Cell-Surface Display of Heterologous Proteins: From High-Throughput Screening to Environmental Applications

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Abstract: A variety of expression systems for the display of either short peptides or fully folded proteins on *E.coli* and, to a lesser extent, on Gram-positive bacteria have been developed. The expression of proteins on the surface of microbial cells has proved extremely important for numerous applications ranging from combinatorial library screening and protein engineering, to whole cell biocatalysts and adsorbants for bioremediation purposes. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **79:** 496–503, 2002.

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INTRODUCTION

Ever since the introduction of recombinant DNA techniques in the late '70s, various proteins have been successfully expressed in different host organisms including bacteria, yeasts, fungi, mammalian cells, and plants. One of the most exciting developments in recent years has been the possibility to directly target proteins on the cell surface of different host organisms. This breakthrough technology is generating intriguing opportunities for applications including bacterial vaccines, high throughput screening of peptide and enzyme libraries, whole cell sorbents, recombinant biocatalysts, and cell-based diagnostics. In this article, we will highlight the technologies available for surface display and certain applications and future uses of particular relevance to biochemical engineering.

Biochemical engineers have played a pivotal role in the development of microbial display systems and in exploring the technological applications of cell surface engineering. The entire community of biochemical engineers working in this field, including the two authors, benefited greatly from Professor Bailey's encouragements and enthusiastic support. Jay helped open the way for the integration and acceptance of molecular biology into biochemical engineering. We will always be indebted to him.

DISPLAY OF HETEROLOGOUS POLYPEPTIDES IN GRAM-NEGATIVE BACTERIA

Methods for the display of recombinant proteins on the surface of Gram-negative bacteria have been the subject of several recent reviews (Earhart, 2000; Georgiou et al., 1997; Lang et al., 2000; Westerlund-Wikstrom, 2000). Therefore, we will present only the salient features of different strategies for protein display, emphasizing features relevant to cell surface engineering.

The display of proteins on the surface of Gram-negative bacteria requires a mechanism by which a desired, recombinant polypeptide: (1) is exported from the cytoplasm, a process typically involving the participation of the protein secretory apparatus of the cell, (2) is targeted to the outer membrane, and (3) can transverse the outer membrane so that it anchors to the external surface. Unfortunately, the mechanisms that dictate targeting and insertion of proteins within the outer membrane are not well understood. Moreover, the incorporation of aberrant proteins within the outer membrane can be toxic to the cell. Poor exposure of surface-anchored heterologous polypeptide sequences, typically resulting from steric effects caused by the lipopolysaccharide layer on the outer membrane, can prevent the interaction of surface-displayed heterologous proteins with antibodies and small molecule ligands. Steric constraints can be avoided by fusing recombinant "passenger" proteins onto components of cell surface appendages, such as flagella or pili, protruding far away from the bacterium surface. However, the fusion of heterologous polypeptides to subunits of cellular appendages must not disrupt their ability to self-assemble into a supramolecular structure. In most cases for example, with the flagellin protein, the major component of the bacterial locomotion apparatus, self-assembly requires an intact N- and C-terminus. This means that guest polypeptides can only be inserted within a permissive loop of a bacterial surface appendage protein component. Unfortunately, the large majority of full-length proteins cannot be readily inserted within a surface loop of another protein without disruption of the tertiary structure. For this reason the use of cellular appendages as display vehicles for cell surface engineering is limited to the insertion of relatively unstructured polypeptides that do not perturb the proper assembly of surface appendages.

The de-novo design of polypeptides capable of localizing and properly inserting within the outer membrane of Gram-negative bacteria has so far proven to be an intractable

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problem. For this reason it has been necessary to exploit native, integral outer membrane proteins as vehicles for display purposes. The utility of most major *E. coli* outer membrane proteins has been evaluated (for recent examples, see Camaj et al., 2001; Chang et al., 1999; Etz et al., 2001; Lang, 2000; Lang et al., 2000; Xu and Lee, 1999). These include LamB, OmpA, OmpC, OmpS, FhuA, the lipoprotein TraT and others for cell surface engineering. However, in an analogy with flagella or pili components, in most outer membrane proteins regions at both the N- and C- termini are required for outer membrane targeting (Georgiou et al., 1997). As a result, it is not possible to construct an end-to-end fusion with a passenger polypeptide without interfering with insertion into the membrane. Therefore, integral outer membrane proteins have proven to be useful only for the display of peptides.

To bypass the above limitations, Francisco et al. (1992) constructed a tripartite fusion consisting of the outer membrane localization domain of the major *E. coli* lipoprotein Lpp joined with a portion of the integral membrane protein OmpA that, in turn, exposed C-terminally fused recombinant polypeptides on the cell surface. The Lpp-OmpA system was the first successful approach for displaying full-length heterologous proteins on the surface of *E. coli* and has since been used extensively for cell surface engineering purposes with dozens of heterologous proteins including enzymes (cellulases, esterases, and organophosphate hydrolases), scFv enzymes, β-lactamase, thioredoxin, and others (Earhart, 2000; Richins et al., 1997).

Pan and co-workers have exploited the *P. syringiae* ice nucleation protein (inp) for the display of viral antigens and enzymes (Jeong et al., 2001; Jung et al., 1998; Kim et al., 2000; Lee et al., 2000,). The mechanism for the incorporation of inp fusions on the cell surface is not clear but an advantage of this system is that it does not seem to adversely affect the growth of the host bacteria. Recently, a different ice nucleation protein (InaV) from *Pseudomonas syringae* INA5 was used for display and was shown to result in better cell growth and more stable incorporation of proteins on the *E. coli* surface when compared to earlier technologies (Shimazu et al., 2001).

The immunoglobulin A1 protease-like autotransporters are multidomain proteins consisting of a C-terminal autotransporter domain that promotes the translocation of N-terminally attached passenger domains across the cell envelopes of Gram-negative bacteria (Jose et al., 1995). In early studies, Meyer and co-workers utilized the *Neisseria* IgA protease autotransporter protein for the display of small heterologous proteins such as the cholera toxin B subunit. However, this system proved to be incompatible with surface expression of most proteins, particularly those containing disulfide bonds. More recently, the Meyer group demonstrated that the autotransporter domain of the *E. coli* adhesin involved in diffuse adherence (AIDA-I) is much more permissive for the surface display of peptide antigens and recombinant proteins such as β-lactamase (Lattemann et al., 2000; Maurer et al., 1997). AIDA-I fusion proteins have been shown to properly assemble on the outer membrane without any adverse effects on cell growth, a feature significant for applications such as combinatorial library screening and vaccine development. Finally, Wentzel et al. (2001) engineered a surface display system that capitalizes on the EaeA intimin from enterohemorrhagic *E. coli* O157:H7. This adhesin contains an N-terminal transporter domain, which resides in the bacterial outer membrane and promotes the translocation of C-terminally attached passenger domains across the bacterial cell envelope. The authors reported that several proteins including a trypsin inhibitor knotin, interleukin 4, and a Bence-Jones protein could be displayed on the *E.coli* cell surface as C-terminal fusions to the EaeA intimin. Notably, intimin fusions represent the only display system other than Lpp-OmpA that has been used for high-throughput combinatorial library-screening experiments in conjunction with flow cytometry (Christmann et al., 2001).

BACTERIAL CELL-SURFACE DISPLAY IN COMBINATORIAL LIBRARY SCREENING

Drug discovery, directed protein evolution, and protein engineering in general rely extensively on the screening of combinatorial polypeptide libraries. These applications involve, first the generation of a library of protein-expressing clones using molecular biology techniques, and second, the use of a technology for the facile isolation of proteins that exhibit ligand-binding, catalytic activity or other desirable biochemical properties. The screening of large libraries is greatly simplified by establishing a direct physical link between a gene, the protein it encodes, and a desired function. Such a link can be established using a variety of in vivo display technologies that have proven invaluable for mechanistic studies, for biotechnological purposes, and for proteomics research (Georgiou, 2000; Hayhurst and Georgiou, 2001; Hoess, 2001; Wittrup, 2001,).

The value of library screening methodologies is made manifest by the popularity of phage technology, the first truly high throughput technique for in vitro screening that currently forms the basis of a multibillion-dollar enterprise. In phage display, an ensemble of filamentous virus particles, each displaying a different polypeptide, is first created through relatively straightforward molecular biology procedures. Subsequently the library is screened, typically for binding to an immobilized ligand using a series of adsorption-desorption cycles. The relative simplicity of library screening by phage display is part of the reason for its widespread use in biotechnology research. However, phage display is not particularly amenable to the isolation of protein catalysts, the screening process lacks quantification and finally, expression biases and multivalency effects often complicate the isolation of useful proteins having specified ligand-binding characteristics (Olsen et al., 2000a, Wittrup, 2001).

Most, if not all, of the limitation of phage display can be addressed by anchoring protein libraries on the surface of

microbial cells. Importantly, the larger size of microbial cells enables the screening of libraries using fluorescence-activated cell sorting (FACS) a technology offering very high throughput and the ability to determine the functional properties of each and every clone in a library. Proteins displayed on the surface of bacteria are readily accessible to fluorescent probes. Furthermore, the molecular architecture of cell surfaces can be engineered in a variety of ways to facilitate the quantitative capture of fluorescent reaction products, a feature that has proven invaluable for the screening of enzyme libraries (Georgiou, 2000).

The utility of flow cytometry as a tool for screening of cell surface expressed protein libraries was first demonstrated by Georgiou and coworkers in 1993. They showed that cells displaying scFv antibodies using the Lpp-OmpA system could be readily enriched from a 10^5 excess of control bacteria (Francisco et al., 1993). Since then, Lpp-OmpA fusions have been used to screen libraries of antibodies and other proteins (Christmann et al., 1999, Daugherty et al., 2000a).

Daugherty et al. (1998) presented evidence that flow cytometric screening can identify the "best" clone in a library, in that case the clone that bound to a desired ligand with the highest affinity. Specifically, a library was constructed by randomizing four CDR 3 residues at or near the V_H-V_L interface of an anti-digoxin scFv antibody displayed on *E. coli* as an Lpp-OmpA fusion. After four rounds of enrichment under stringent conditions that selected for maximal affinity, a clonal population was enriched. The isolated scFv was shown to have the same amino acid sequence as the wild-type residues but was encoded by a different sequence at the DNA level. Even though the frequency of this alternate DNA sequence encoding the wild-type amino acids was only about $1{:}10^6$, it was nonetheless enriched to homogeneity and selected from other clones having only slightly lower affinity. In subsequent studies the same researchers reported the isolation of numerous mutants with subnanomolar affinities to digoxin and other haptens from libraries generated either by localized randomization of specific residues or by error-prone PCR of the entire scFv gene (Daugherty et al., 1998, Daugherty et al., 2000b).

Kolmar and coworkers used the IgA-b autotransporter protein of Gram-negative bacteria fusions to display libraries of EET-II, a 28 amino acid polypeptide of the squash family of trypsin inhibitors (Wentzel et al., 1999). Functional EETI-II was detected on the surface of *E.coli* indicating correct formation of the three disulfide bonds that form a cysteine knot motif. A library of 5×10^7 clones was created by randomizing a four amino acid loop and screened first by affinity adsorption on trypsin-derivatized magnetic beads followed by flow cytometric sorting using a high throughput Cytomation MoFlo instrument. Because the particular expression system used for display on the *E. coli* surface resulted in cell lethality (the Neisseria IgA-b autotransporter), the EETI-II genes in highly fluorescent but non-viable cells in the sort solution had to be rescued by PCR (Wentzel et al., 2001). The same research group subsequently demonstrated that cell lethality observed with IgA-b autotransporter fusions could be prevented by using Lpp-OmpA for protein display on *E. coli*. In this manner, clones expressing high affinity variants could be isolated without the need for PCR and subcloning (Christmann et al., 1999).

Recently, Kolmar and coworkers developed a surface display technique for mapping the epitopes of monoclonal antibodies using flow cytometry. A random library of peptides from the classical swine fever virus envelope protein E (CSFV-E) was expressed as a fusion to a domain of the EaeE intimin. Peptide-expressing clones that reacted with fluorescently labeled monoclonal antibodies to CSFV-E could then be isolated by FACS and identified by DNA sequencing (Christmann et al., 2001). Subsequently, epitope-expressing bacteria were used as an affinity reagent for the purification of a monospecific antibody population from polyclonal sera.

Bacterial cell surface display coupled with flow cytometric screening has also been adapted to the screening of enzyme libraries (Olsen, 2000b). In fact, this technology currently represents the only general approach for the quantitative interrogation of enzyme catalytic activity at the single cell level and in very large populations of mutants. The display of enzymes on the bacterial surface provides free access of synthetic substrates to the enzyme. Moreover, as was mentioned above, bacterial surfaces have a number of unique features the most important of which is negative charge that can be exploited for the efficient retention of the reaction products of enzymatic reactions. The ability to form a physical link between a fluorescent product of a reaction and the cell that expresses the respective enzyme on its surface proved to be key for the quantitative determination of catalytic activity at the single cell level. Georgiou, Iverson and coworkers developed cell surface-associating substrates that also exhibit fluorescence resonance energy transfer (FRET). The first generation of such substrates consisted of: (i) a fluorophore (Fl); (ii) a positively-charged moiety; (iii) the scissile bond to be cleaved by a desired enzyme and (iv) a quenching fluorophore that acts as an intramolecular FRET partner (Q). Since the surface of *E. coli* is negatively-charged (z potential -25 to -30 mV) the positively-charged FRET substrates associate with the cell surface in low ionic strength solutions. Enzymatic cleavage of the scissile bond separates the Fl and Q moieties, disrupting intramolecular FRET quenching. The product containing the (Q) fluorophore has no net charge, and presumably diffuses away from the cell while the product containing the fluorescent group, in this case BODIPY™, remains cell-associated. As a result, the cells become fluorescently labeled in proportion to substrate turnover. This approach was shown to allow the efficient enrichment of cells expressing enzyme catalysts over background cells (>5,000 fold per round of screening). An enzyme mutant showing a 60-fold increase in catalytic activity towards a non-preferred substrate was isolated following a single round of screening of a library of about 2 million cells

(Olsen, 2000b). In more recent studies, the same researchers employed two-color sorting to simultaneously screen mutant libraries for clones that exhibit cleavage of a desired substrate and lack of reactivity towards a second substrate. A dramatic switch in catalytic substrate selectivity, far greater than what had ever been obtained previously in protein engineering experiments, has been obtained using this approach (Olsen, M., Gam, J., Georgiou, G. and B.L. Iverson, in preparation).

The utility of cell surface displayed libraries in biotechnology and basic biology investigations extends well beyond the use of flow cytometry. Immobilized ligands have been employed for the separation of bacteria expressing surface receptors for genetic and biochemical studies dating back almost 20 years. In 1982 Ferenci and Lee used amylose resins to isolate *E. coli* cells expressing mutants of the outer membrane maltodextrin protein (Ferenci and Lee, 1982). A few years later, Brown (1992) displayed peptide libraries on externally exposed loops of LamB and isolated bacteria that could specifically bind to iron oxide particles but not to other metals. This work represented a landmark first demonstration of how combinatorial protein libraries can be used for applications related to materials development.

The display of peptides on the *E. coli* flagella has been used extensively for analyzing interactions of proteins with peptide ligands (see Westerlund-Wikstrom et al., 2000 for a recent review). Permissive surface loops of flagellin, the major component of the bacterial flagella which is present in thousands of copies per flagella filament in motile cells are exploited to insert thioredoxin which in turn serves as a scaffold = for the presentation of heterologous peptides of up to 300 amino acids. In other words, the displayed peptide is "nested" within thioredoxin which in turn is inserted within flagellin. If desired, two (or more) different thioredoxin-peptide fusions can be incorporated within a single flagella (Tanskanen et al., 2000). Flagellated cells displaying peptide epitopes are enriched by binding to immobilized ligands under carefully controlled conditions to prevent shearing of the long flagella filaments. This clever and powerful technique has been used for epitope mapping and the identification of functional regions in bacterial adhesins (Brown et al., 2000, Westerlund-Wikstrom et al., 2000). Recently, Tripp et al. (2001) used a two-step selection process to isolate "switch" peptides that exhibited reversible binding to antibodies controlled by pH or metals. Such switch peptides hold great promise for the development of reagents for affinity chromatographic separations.

An innovative method for epitope mapping was reported recently by Camaj et al. (2001). Peptides are inserted within a permissive loop of the outer membrane protein A (OmpA) that serves as the receptor for the lytic phage K3. Antibody binding to bacteria displaying peptide epitopes occludes K3 from binding to OmpA resulting in protection from phage-mediated lysis. In this manner, cells displaying an epitope recognized by antibody survive the phage challenge and are enriched from the rest of the library.

In a somewhat different vein, the display of hydrolytic enzymes on bacteria can be exploited to degrade impermeable macromolecules into compounds that can be utilized for cell growth. For example, Kim et al. (2000) used ice nucleation protein fusions to display carboxyemethyl cellulase libraries on *E. coli*. Enzyme mutants with a higher catalytic activity were isolated by selecting for faster growth and larger colony size on agar plates.

A different application of bacterial surface display in combinatorial library screening applications was demonstrated by Benhar and coworkers (2000). These researchers developed a technique they termed "delayed infectivity panning" which capitalized on bacterial display of a target antigen to improve the efficiency of screening phage libraries of scFv antibodies. Antigen-displaying bacteria are grown under conditions that prevent the expression of the normal receptor for filamentous bacteriophage attachment. The bacteria are used to capture antibody-displaying phage via antigen-antibody interactions. A switch to permissive conditions for the expression of the receptor allows cell-bound phage to infect the cell and in turn results in the enrichment of the desired antigen-binding clones by factors as high as 10^6. This innovative application of bacterial surface display was shown to greatly facilitate the isolation of scFv antibodies to a variety of important antigens (Benhar et al., 2000).

Surface Engineering for Environmental Applications

Biosorbents for Heavy Metal Removal

The discharge of heavy metals from agricultural, industrial, and military operations has serious adverse effects on the environment (Nriagu and Pacyna, 1989). Conventional technologies are often inadequate to reduce heavy metal concentrations in waste water to acceptable regulatory standards. Recent research has focused on the development of novel bioadsorbents with increased affinity, capacity, and selectivity for target metals.

Eukaryotes limit the concentrations of reactive free metal ions by intracellular sequestration. Glutathione (GSH), GSH-related phytochelatins (PCs) and cysteine-rich metallothioneins (MTs) (Winklemann and Winge, 1994) are the main metal sequestering peptides used by cells to immobilize metal ions. Earlier attempts to produce these peptides in *E. coli* as a mean to increase their metal-binding capability were successful in some cases (Romeyer et al., 1988; Pazirandeh et al., 1995). *E. coli* overexpressing *Neurospora crassa* MTs have been shown to accumulate copper, cadmium and other metals effectively (Pazirandeh *et al.*, 1995). However, expression of such cysteine-rich proteins is not devoid of problems because of the predicted interference with the redox pathways in the cytosol (Bardwell, 1994). More importantly, intracellular expression of MTs may prevent the recycling of the bioadsorbents because the accu-

mulated metals cannot be released easily (Gadd and White, 1993).

One clever solution to bypass this transport problem is to express MTs on the cell surface. Sousa et al. (1996) demonstrated this possibility by inserting MTs into the permissive site 153 of the LamB sequence. Expression of the hybrid proteins on the cell surface multiplied the natural Cd^{2+} accumulation ability by more than 20-fold. In addition to naturally occurring peptides, the *de novo* design of metal-binding peptides is an attractive alternative as they offer the potential of improved affinity and selectivity for heavy metals. Peptides with an abundance of cysteine or histidine residues, for example, are known to bind Cd^{2+} and Hg^{2+} with a very high affinity. Sousa and coworkers (1996) first applied this approach for improved heavy metal sequesteration by inserting one or two hexahistidine clusters onto the outer membrane LamB protein. Strains with surface-exposed histidines accumulated greater than 11-fold more Cd^{2+} than cells expressing the LamB protein without the insert. Novel metal-binding peptides could also be selected from a phage display library (Mejare et al., 1998). The peptide His-Ser-Gln-Lys-Val-Phe, which exhibits the strongest affinity for Cd^2, was cloned into *E. coli* as a fusion to the cell surface exposed area of the outer membrane protein OmpA. Cells expressing this peptide showed increased survival in growth medium containing toxic levels of $CdCl_2$, demonstrating the binding of Cd^{2+} by the surface-exposed peptide. Peptide sequences have also been selected from a fimbriae display system, which conferred recombinant *E. coli* the ability to adhere to different metal oxides (Schembri et al., 1999). Similarly, cells expressing other histidine (Gly-His-His-Pro-His-Gly) or cysteine (Gly-Cys-Gly-Cys-Pro-Cys)-rich peptides, which bind mercury with much higher affinity than other divalent heavy metal, have been anchored on the surface of *E. coli* (Kotrba, et al., 1999). Display of these peptides again increased the bioaccumulation of Cd^{2+} although to a lesser extent than MTs.

Another interesting class of metal-binding peptides known as synthetic phytochelatins (ECn) with repetitive metal-binding motif $(Glu\text{-}Cys)_n Gly$ have recently been developed and were shown to have improved Cd^{2+} binding capability over that of MTs (Bae et al., 2000). The measured Hg^{2+} and Cd^{2+} binding stoichiometry of 20 and 10, respectively, were significantly higher than the typical values reported for MTs (Bae et al., 2001). As a result, the Cd^{2+} binding capability of cells expressing EC20 was almost twice the amount obtained using MTs.

Besides lab-born *E. coli* strains, metal-binding peptides have also been expressed on the surface of soil bacteria that are known to survive in contaminated environments for an extended period. The mouse MT was fused to the autotransporter b-domain of the IgA protease from *Neisseria gonorrhoeae* and displayed on the surface of *Pseudomonas putida* (Valls et al., 2000a) and *Ralstonia metallidurans* CH34 (Valls et al., 2000b), resulting in a threefold increase in binding of Cd^{2+}. This modest increase in binding was sufficient to improve growth and chlorophyll production of the tobacco plant *Nicotiana betamiana* in contaminated soil (Valls et al., 2000b). A genetically engineered *Moraxella sp*. with surface-expressed EC20 has also been developed with almost 10-fold improvement in mercury binding (Bae et al., 2002). This higher level of improvement again reflects the improved binding capacity of EC20 over that of MTs.

Whole Cell Biocatalysts for Detoxification of Organic Contaminants

Neurotoxic organophospates are used extensively as agricultural and domestic pesticides and are one of the most toxic compounds known. Organophosphorus hydrolase (OPH) isolated from soil microorganisms has been shown to degrade these pesticides effectively. However, the use of OPH for detoxification has always been limited by the high cost associated with purification. Whole cell detoxification is more cost effective, however, it is limited by the transport barrier of organophosphates across the cell membrane. Surface expression of OPH can circumvent transport limitations imposed by cell membranes in much the same way that surface expression of metallothioneins enhanced the metal binding capability of cells. Whole cells expressing OPH on the cell surface degraded parathion and paraoxon 7-fold faster compared to whole cells expressing OPH intracellularly (Richins et al., 1997). The resulting live biocatalysts were also considerably more stable and robust than purified OPHs, retaining 100% activity over a period of one month when maintained at 37°C (Chen and Mulchandani, 1998). Immobilization of these novel biocatalysts by physical adsorption onto solid supports provides an attractive means for pesticide detoxification in place of immobilized OPH (Mulchandani et al., 1998). However, a gradual cell detachment from the support reduced the effectiveness of the immobilized-cell system for long-term operation. A significant improvement, both in terms of economics and technology could be achieved with reversible and specific adhesion to the support.

Specific adhesion of whole cells to cellulosic materials with high affinity has been demonstrated by anchoring the cellulose-binding domain (CBD) from *Cellulomonas fimi* on the surface of *E. coli* (Francisco et al., 1993). This was exploited to enable very strong attachment of the organophosphate-degrading cells to cellulose supports for long-term usage (Wang et al., 2002). Two different surface anchors (Lpp-OmpA and INPNC) were employed to target OPH and CBD onto the cell surface, respectively, in order to minimize direct competition of the same translocation machinery. Whole-cell immobilization with surface-anchored CBD was very specific, forming essentially a monolayer of cells onto different supports as shown by electron micrographs. Immobilized cells degraded paraoxon rapidly and retained almost 100% efficiency over a period of 45 days. This is also the first reported genetic co-immobilization of two functional moieties onto the surface of *E. coli*.

Although the enzymatic hydrolysis of organophosphates

such as parathion and methyl parathion reduces the toxicity by nearly 120-fold (Munnecke, 1979), this results in the formation of *p*-nitrophenol (PNP) (Munnecke and Hsieh, 1976), which is still considered a priority pollutant by the U.S. EPA. A novel approach was developed to enable the simultaneous degradation of organophosphates and PNP by anchoring OPH on the surface of a native PNP degrader, *Moraxella* sp (Shimazu et al., 2001). The result is a single microorganism that is endowed with the capability to rapidly degrade organophosphate pesticides and PNP. This is also the first report on the functional expression of enzymes on the surface of Gram-negative bacteria other than *E. coli.*

Although OPH hydrolyzes a wide range of organophosphates, the effectiveness of hydrolysis varies dramatically. For example, some highly used organophosphorus insecticides such as methyl parathion, chlorpyrifos and diazinon are hydrolyzed 30-1000 times slower than the preferred substrate, paraoxon. Sequential cycles of DNA shuffling and screening were used to "fine tune" and enhance the activity of OPH towards poorly degraded substrates. Because of inaccessibility of these pesticides across the cell membrane, OPH variants were displayed on the surface of *E. coli* using the truncated ice-nucleation protein in order to isolate novel enzymes with truly improved substrate specificities (Cho et al., 2002). Two rounds of DNA shuffling and screening were carried out and several improved variants were isolated. One variant 22A11, in particular, hydrolyzes methyl parathion 25-fold faster than the wild type. Because of the success we achieved with directed evolution of OPH for improved hydrolysis of methyl parathion, this method can be extended in creating other OPH variants with improved activity against poorly degraded nerve agents such as sarin and soman.

In addition to detoxification, surface engineering can also be used as whole cell immunosorbents for the removal of toxic contaminants. A scFv fragment of the anti-atrazine antibody was successfully anchored on the surface of *E. coli* using the PAL lipoprotein anchor (Dhillon et al., 1999). However, the severe reduction in cell growth and the very low level of surface expression may preclude their practical usage. Other robust surface expression systems such as the ice nucleation protein anchor may be used to improve the overall efficiency.

Surface Engineering for Gram-Positive Bacteria

Although initial efforts with surface engineering have been focused on Gram-negative bacteria, several reports with Gram-positive bacteria have also been reported. In most cases, surface display of proteins were made possible by taking advantage of the anchoring mechanism of *Staphylococus auresus* protein A (SpA) (Schneewind et al., 1995). The C-terminal anchoring region of SpA consists of a charged repetitive region that interacts with the peptidoglycan cell wall and a cell-surface-bound receptor containing a LPXTG motif.

The first successful report of surface expression in Gram-positive bacteria was demonstrated by Hannsson et al. (1992) by anchoring a malaria blood-stage antigen and the albumin-binding reporter protein to the cell surface of *Staphylococcus xylosus.* Since this Staphylococccus strain is non-pathogenic and is safe to administer by mucosal or subcutaneous routes, this initial report provides the possibility of using recombinant *Staphylococcus* as a delivery system for oral vaccination (Nguyen et al., 1993). Mice were immunized orally with *S. xylosus* cells displaying an ABP-G3-XM receptor and antibodies could be detected in the mice even after 143 days. Subsequently, Samuelson et al. (1995) also demonstrated the surface expression of proteins in a related *Staphylococcus carnosus.* Interestingly, the antibody response elicited by immunization with recombinant *S. carnosus* was higher than *S. xylosus*. This was attributed to the higher level of antigen displayed (Andreoni et al., 1997). Immunization response was further improved by the co-expression of a fibronectin-binding domain that is capable of binding to the mucosal epithelium (Liljeqvist et al., 1999).

In addition to antigens, various other proteins have been anchored on the surface of Gram-positive bacteria. Most noticeable is the functional expression of single-chain antibodies (Gunneriusson et al., 1996). This opens up the opportunities of using these recombinant *Staphylococci* for high throughput screening of novel binders (Nord et al., 1997) or as whole cell diagnostic devices (Stahl and Uhlen, 1997). Novel affibodies have been successfully selected to diverse targets (Hansson et al., 1999; Gunneriusson et al., 1999).

Although the use of Gram-positive bacteria as enzyme carriers or for environmental applications has only been demonstrated for a limited number of systems, they are potentially attractive because of the thick cell wall surrounding the cells. In a pioneer study, β-lactamase and a lipase were targeted on the outer surface of *S. carnosus* (Struass and Gotz, 1996). Immobilization of Gram-positive bacterial on to solid supports has also been demonstrated by targeting the cellulose-binding domain (Lehtio et al., 2001) and streptavidin (Steidler et al., 1998) on the cell surface. When combined with surface-displayed scFv or metal-binding peptides (Samuelson et al., 2000), these strategies provide an ideal immobilization tool for the fabrication of whole-cell biosensors suitable for medical or environmental diagnostics.

Concluding Remarks

This paper is dedicated to Professor James (Jay) E. Bailey. Jay is one of the first in chemical engineering to integrate advanced techniques in molecular biology to different aspects of his projects. Jay, being a pioneer in the area of protein expression, would certainly enjoy the recent explosion in surface display technologies and their wide-range applications to different areas of biotechnological importance.

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