotic binding protein	GFP	*In vivo* monitoring	Yu *et al.* (2011)

successful to display three to four of the major antigens of various influenza viruses on the VLPs to induce a broad array of immunity and confer a protection against influenza challenges (Bright *et al.*, 2008; Ross *et al.*, 2009; Wu *et al.*, 2010) (Table 1).

Cell surface display

Gram-negative bacterial cell display

In 1986 the first reports about bacterial display of foreign peptides/proteins were published and gave way to new insights, developments and applications of bacterial cells (Charbit *et al.*, 1986; Freudl *et al.*, 1986). In the following decades, applications such as vaccine delivery vehicles, library and selection devices, cellular adsorbents and biocatalysts were described (Ståhl and Uhlén, 1997). The first used outer membrane proteins LamB (maltoporin), OmpA (outer membrane protein A) and PhoE (phosphate-inducible porin) were not applicable for the display of larger proteins (Benhar, 2001). Appendices like flagellae and fimbriae were presented as alternatives for the surface display of proteins and also offered the possibility to overcome steric hindrances occurring in the outer membrane. Flagellae are mainly composed of the major flagellin protein FliC (Wilson and Beveridge, 1993). The N- and C-terminal regions of FliC are highly conserved, whereas the central region is variable and not essential for the filament structure (Wilson and Beveridge, 1993) and therefore employed for the display of foreign polypeptides and proteins. The first example was the successful surface display of a hen egg-white lysozyme epitope within FliC (Kuwajima *et al.*, 1988). Various proteins have been displayed using this technique, also thioredoxin that was used for library display and protein–protein interactions studies (Lu *et al.*, 1995). Fimbriae are long filamentous adhesins

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required for targeting and colonization of specific host regions and mainly consist of the major subunit fimbrillin. The hypervariable regions of this protein are potential insertion points for the display of foreign epitopes and antigens (Samuelson *et al*., 2002). Georgiou and co-workers developed a very robust and widely applied display system based on the lipoprotein Lpp and the outer membrane protein OmpA. The first nine residues of the Lpp signal peptide are fused to the residues 46–159 of OmpA and thus allow surface display of a protein fused to the C terminus of the anchoring chimera (Francisco *et al*., 1992). This system allowed the display of full-length proteins on the surface of *E. coli*. Proteins such as cellulases, esterases, organophosphate hydrolases, scFv antibodies/enzymes, β-lactamase or thioredoxin have been successfully displayed using the Lpp-OmpA system (Chen and Georgiou, 2002). Recently, a different display system utilizing the ice nucleation protein (INP) from *Pseudomonas syringae* has been developed (Jung *et al*., 1998). The N-terminal region anchors in the membrane via glycosylphosphatidylinositol (GPI). INP can be used as full-length anchor, but more frequently either only the N-terminal region or a fusion of N- and C-terminus are used. Some results suggested the addition of the C-terminal domain might stabilize the fusion protein, depending on the fusion partner. For example, the production of GFP was unaffected by addition of the C-terminal domain, whereas organophosphorus hydrolase production and activity was significantly enhanced (Li *et al*., 2003). The INP system could also be established in *Pseudomonas putida* (Jung *et al*., 2006). A second INP system is based on the INP from *P. syringae* INA5. It is employed the same way as the previously described system based on InaK. Compared with Lpp-OmpA and InaK as anchors, the InaV system led to five times higher activity of the fusion partner organophosphorus hydrolase. Also, no lysis or growth inhibition of the host could be detected as compared with using Lpp-OmpA (Shimazu *et al*., 2001).

A display system with broader versatility might be the use of bacterial autotransporters. The natural occurring passenger domain can be replaced by a heterologous protein to be displayed on the cell surface. As autotransporters are widely distributed and show high homology among Gram-negative bacteria, one construct could be applied in several hosts (Rutherford and Mourez, 2006).

Bacterial ghost based delivery and display

Bacterial ghosts are non-living, empty cell envelopes of Gram-negative bacteria origin. These empty shells are the result of the expression of the bacteriophage ΦX174 and the resulting protein E-mediated cell lysis (Lubitz *et al*., 1984; Witte *et al*., 1990). The protein E-derived lysis is leading to the formation of a specific transmembrane tunnel structure. Due to the osmotic pressure difference, the cytoplasmic content is banished and empty bacterial cell envelopes can be retrieved (Blasi *et al*., 1989; Witte *et al*., 1992). Additionally, a staphylococcal nuclease is employed to degrade host and plasmid DNA (Haidinger *et al*., 2003). Therefore, bacterial ghosts are safe as no pathogenic islands or antibiotic resistances can be transferred by horizontal gene transfer (Lubitz *et al*., 2009). The bacterial ghosts exhibit the natural non-denatured surface components of the original Gram-negative bacterial cells with all targeting functions and can be applied for foreign protein display as described for genetically active Gram-negative bacterial cells (Eko *et al*., 2000). Most commonly, bacterial ghosts are applied as vaccines or gene delivery vehicles (Jalava *et al*., 2002). The lipopolysaccharides (LPS) of the bacterial ghosts are not limiting their use and application as candidate vaccines as no significant fever responses due to the endotoxins could be observed (Mader *et al*., 1997). Due to the intact outer membrane, bacterial ghosts are recognized by the innate immune system. They are ingested by dendritic cells and macrophages and are able to generate cellular and humoral immune responses without exogenous adjuvants (Haslberger *et al*., 2000; Jalava *et al*., 2003). Bacterial ghost vaccine preparations are stable for many years at ambient temperatures as they are freeze-dried and therefore do not require a cold chain storage system (Szostak *et al*., 1996). A simple but effective application is the use of bacterial ghosts as candidate vaccines against pathogenic Gram-negative bacteria. Ghosts of the enterohaemorrhagic strain *E. coli* O157 : H7 have been prepared and applied in a mouse model. A single dose of the orally applied vaccine was sufficient to induce protection even against a lethal challenge (Mayr *et al*., 2005). Same results could be observed with intragastric immunization of mice where the mixed immune response was dominated by humoral immunity (Cai *et al*., 2010). Rabbits orally vaccinated with *Vibrio cholerae* ghosts also showed protection against lethal challenge (Eko *et al*., 2003b). Also, *V. cholerae* ghosts engineered to display *Chlamydia trachomatis* antigens induced a Th1 response specific to *Chlamydia* infection (Eko *et al*., 2003a). Bacterial ghosts are also applied as DNA vaccines. The antigen-encoding plasmid DNA can be loaded *in vitro* in the bacterial ghost structures as shown with *E. coli* and *Pasteurella haemolytica* ghosts, loaded with plasmids encoding GFP (Ebensen *et al*., 2004; Paukner *et al*., 2005). The loaded DNA was localized to the inner lumen of the ghosts as shown with confocal laser scanning microscopy to exclude outer surface association of the DNA. Green fluorescence could be observed in macrophages and dendritic cells, proving the efficient targeting and gene transfer of the loaded bacterial ghosts. Murine vaccination studies revealed a modulation of the immune response from a mixed Th1/Th2 to a more

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dominant Th2 pattern (Ebensen *et al*., 2004). More sophisticated is the *in vivo* loading of the ghost particles by using the so-called self-immobilizing plasmid system (pSIP) (Jechlinger *et al*., 2004). A DNA binding protein anchored to the inner membrane is interacting with the corresponding operator region on the *in vivo* loaded plasmid DNA. By interaction of the different proteins, the loaded DNA is protected from the nuclease in the ghost generating step. After transfection and induction the operon repressor is released and the target gene expressed (Mayrhofer *et al*., 2005). This system allows for a one-step process of bacterial ghost generation and DNA loading.

Gram-positive bacterial cell display

Escherichia coli is the most frequently employed host organism for bacterial cell surface display and although most genetic engineering tools and techniques are designed for this organism, Gram-negative bacteria also have disadvantages, e.g. transport over two membranes or LPS-toxicity. In recent years much progress was made especially overcoming low transformation efficiencies with Gram-positive host bacteria and therefore it became feasible to also employ 'generally regarded as safe' (GRAS) organisms for biomedical applications. The most frequently used display system in Gram-positive bacteria is engaging the cell wall binding ability of protein A from *S. aureus* (Schneewind *et al*., 1995). The protein A cell wall sorting signal comprises a LPXTG sequence motif followed by a stretch of hydrophobic membrane-spanning residues and six or seven mostly positively charged amino acid residues at the extreme C terminus serving as retention signal to prevent the secretion of the protein into the surrounding medium (Schneewind *et al*., 1993). A transpeptidase (sortase) recognizes the LPXTG motif and catalyses the covalent anchoring of protein A to the cell wall. This anchoring mechanism can be conferred to several Gram-positive host organisms. For example *Lactococcus lactis* and was employed for the functional display of streptavidin monomer (Steidler *et al*., 1998). This system was also utilized for the first use of a Gram-positive host – *Staphylococcus carnosus* – for library screening and selection of affinity proteins (Kronqvist *et al*., 2008). Gram-positive bacteria are also considered for application as biosorbents in bioremediation of heavy metals. Therefore, especially staphylococci are engineered to display metal-binding motifs as fusions with the protein A cell wall anchor, e.g. polyhistidyl residues or cellulose-binding domains engineered to bind divalent ions such as Ni^{2+} were successfully displayed (Samuelson *et al*., 2000; Wernérus *et al*., 2001). The lysine motif domain LysM can be used for non-covalent cell wall binding and exposure of a heterologous fusion protein. (Shao *et al*., 2009) developed a display system for *Bacillus thuringiensis* using the LysM domain of a peptidoglycan hydrolase and GFP as well as a laccase as reporter. Also, the C-terminal region of the *S. aureus* fibronectin-binding protein B (FnBPB) was N-terminally functionalized for the display of a lipase or β-lactamase in *S. carnosus*. The FnBPB variant contained the original cell wall sorting signal and cell wall spanning region and was sufficient for the functional immobilization of normally soluble enzymes without influencing their catalytic activity (Strauss and Götz, 1996). The streptococcal M6 protein was used as anchor to C-terminally displayed cohesin modules and thus a complex of enzymes similar to cellulosomes on the surface of *L. lactis* exploiting the cohesin-dockerin system was established. These authors envisaged the display of multiple enzymes on the same cell surface applying their system with the corresponding number of cohesin scaffolds on a single cell wall anchor (Wieczorek and Martin, 2010).

S-layers

S-layers are crystalline arrays of protein and glycoprotein subunits and are widely distributed among all phylogenetic branches of Archaea and Bacteria (Engelhardt and Peters, 1998; Sleytr and Beveridge, 1999). The individual subunits are linked to each other as well as to the cell wall peptidoglycan in Gram-positive bacteria or the LPS of the outer membrane of Gram-negative bacteria by non-covalent forces (Sleytr and Beveridge, 1999). S-layer proteins are assembled in a two-dimensional lattice with oblique, tetragonal or hexagonal symmetry (Mobili *et al*., 2010). In most species S-layers are monomolecular and consist of only one type of protein, in some such as *Clostridium difficile* two different proteins derived from the same precursor assemble to the S-layer structure (Calabi *et al*., 2001). Several specific functions are assigned to the individual surface layers, e.g. protective coats, molecular sieves, molecule and ion traps, cell adhesion, surface recognition or virulence factors (Mobili *et al*., 2010). One of the most remarkable and biotechnological exploited property of S-layer proteins is the ability to self-assemble in suspension (Sleytr *et al*., 2007a,b). For biotechnological approaches natural S-layer hosts are employed but S-layer comprising fusion proteins are also recombinantly expressed, e.g. in *Bacillus subtilis*, which is GRAS approved but has no intrinsic S-layer on its cell surface (Fu *et al*., 2006). The most commonly employed S-layer protein from a Gram-positive is SbpA of *Lysinibacillus sphaericus* CCM2177. In order to develop a new vaccine for immunotherapy of atopic allergies, the major birch pollen allergen Bet v1 was fused to SbpA and the fusion protein recombinantly produced in *B. subtilis* (Ilk *et al*., 2011). Previous studies have shown the feasibility regarding immunomodulatory capacities of such vaccines but protein expression has always been

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carried out in *E. coli*, thus an additional, time-consuming endotoxin-removal step was required (Breitwieser *et al.*, 2002; Ilk *et al.*, 2002). In a recent study, the endotoxin-free fusion protein was secreted due to the amyQ signal peptide from *Bacillus amyloliquefaciens* and self-assembled but not crystallized in solution. Purification of the fusion protein from the culture medium was achieved by exploiting the specific binding mechanism between the N-terminus of SbpA and the secondary cell wall polymer – the natural anchor – in the peptidoglycan-containing sacculi of *Ly. sphaericus* (Ilk *et al.*, 1999). The immune reactivity of the putative vaccine could be demonstrated in immunoblotting experiments with serum samples from patients suffering from birch pollen allergy (Ilk *et al.*, 2011). SbpA has also been used, e.g. for the functional display of two copies of the IgG-binding domain Z of protein A from *S. aureus* (Völlenkle *et al.*, 2004). The most commonly employed S-layer protein of Gram-negative bacterial origin is RsaA from *Caulobacter crescentus*. Despite being a Gram-negative host, the LPS structure of *C. crescentus* is quite unusual and less toxic compared with enteric bacteria (Smit *et al.*, 2008). In one of the first studies it could be demonstrated that RsaA as a member of S-layer proteins can be employed for heterologous peptide display on bacterial cell surfaces (Bingle *et al.*, 1997). Later, the IgG-binding domain of protein G was displayed as RsaA fusion in order to develop a low-cost antibody binding reagent (Nomellini *et al.*, 2007). Recently, efforts have been undertaken to develop a RsaA based HIV-specific microbicide. The domain 1 of the HIV receptor and a ligand for the HIV co-receptor have been displayed on *C. crescentus* and when applied simultaneously showed additive microbicide effects and could effectively block infection in an HIV pseudovirus assay system (Nomellini *et al.*, 2010).

Yeast cell display

Saccharomyces cerevisiae is the most frequently used eukaryotic display system, has GRAS status and can be cultivated to high cell densities in inexpensive media. There are two main display systems for yeast, the agglutinin system and the flocculin system. In most cases when applying the agglutinin system the protein of interest is fused to the C-terminus of Aga2, which is bound at the cell surface to Aga1 that is cell wall bound via a GPI anchor. Although the anchoring motif and the protein to be displayed are separated, the interaction of the two carrier proteins secures the surface display. The second version is employing α-agglutinin (Agα1). The protein of interest is fused between the signal sequence and the C-terminal half of Agα1, which contains the GPI anchor for cell wall binding (Kondo and Ueda, 2004). α-agglutinin has been used for, e.g. the display of the ZZ domain for IgG purification and immunoassays (Nakamura *et al.*, 2001; Shimojo *et al.*, 2004). *S. cerevisiae* allows the display of correctly folded eukaryotic proteins that failed in *E. coli*, e.g. a lot of allergens have conformational IgE epitopes that disappear in an incorrectly folded protein. Three components of the wasp venom (phospholipase A1, antigen 5, hyaluronidase) were tested for expression and surface display in *S. cerevisiae* using the Aga1/Aga2 system. All three fusion proteins were expressed and surface displayed but in various levels. Only antigen 5-expressing cells could be tested positive for allergen-specific histamine release from human cells and IgE binding as determined by FACS (Borodina *et al.*, 2010). Several researchers also investigated the display of cohesins on the yeast surface: one anchor – Aga1/Aga2 for yeast display – will allow the display of a respective number of cohesins that will concomitantly bind a series of enzymes for the display of cellulosome-like structures, e.g. for the ethanol production based on cellulose (Tsai *et al.*, 2009; Wen *et al.*, 2010).

The flocculin system can also be applied following two different strategies. Either the GPI anchor of the C-terminal half of the FLO1 protein is used and as described for the Agα1 system, the protein of interest is fused between the signal sequence and the anchoring motif. A second approach employs the adhesive ability of the flocculation domain of FLO1, a fusion of this domain to the N-terminus of the protein of interest will non-covalently bind the heterologous fusion protein to the cell wall via interaction with mannan chains (Kondo and Ueda, 2004). The latter system has been suggested to be advantageous for the display of enzymes with a C-terminally located active site, e.g. lipases (Matsumoto *et al.*, 2002) (Fig. 1).

Endospores

Endospores are formed by members of the genera Bacillus and Clostridia to survive unfavourable conditions and nutrient depletion (Henriques and Moran, 2007) and are encapsulated in a protein shell or coat. In most cases this coat is the outermost spore structure, e.g. in *B. subtilis*, the most studied endospore forming organism (Driks, 1999). Of the at least 20 polypeptides of the coat some like CotA, CotB, CotC, CotF and CotG have been identified as surface proteins although in some cases the exact location remains still unclear (Isticato *et al.*, 2001). So far, mainly CotB, CotC and CotG have been identified as suitable anchors. The first example of spore surface display used CotB as anchor and the C-terminal fragment of the tetanus toxin (TTFC), which has been used successfully before as model antigen (Medaglini *et al.*, 2001). During this study, the 459 amino acid TTFC was fused to the N-terminus and the truncated C-terminus of CotB and also inserted into the middle of CotB. Flow cytometry

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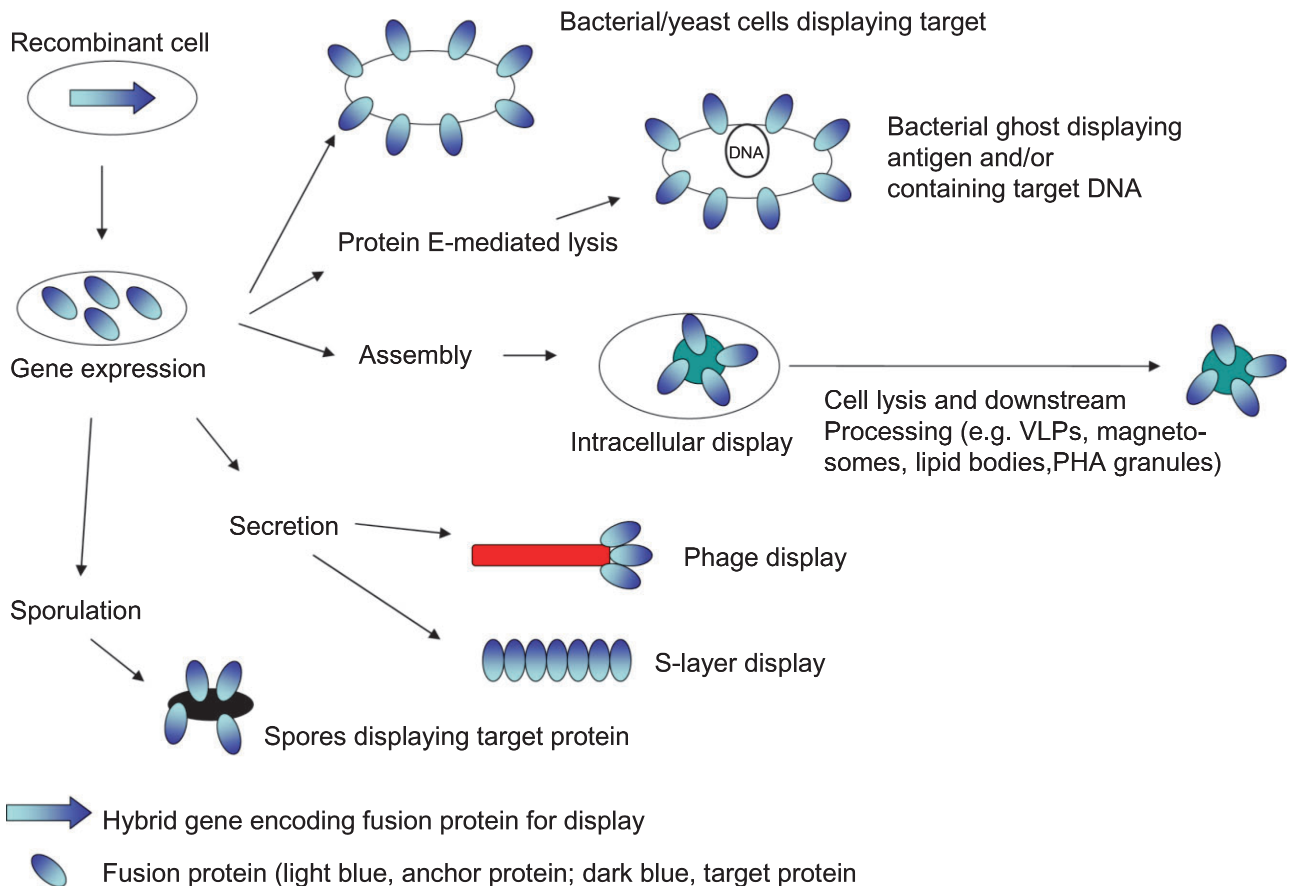

Fig. 1. Schematic overview of different display principles.

confirmed the surface display of all three fusion proteins (Isticato *et al*., 2001). For further vaccine delivery studies only spores displaying the C-terminal CotB-TTFC fusion were used. Oral and intranasal immunization of mice provoked mucosal IgA and systemic IgG responses and confirmed the potential use of endospores displaying surface-expressed antigens as vaccine delivery vehicles (Kim and Schumann, 2009). TTFC has also been displayed as CotC fusion as well as the heat-labile toxin of *E. coli*, and again, both antigen-displaying spores showed positive immune responses in mice experiments (Mauriello *et al*., 2004). Low-cost production, heat stability and proven convenient oral delivery route of spore vaccines triggered thoughts of the 'ideal' strategy to use in developing countries and for military personnel (Duc *et al*., 2007). As the spore core itself is metabolically inactive (Henriques and Moran, 2000), mainly the spore surface layers are of biotechnological interest (Knurr *et al*., 2003; Dickinson *et al*., 2004) but gastrointestinal spore germination in connection with vaccination has also been investigated (Duc *et al*., 2003a,b; Hong *et al*., 2005). Duc *et al*. developed different application routes for spore based anthrax vaccines in contrast to the currently used acellular vaccine vehicles. The protective antigen (PA) was displayed as CotB or CotC fusion on the spore surface as well as overexpressed in the vegetative cell or germinated spore. Studies confirmed that the PA is only functional when secreted from the cell or displayed on the spore surface. N-terminally truncated forms of PA lacking the signal peptide could not confer protection against anthrax (Duc *et al*., 2007). Additionally, findings by the same group were showing that *B. subtilis* spores can germinate in the murine gut (Casula and Cutting, 2002; Duc *et al*., 2003a) and opened up a second way of application of spore based vaccines.

Two other coat proteins, CotG and CotE, have been used for the immobilization of β-galactosidase from *E. coli*, in both cases fused to the C-terminus of the anchor protein (Ban *et al*., 2003) and positive results indicated that the spore display systems might not be limited regarding size or multimeric nature of the target protein (Kim and Schumann, 2009).

The pathogens *Bacillus cereus, Bacillus anthracis* and *Bacillus thuringiensis* show an additional spore layer, the exosporium (Ricca and Cutting, 2003). Therefore, a different way of display has been explored, e.g. using the

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Bacillus thuringiensis protoxin as anchor for the display of GFP and scFv (Du *et al*., 2005). The protoxin is not essential for the functionality and integrity of the *Bacillus thuringiensis* spore, whereas the native as well as the functionalized Cot proteins are anchored to the *Bacillus subtilis* spore and part of the spore coat protein network (Kim *et al*., 2006; Kim and Schumann, 2009). GFP and β-galactosidase have also been fused to the C-terminal region of the *Bacillus thuringiensis* exosporium protein InhA (Park *et al*., 2009). Surface display and functionality could be confirmed and the authors proposed this method of display advantageous over the *cot* gene based method for the same reasons as Du *et al*.

Magnetosomes

Magnetosomes are intracellular crystal particles of magnetite, Fe_3O_4. These nanometre sized iron minerals are enveloped by a membrane and allow magnetotactic bacteria, e.g. *Magnetospirillum magneticum* and related bacteria, to migrate along magnetic field lines especially in oxygen gradients in aquatic environments (Blakemore, 1975). Magnetosome-specific genes are clustered in a so-called 'magnetosome island' in different species of magnetotactic bacteria. Mostly, magnetosome transmembrane proteins such as Mms13 and Mms16 are used as anchoring motifs for functional display of peptides, proteins, enzymes. Although Mms16 has been employed for functional display (Yoshino *et al*., 2004), there is evidence of its being only an artefact due to unspecific absorption during isolation as the *mms16* gene is not located in the magnetosome island in the studied organism *Magnetospirillum gryphiswaldense* (Schultheiss *et al*., 2005). However, Mms13 could be shown to be tightly bound to the magnetite surface (Tanaka *et al*., 2006) and luciferase fusion protein studies demonstrated that the Mms13 C-terminus is localized to the magnetosome surface (Yoshino and Matsunaga, 2006). Magnetic cell separation using immunomagnetic particles is one of the routinely used current cell separation techniques and is considered simple, rapid and specific. Previously, MagA – a proton/iron ion antiporter protein (Nakamura *et al*., 1995) – has been used for the immobilization of the IgG binding domain ZZ from protein A (Kuhara *et al*., 2004). After identification of Mms13 as more suitable anchor protein, the experiments were repeated and Mms13 fusions performed significantly better (Matsunaga *et al*., 2006). Protein G was also displayed via Mms13 in *M. magneticum* AMB-1 and successfully used for the direct magnetic separation of immune cells from whole blood (Takahashi *et al*., 2009).

PHA granules

A wide variety of bacteria and even some archaea are able to form PHA inclusions as carbon and energy reserve material (Rehm, 2010). These inclusions are water-insoluble and spherical and often referred to as PHA granules (Rehm, 2006a). The key enzyme of the PHA granule formation is the PHA synthase, which catalyses the synthesis of PHA and mediates formation of intracellular PHA inclusions as well as it remains covalently bound i.e. immobilized to the surface of the emerging granule (Rehm, 2006b; 2007). PHA granules are not only surrounded by PHA synthase proteins but also various other proteins, which are non-covalently attached, such as, e.g. the structural proteins called phasins, PHA depolymerases or regulatory proteins (Grage *et al*., 2009). All these granule associated proteins were tested as suitable surface anchors for the immobilization of target proteins.

In one of the simplest approaches, the N-terminal granule-binding domain of the phasin PhaF from *P. putida* GPo1 was co-expressed with the protein of interest as functional fusion protein under PHA accumulating conditions. The anchoring domain – also called BioF – was employed as purification tag in analogy to other commercial protein tags and allowed purification of the protein of interest with the PHA granules as matrix material (Moldes *et al*., 2004). It is also possible using the whole phasin protein as anchoring sequence. In order to achieve higher purity of the isolated protein, inteins (intervening proteins) (Banki and Wood, 2005) were employed as linker sequences between a phasin and the protein of interest. The phasin, in most cases PhaP, allowed hydrophobic attachment to the natively formed granules inside the cells. For better binding to the PHA matrix, i.e. to reduce leakage, multiple copies of PhaP had to be used. The protein of interest could be purified using the self-cleaving intein linker. This system was developed for the natural host *Ralstonia eutropha* as well as in recombinant *E. coli* and is not necessarily combined with PHA granule isolation before protein purification and release (Banki *et al*., 2005; Barnard *et al*., 2005). Also, the production of PhaP-intein tagged fusion protein and PHA purification matrix can be separated from each other. The PHA matrix particles can also be made from already purified PHA. The crude extract containing the tagged protein of interest is incubated with the artificial PHA particles where phasin mediated binding followed by intein cleavage is leading to protein purification (Wang *et al*., 2008). A few years later, it was demonstrated that a single copy of PhaP is sufficient for extensive display of desired fusion proteins to native PHA granules. Also, the correct folding of eukaryotic proteins – here interleukin-2 and myelin oligodendrocyte glycoprotein – fused to PhaP and produced in *E. coli* could be shown in the first time FACS application of functionalized PHA granules (Bäckström *et al*., 2007). Interestingly, the N-terminus as well as the C-terminus of the anchoring PhaP can be used for functionalization without

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interference with PHA granule-binding (Bäckström *et al*., 2007), therefore even the display of two functionalities with the same PhaP anchor is possible as demonstrated in GFP-PhaP-myelin oligodendrocyte glycoprotein fusion protein displaying PHA granules (Atwood and Rehm, 2009).

In analogy to the BioF system described for phasins, the substrate binding domain of the PHA depolymerase from *Alcaligenes faecalis* was employed as protein purification tag for the enhanced green fluorescent protein (EGFP) and other reporter proteins (Lee *et al*., 2005). Although described as functional, other PHA displaying systems show wider applicability and are more exploited than the above-described depolymerase substrate binding domain system.

Only recently, the regulatory protein PhaR was described as affinity tag for protein purification. Again, an intein linker between PhaR and the target protein was employed for final release of the protein of interest, e.g. EGFP. The fusion protein production was hosted by recombinant *E. coli*, the PHA matrix nanoparticles were produced separately (Zhang *et al*., 2010). PhaP and PhaR fusions or parts thereof also found application in a transferable *E. coli* two-hybrid system. In short, the DNA-binding domain of PhaR is fused to the 'bait', the 'prey' is fused to PhaP. Upon formation of PHA granules, both fusion proteins will start interacting with each other, resulting in weaker interaction of the DNA-binding domain with its natural target the *phaP* promoter region. The resulting expression of the marker gene will directly indicate the interaction strength of 'bait' and 'prey' (Wang *et al*., 2011).

The most common PHA display system is based on the PHA synthase PhaC as surface anchor. While the above-described structural and regulatory proteins used as anchor proteins are non-covalently attached to the PHA surface, the synthase is covalently bound to the polyester core. Therefore, the displayed fusion protein is much more tightly connected to the support matrix. Most frequently, the target protein is fused to the N-terminus of the PHA synthase. Multiple examples show the diverse applicability of engineered PHA particles, e.g. the display of an α-amylase (Rasiah and Rehm, 2009), several variants of streptavidin (Peters and Rehm, 2008) or a scFv antibody (Grage and Rehm, 2008). The most successful application so far has been the display of the ZZ domain of protein A (Brockelbank *et al*., 2006). These engineered polyester particles performed equally compared with commercially available protein A beads and demonstrated the broad capacity of this display technology (Lewis and Rehm, 2009; Rehm *et al*., 2011). Until recently, proteins that needed a free C-terminal end for functionality could only be surface displayed on PHA particles using one of the non-covalently attached anchoring motifs. The lack of an engineering strategy for the synthase carboxy end could be overcome and thus the applicability of the whole technology broadened. Now, several functionalities can be displayed using the same anchoring motif – the PHA synthase – which allows the production of multifunctional PHA particles (Jahns and Rehm, 2009). So far this has only been possible by use of several anchoring motifs on the same particle surface or by use of the non-covalently attached phasin as mentioned above (Jahns *et al*., 2008; Atwood and Rehm, 2009). Efforts have been undertaken to also establish this recombinant protein production system in the Gram-positive host *L. lactis* to circumvent issues with pyrogen toxicity (Mifune *et al*., 2009).

Almost all display systems aim on applicability in biomedicine, as tools in bio-diagnostic assays and even more as vaccination vehicles. PHA particles applied for biomedical purposes are mostly artificially derived from isolated polyesters and fusion proteins are produced in endotoxin-free host organisms. This strategy led to the development of an endotoxin removal assay based on PhaP-tagged human LPS binding protein produced in *Pichia pastoris* (Li *et al*., 2011). To simulate targeted drug delivery, PHB-copolymer nanoparticles were loaded with a lipid-soluble dye to mimic the drug load and covered with recombinant PhaP-tagged human epidermal growth factor or mannosylated human α1-acid glycoprotein. Macrophages recognized the displayed proteins and receptor mediated endocytosis could be successfully established, proving the capacity of these particles for targeted drug release (Yao *et al*., 2008; Xiong *et al*., 2010). Vaccine vehicles based on PHA particles have been also developed, e.g. particles displaying a combined PhaC-tagged antigen from *Mycobacterium tuberculosis* could be successfully expressed in recombinant *E. coli* and displayed on *in vivo* produced PHA particles. Animal studies revealed no toxicity albeit the Gram-negative production host and immunological data unveiled a stronger response of the immobilized antigen compared with the free fusion protein alone (Parlane *et al*., 2009). These results were significant for the conception of polyester based vaccines as new emerging life science tools.

Although there are several functional display systems established in PHA research, some controversy occurs with respect to the method of PHA matrix production. Some groups prefer the *in vitro* production of PHA particles – pure PHB or co-polymers – and argue that the *in vivo* accumulation and isolation of fusion protein displaying particles would be a rather complicated and time-consuming process as mild and laborious methods have to be employed to prevent destruction and inhibition of the surface exposed functionality (Zhang *et al*., 2010). On the other hand, the *in vitro* formulation of PHA particles is frequently accumulating high amounts of solvent waste and depending on the application requires a laborious

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purification process. Successful examples also underline the merit of *in vivo* one-step production, especially when the PHA based immobilization is leading to the production of a target protein that is commonly known to form inclusion bodies as shown with the PhaP-tagged plasminogen activator (Geng *et al*., 2010). The authors explicitly stated the PHA particle based production of their protein as easy and efficient. One-step production also allows specific design and tailoring of the final product as demonstrated in the vaccine development without the obligation of additional steps like separate protein production, purification and conjugation or cross-linking (Parlane *et al*., 2009).

Lipid bodies

Neutral lipid inclusions are the major source of stored energy in eukaryotes. Triacylglycerols (TAGs) are stored as intracellular lipid droplets or lipid bodies (Murphy and Vance, 1999). These spherical structures serve as energy reserves and are essential for the intracellular energy metabolism and homeostasis (Bell and Coleman, 1980). In prokaryotes energy is mostly stored in PHA inclusions (see above). Nevertheless, some bacteria are also able to synthesize TAG inclusions (Alvarez and Steinbüchel, 2002). Structurally, bacterial and eukaryotic TAG inclusions are related: a neutral lipid matrix, composed of TAG or wax esters, is surrounded by a monolayer of phospholipids. Additionally, only eukaryotic lipid bodies show proteins embedded into the phospholipidlayer (Kalscheuer and Steinbüchel, 2003; Wältermann *et al*., 2005). Known eukaryotic lipid body associated proteins were heterologously expressed in prokaryotes able to synthesize TAG inclusions in order to study targeting and binding mechanisms. As these proteins successfully bound to prokaryotically derived lipid bodies it was hypothesized to employ heterologously expressed eukaryotic TAG binding proteins as surface anchors for biotechnological interesting applications of lipid inclusions displaying enzyme activities (Hänisch *et al*., 2006a). Already, the functionalization of bacterial TAG inclusions could be shown by employing the non-specific binding to lipid bodies of the PHA-binding phasin PhaP1 from *R. eutropha* H16 (Hänisch *et al*., 2006b). Fusion proteins comprising the EGFP or β-galactosidase could be functionally displayed on TAG inclusions using the PhaP1 anchor. Eukaryotic lipid bodies or lipid droplets are also investigated regarding functionality and targeting. In *Drosophila* studies the lipid droplet targeting domain of the Klar protein could be identified in the C-terminal region of the protein (Guo *et al*., 2005). A fusion of this previously identified domain to GFP was targeted to lipid droplets and allowed monitoring of lipid droplet formation and movement in living tissues of *Drosophila* (Yu *et al*., 2011).

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