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.

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 (McCafferty 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
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 pII 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 (Garce and Gissmann, 2004). Capsid proteins display basic domains facing the inside of the particle and are therefore predesigned 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 focusing 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...
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).

Table 1. Examples for different surface display systems.

| Display system | Anchor | Anchor | Fusion partner | Application | References |
|----------------|--------|--------|----------------|-------------|------------|
| Phage          | pIII   | M13 coat protein | scFv | Antibody engineering | McCafferty et al. (1990) |
| Virus-like particles | VP1 | Polymya virus coat | Z domain of protein A | Antibody purification | Gleiter and Lilie (2001) |
| Gram-negative cells | LamB | Maltoporin | Peptides | Library screening | Benhar (2001) |
| Bacterial ghosts | E. coli O157:H7 antigens | Vibrio cholerae antigens | DNA-binding protein of MS2 | DNA | DNA vaccine | Mayrhofer et al. (1986) |
| Gram-positive cells | Protein A | Cell wall anchor (covalent) | Streptavidin | Biotin-binding | Steidler et al. (1998) |
| LysM | Cell wall anchor (non-covalent) | Metal binding peptides | Enzyme display | Bioassays | Shao et al. (2009) |
| FnBPB | Cell wall binding | Lipase/lactamase | Bioassays | Enzyme display | Strauss and Götz (1996) |
| S-layers | SbpA | Lysinibacillus sphaericus | Bet v1 | Vaccines | Ilk et al. (2011) |
| Yeast | Agox1 | α-agglutinin | Z domain of protein A | Immunoassays | Shimojo et al. (2004) |
| Endospores | CotB, C, D | Coat proteins | Toxins/antigens | Vaccines | Istitato et al. (2001) |
| Magnetoosomes | Mms13 | Transmembrane protein | Protein A/G | IgG purification | Matsunaga et al. (2006) |
| PHA beads | PhaF | Phasin | Enzymes | Protein purification | Moldes et al. (2004) |
| Lipid bodies | PhaP1 | Phasin | Enzymes | Protein purification | Banki and Wood (2005) |

**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; Freudi et al., 1986). In the following decades, applications such as vaccine delivery vehicles, library and selection devices, cellular adsorbents and bio-catalysts were described (Stähli 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., 1997). 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...
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 (Haideringer 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...
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 as 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 Ni²⁺ 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. camosus*. 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
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 *L. 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 $\alpha$-agglutinin (*Ago1*). The protein of interest is fused between the signal sequence and the C-terminal half of Ago1, which contains the GPI anchor for cell wall binding (Kondo and Ueda, 2004). $\alpha$-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 Aga1 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 (Istitaco et al., 2001). So far, mainly CotB, CotC and CotG have been identified as suitable anchors. The first example of sparse 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
confirmed the surface display of all three fusion proteins (Istitato 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 (Knutr 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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**Fig. 1.** Schematic overview of different display principles.
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, Fe₃O₄. 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 GP01 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
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 Escherichia 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 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
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 phospholipid layer (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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