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review

Microbial Anchoring Systems for Cell-Surface Display of Lipolytic Enzymes

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Summary

Studies of microbial cell envelopes and particularly cell surface proteins and mechanisms of their localization brought about new biotechnological applications of the gained knowledge in surface display of homologous and heterologous proteins. By fusing surface proteins or their anchoring domains with different proteins of interest, their so-called genetic immobilization is achieved. Hybrid proteins are engineered in a way that they are expressed in the host cells, secreted to the cell surface and incorporated into the wall/envelope moiety. In this way, laborious and often detrimental procedure of chemical immobilization of the protein is avoided by letting the cells do the whole procedure. Both bacterial and yeast cells have been used for this purpose and a number of potential biotechnological applications of surface-displayed proteins have been reported. Among the most frequently used passenger proteins are lipolytic enzymes, due to their great technological significance and numerous important applications. In this review, our current knowledge on mechanisms and molecular systems for surface display of lipolytic enzymes on bacterial and yeast cell surfaces is summarized.

Key words: surface display, genetic immobilization, lipolytic enzymes, bacterial envelope, yeast cell wall

Introduction

In the last decade, studies of recombinant proteins and peptides displayed on the bacterial or yeast surfaces have proven to be a promising and powerful tool for large scale industrial applications (*1*). Examples range from the development of microbial whole cells biocatalyst, biosorbent, biosensor and biostimulants (*1,2*) to the design and screening of protein and peptide libraries (*3*). Surface display of active proteins on living cells offers several distinct advantages over purified enzymes. When connected to the matrix, enzymes have shown better stability. For example, the whole-cell biocatalyst displaying nitrylase could be stored for extended periods of time (six months) without significant loss of enzymatic activity (*4*). Thus, cells displaying active enzymes can be repeatedly used in the same or different industrial processes, while in most cases purified enzymes cannot be used in repeated reactions (*1*). Additionally, a large number of active molecules (>10^5 per cell) can be expressed on the host surface without affecting cell viability (*5*). When surface-immobilized enzymes are used, substrates do not need to cross membrane barriers, *i.e.* enzymes are free to access any externally added substrate. Thus, often complex and expensive purification of the enzymes used on an industrial scale is bypassed. In addition, the mul-

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ti-step transformation can be performed using microbial cells displaying different enzymes that catalyze cascade reactions (*6*). Finally, enzyme display systems can be used for high throughput screening of enzyme libraries obtained by directed evolution (*7,8*).

Protein-anchoring system was used for the first time to display antigens on the virion surface (*9*) but the size of proteins which could be displayed in such system was rather limited. Bacterial display was introduced in 1986 (*10,11*), allowing the surface display of larger proteins. Since then, various microorganisms have been used as hosts for surface display of recombinant proteins and peptides, including Gram-positive and Gram-negative bacteria and fungi (*1,6,12,13*). Microbial cell surface display is accomplished by expressing a protein of interest (passenger protein) fused to various carrier proteins that possess anchoring motifs. These proteins facilitate export and anchor of passengers to the cell surface. To ensure proper cell surface exposure, the passenger could be fused to N- or C-terminus of the carrier protein. In some cases, insertional fusion could be applied as a strategy, *i.e.* passenger can be inserted into a surface-exposed region of the carrier (*6*). In addition, to allow proper functional display, the passenger and the carrier are often separated by a spacer of appropriate size.

In spite of potential industrial and environmental applications of the surface display technology, some challenges of this approach still remain to be solved. These include display of large functional proteins complexes, of the cofactor containing enzymes and co-display of multiple enzymes (*6*). More studies are needed to bring the whole-cell biocatalysts displaying enzymes from the laboratory to industrial scale (*1*). In addition, some of the limitations and drawbacks of the anchoring systems are mentioned here. These correlate with the complexity of the host cell surface, translocation mechanisms (*14*), passenger-specific foldases (*15*), proteolytic activities (*16, 17*), or specifically with yeast system translocation into the endoplasmic reticulum (ER) (*18*), processing and folding in the ER and Golgi (*19*), and secretion (*20*). In this respect, *Saccharomyces cerevisiae* has some advantages over bacteria as a potential host for expression of mammalian proteins, since it possesses protein folding and secretory machinery similar to those of higher eukaryotic cells, and enables proper post-translational modifications and folding of many mammalian proteins. Besides, most yeast strains do not secrete significant amounts of proteases, thus no particular proteolytic mutants are required. Still, one has to bear in mind that surface display requires formation of hybrid proteins and the experience in many laboratories, including ours, shows that in many cases proteins are properly exported and embedded at the cell surface but not in their active conformation. Improper folding may particularly be the problem in the case of lipases with lower solubility in water.

Lipolytic enzymes in general have a great potential for biotechnological applications. This superfamily comprises different classes of enzymes such as esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), which are widely applied for the hydrolysis of ester bonds and transesterifications (*21*). Thus, over the past few decades these enzymes have been used in a wide range of industrial applications, including production of food, flavours, detergents, textiles, fine chemicals, and in bioremediation (*22,23*). Most lipolytic enzymes used in industry are of microbial origin. Microbial enzymes exhibit great variety of catalytic activities, they are proven to be more stable and their production is more convenient (*22*). Many of them display robust performance under extreme conditions. Extensive studies have been undertaken to elucidate mechanism of reactions of lipolytic enzymes, 3D structures and physiological functions because improvement of catalytic reactions could lead to a better industrial exploitation. New and emerging industrial applications and global enzyme market drive the demand for high-yield production and discovery of novel enzymes or genetically engineered variants associated with improved or novel properties.

Over a decade we have been interested in studying bacterial GDSL lipolytic enzymes (*24–26*). These enzymes possess flexible active sites that change conformation in the presence of substrates and therefore display broad substrate specificity. In addition, they show good stability and activity in various organic solvents and in a broad range of pH and working temperatures (*24,27–29*). Our recent bioinformatic analysis of Gram-positive, actinobacterial genomes pointed out that these bacteria possess a comparable number of extracellular and intracellular GDSL enzymes, suggesting their importance in intracellular metabolism as well as in extracellular degradation of complex organic matter in the bacterial environment. Gram-negative bacteria also possess numerous GDSL enzymes. For this review, the most relevant are surface attached GDSL enzymes, *i.e.* autotransporters (*3*). The use of their domains for displaying different classes of enzymes will be described here in more detail.

Surface display of lipolytic enzymes is reported as a powerful technique that has been applied in various organisms. In this study we review all reported bacterial and yeast anchoring systems used to display functional lipolytic enzymes on the cell surface. Basic characteristics of anchoring systems and various passengers are summarized in Tables 1–3 (*1,3,6–8,13,17,30–89*). Moreover, described examples show that the whole-cell biocatalysts represent promising industrial systems for many biotechnological applications. This review is aimed not only for readers experienced with lipolytic enzymes, but also to trigger ideas for other potential uses of the surface display methodology.

Architecture of the Bacterial Cell Surface and Systems Used to Display Lipolytic Enzymes

This chapter considers the nature of bacterial cell envelope architecture associated with the anchoring systems used to display lipolytic enzymes on the surface of bacteria belonging to the phyla Firmicutes (*Bacillus subtilis* and *Staphylococcus carnosus*) and γ-Proteobacteria (*Escherichia coli* and *Pseudomonas putida*). These species are typical examples of Gram-positive and Gram-negative bacteria, although such grouping is oversimplified considering the present knowledge and diversity of bacterial cell envelope architecture (*90,91*). However, the major basis of Gram staining is founded in the structural differences of cell envelopes. The cell envelope of Gram-

Table 1. Lipolytic enzymes immobilized at the bacterial cell surface

Anchor	Fusion	Passenger	Application	Basic information	Ref.
Gram-negative bacteria					
Escherichia coli					
Truncated AIDA-I/ *E. coli*	N-terminal	Carboxylesterase EstA/ *B. gladioli*	Functional expression studies	Fusion protein consists of CTB signal peptide, EstA, and AIDA-I autotransporter with linker region.	(*30*)
Truncated AIDA-I/ *E. coli*	N-terminal	GDSL esterase ApeE/ *S. enterica*	Analysis of substrate preferences	Development of screening technology with pH sensor microplates. Esterase fusion altered enzyme activities.	(*31*)
Inactive EstA/ *P. aeruginosa*	N-terminal	Lipase LipA/*B. subtilis* Lipase/*S. marcenscens*	Translocation studies	Full-length EstA (686 amino acids, aa) efficiently displayed passenger (613 aa) of nearly its own size albeit with a reduced yield.	(*32*)
Inactive EstA/ *P. aeruginosa*	N-terminal	Chaperone LipH/ *P. aeruginosa*	Lipase renaturation	The cell-surface display of a lipase functional chaperone could be used as whole cell 'refolding microparticles'.	(*33*)
Truncated EstA (EstATu)/*P. putida*	N-terminal	Lipases: – PAL/*P. aeruginosa* – BCL/*B. cepacia* – PFL/*P. fluorescens*	Functional expression with chaperones	Coexpression with chaperones was essential for PAL and BCL activities. Signal sequence, OmpA increased expression level of PFL and PAL.	(*15*)
INP and INPNC/ *P. syringae*	C-terminal	Lipase TliA/ *P. fluorescens*	Directed evolution	INPNC-TliA showed 2× higher activity than INP-TliA.	(*34*)
INPNC/ *P. syringae*	C-terminal	Carboxylesterase CaE B1/*Culex* sp. (mosquito)	Bioremediation of organophosphorus pesticides	High biodegradation activity, superior stability.	(*35*)
Truncated FadL/ *E. coli*	C-terminal	Lipase/*Bacillus* sp.	Enantioselective biocatalysis	The lipase was fused to the external loop of $FadL^{Arg384}$.	(*36*)
Truncated OmpC/ *S. typhimurium*	C-terminal	Lipase TliA/ *P. fluorescens*	Enantioselective biocatalysis	Lipase exhibited broad substrate specificity, reusability and stability (120 h).	(*37*)
Truncated OmpC/ *E. coli*	C-terminal	Lipase TliA/ *P. fluorescens*	Functional coexpression	Coexpression with *gadBC* increased lipase activity 48 times.	(*38*)
Truncated OmpX/ *E. coli*	C-terminal	Lipase TliA/ *P. fluorescens*	Translocation and functional studies	In the extracellular loops of OmpX fusion site, Lys^{122} was slightly better than Val^{160}.	(*39*)
Truncated OmpW/ *E. coli*	C-terminal	Lipase TliA/ *P. fluorescens*	Enantioselective biocatalysis	Surface-displayed TliA was an efficient enantioselective biocatalyst.	(*40*)
Truncated OprF/ *P. aeruginosa*	C-terminal	Lipase TliA/ *P. fluorescens*	Translocation/ Enantioselective biocatalysis	$OprF^{Glu164}$-TliA exhibited the highest lipase activity for a week in aqueous and organic solvent (over 80 %).	(*41*)
PgsA/*B. subtilis*	C-terminal	Lipase B (CALB)/ *C. antarctica*	Enantioselective transesterification	Useful to display larger enzymes and to support protein folding.	(*42*)
Pseudomonas putida					
Truncated OprF/ *P. aeruginosa*	C-terminal	Lipase TliA/ *P. fluorescens*	Translocation/ Enantioselective biocatalysis	Hybrid protein $OprF^{Val188}$-TliA showed the highest activity.	(*43*)
INP and INPNC/ *P. syringae*	C-terminal	Lipase TliA/ *P. fluorescens*	Functional studies	INPNC-TliA exhibited better activity in the presence of organic solvent.	(*44*)
Gram-positive bacteria					
Bacillus subtilis					
CWB_b (truncated CwlB)/*B. subtilis*	C-terminal	Lipase LipB/ *B. subtilis*	Translocation studies	Lipase was unstable in the wild-type strain (*17*) and not accumulated in high yield.	(*45*)
CWB_b/*B. subtilis*	C-terminal	Lipase LipB/ *B. subtilis*	Translocation studies	Mutant *B. subtilis wrpAsigD* exhibited best accumulation of CWB_b-LipB.	(*17*)
CWB_b/*B. subtilis*	C-terminal	Lipase CutL/ *A. oryzae*	Translocation studies	*B. subtilis wrpAsigD* was a suitable host for CWB_b-CutL display.	(*46*)
CWB_b and CWB_c (truncated CwlC)/ *B. subtilis*	N- and C-terminal	Lipases: CWB_b-CutL/*A. oryzae* LipB-CWB_c /*B. subtilis*	Translocation studies	*B. subtilis* accumulated considerable amounts of both lipases on the cell surface.	(*47*)

Tab. 1 – continued

Anchor	Fusion	Passenger	Application	Basic information	Ref.
Staphylococcus carnosus					
Truncated FnBPB/ *S. aureus*	N-terminal	Lipase/*S. hyicus*	Translocation and functional studies	FnBPB was fully compatible with folding of the lipase to the active conformation if the spacers exceeded the length of approx. 90 aa.	(*48*)
Applied methods for high-throughput screening (HTS) of bacterial surface exposed enzymes					
Functional EstA/ *P. aeruginosa*	Whole gene	EstA/*P. aeruginosa*	HTS-system development	Attachment of biotin tyramide to surface of esterase-proficient bacteria.	(*8*)
Functional EstA/ *P. aeruginosa*	Whole gene	EstA/*P. aeruginosa*	HTS of enantioselective esterase variants	Results are relevant for analysis of massive mutagenesis and HTS applying fluorescence-activated cell sorting (FACS).	(*7*)

-negative bacteria (Fig. 1) is a complex multilayered structure that contains the outer membrane (OM), the peptidoglycan (PG) cell wall, and the cytoplasmic or inner membrane (CM). Due to the presence of two membranes, Gram-negative bacteria are also named diderm bacteria (*91*). The inner membrane is a phospholipid bilayer. In a model organism, *E. coli*, phosphatidyl ethanolamine and phosphatidyl glycerol are predominant phospholipids, phosphatidyl serine and cardiolipin are present in lesser amounts, whereas the minor lipids also include polyisoprenoid carriers (*90*). Peptidoglycan, located between CM and OM, is made up of repeating units of N-acetyl glucosamine/N-acetyl muramic acid cross-linked by pentapeptide side chains. In Gram-negative bacteria, several nanometers thick peptidoglycan contains from one to several layers. The structure is rigid and determines the cell shape. The outer membrane is a lipid bilayer attached to the underlying peptidoglycan by murein lipoprotein (Lpp). The outer leaflet of this membrane does not contain phospholipids like the inner membrane, but it is composed of glycolipids, principally lipopolysaccharides (LPS). It plays a critical role in the barrier function of the OM. Contrary to Gram-negative bacteria, the outer membrane is absent from Gram-positive (monoderm) bacteria (Fig. 1) (*91,92*). To compensate for the lack of OM, these bacteria possess multiple layers of peptidoglycan (30–100 nm), much thicker than that found in *E. coli*. Long anionic polymers, named teichoic acids, are threading through the layers of peptidoglycan, composing up to 60 % of the cell wall mass.

A range of proteins are embedded in the described layers of bacterial cell envelopes, *via* covalent or non-covalent attachment. These proteins significantly contribute to the overall cell envelope architecture and display a variety of functions (*90,92*). Some of them are exploited for cell surface display of heterologous proteins, including lipolytic enzymes (Fig. 1), which will be described in more detail.

Gram-positive bacteria

In general, surface proteins of Gram-positive bacteria are anchored either in the cytoplasmic membrane or in the peptidoglycan layer of the cell envelope. CM-associated proteins with transmembrane domain are attached in a non-covalent manner whereas lipoproteins are covalently attached to long chain fatty acids of the cytoplasmic membrane. PG-associated proteins could be covalently attached through C-terminal LPXTG or related motifs whereas non-covalently PG-embedded proteins possess cell wall-binding domains (CWBDs) (*92*). Anchoring proteins have been successfully used for development of display systems in Gram-positive bacteria (*12*). Table 1 summarizes all reported anchoring domains used to display lipolytic enzymes on the surface of Gram-positive bacteria.

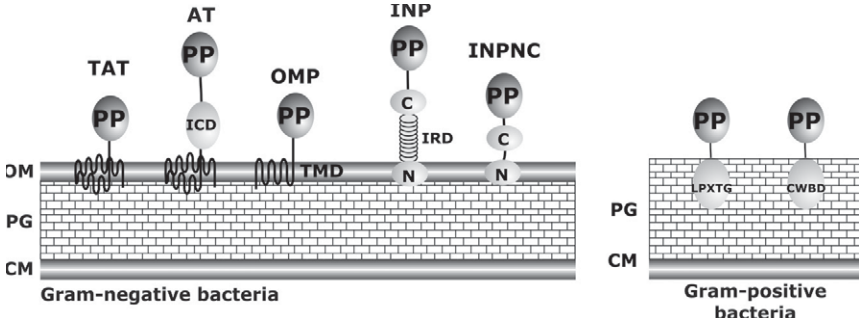

Fig. 1. Schematic representation of the bacterial systems used for the display of lipolytic enzymes. Gram-negative bacteria: TAT=truncated autotransporter (*e.g.* AIDA-I, EstA); AT=autotransporter EstA with inactive catalytic domain (ICD); OMP=outer membrane protein (*e.g.* FadL, OmpC, OmpW, OmpX, OprF) with transmembrane domain (TMD); INP=ice-nucleation protein with internal repeating domain, IRD (*i.e.* N-IRD-C); INPNC=INP with deleted IRD. Gram-positive bacteria: LPXTG=motif for attachment to peptidoglycan; CWBD=cell wall-binding domain (*e.g.* CWB_B and CWB_C). Additional abbreviations: PP=passenger protein, OM=outer membrane, PG=peptidoglycan, CM=cytoplasmic membrane

Table 2. Lipolytic enzymes immobilized at the yeast cell surface

Anchor source	Anchor	Passenger	Comments	Ref.
Saccharomyces cerevisiae				
α-agglutinin/ *S. cerevisiae*	GPI	Lipase B (CALB)/ *Candida antarctica*	Biocatalysts for industrial scale production of ethyl hexanoate in non-aqueous phase.	(*49*)
α-agglutinin/ *S. cerevisiae*	GPI	Lipase B (CALB)/ *Candida antarctica*	The lyophilized CALB-displaying yeasts showed their original hydrolytic activity and catalyzed enantioselective ethyl lactate synthesis.	(*50*)
α-agglutinin/ *S. cerevisiae*	GPI	Mutated lipase B (mCALB)/ *Candida antarctica*	mCALB showed a preference for short chain fatty acids, which is an advantage for producing flavours.	(*51*)
Sed1p, Sag1p/ *S. cerevisiae*	GPI	Lipase B (CALB)/ *Candida antarctica*	The heterogeneous co-displaying yeast system resulted in improved efficiency of enzyme-displaying yeast cells.	(*52*)
FLO1/ *S. cerevisiae*	flocculation domain	Prolipase B (ProCALB)/ *Candida antarctica* CBS6678	Lyophilized whole cells with surface-immobilized ProCALB could be used to catalyze esterification reaction at 60 °C.	(*53*)
FLO1/ *S. cerevisiae*	flocculation domain	Lipase B (CALB)/ *Candida antarctica*	Diploid yeast cells containing eight copies of the expression cassette integrated in the genome showed high hydrolytic activity and could catalyze the polyester synthesis reaction with reduced by-product production.	(*54*)
FLO1/ *S. cerevisiae*	flocculation domain	ROL/ *Rhizopus oryzae*	The ROL-displaying yeast catalyzed optical resolution of the pharmaceutical precursor (*R*,*S*)-1-benzyloxy-3-chloro-2-propyl monosuccinate.	(*55*)
FLO1/ *S. cerevisiae*	flocculation domain	proROL/ *Rhizopus oryzae*	High lipase activity for methanolysis reaction in a solvent-free system. Cells showed strong flocculation ability.	(*56*)
FLO1/ *S. cerevisiae*	flocculation domain	proROL/ *Rhizopus oryzae*	Heterologous enzymes catalyzed an enantioselective transesterification in nonaqueous organic solvent.	(*57*)
α-agglutinin/ *S. cerevisiae*	GPI	ROL/ *Rhizopus oryzae*	Spacers (linker peptides) of different lengths between ROL and the C-terminal half of α-agglutinin increased lipase activity displayed on the cell surface.	(*58*)
α-agglutinin/ *S. cerevisiae*	GPI	Mutated ROL/ *Rhizopus oryzae*	ROL gene was amplified by error-prone PCR, and mutants with increased activity and stability in organic solvent were selected.	(*59*)
α-agglutinin/ *S. cerevisiae*	GPI	Codon optimized ROL/ *Rhizopus oryzae*	Recombinant yeast displayed fully codon-optimized ROL according to the codon bias of *Saccharomyces cerevisiae.*	(*60*)
a-agglutinin/ *S. cerevisiae*	Aga2	Lip7 and Lip8/ *Yarrowia lipolytica*	Recombinant lipases exhibited preference for medium-chain fatty acids and a high thermal stability.	(*61*)
Cwp2/ *S. cerevisiae*	GPI	Lip2/ *Yarrowia lipolytica*	Exhibited better thermostability than free Lip2 and retained 100 % activity in 0.1 % Triton X-100 and 0.1 % Tween 80 for 30 min, and 92 % activity in 10 % DMSO for 30 min.	(*62*)
a-agglutinin/ *S. cerevisiae*	Aga 2	Lipase ANL/ *Aspergillus niger*	The displayed lipase showed a preference for medium-chain fatty acid esters and high thermostability.	(*63*)
FLO1/ *S. cerevisiae*	flocculation domain	LipB52/ *Pseudomonas fluorescens* B52	Immobilized lipases showed high level of enantioselectivity.	(*64*)
α-agglutinin/ *S. cerevisiae*	GPI	Lipase RML/ *Rhizomucor miehei*	Cell surface-displayed lipase showed the highest activity at 45 °C and pH=8.0.	(*65*)
Pichia pastoris				
α-agglutinin/ *S. cerevisiae*	GPI	Lipase B (CALB)/ *Candida antarctica*	Robust biocatalysts for large-scale production of flavour esters in non-aqueous media.	(*66*)
α-agglutinin/ *S. cerevisiae*	GPI	Lipase B (CALB)/ *Candida antarctica*	The synthetic activity and catalytic efficiency of CALB fused with α-agglutinin was shown to be higher than that of the FS fusion protein.	(*67*)
FLO1/ *S. cerevisiae*	flocculation domain			
Sed1p/ *S. cerevisiae*	GPI	Lipase B (CALB)/ *Candida antarctica*	The fusion protein CALB-Sed1 was highly glycosylated and exhibited increased thermostability.	(*68*)
Gcw21p/ *Pichia pastoris*	GPI	Lipase B (CALB)/ *Candida antarctica*	A novel *P. pastoris* display system for biodiesel production.	(*69*)
FLO1-/ *S. cerevisiae*	flocculation domain	LipB52/ *Pseudomonas fluorescens* B52	Immobilized lipases showed high level enantioselectivity. The LipB52 displayed on the *Pichia pastoris* exhibited better stability than on *Saccharomyces cerevisiae* cell surface.	(*64*)

Tab. 2 – continued

Anchor source	Anchor	Passenger	Comments	Ref.
a-agglutinin/ *S. cerevisiae*	Aga2	Lipase GSL/ *Geotrichum* sp.	The GSL-displaying yeast whole cells were used to enrich eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the fish oil by selective hydrolysis.	(*70*)
FLO1/ *S. cerevisiae*	flocculation domain	Lipase LIPY7 and LIPY8/ *Y. lipolytica*	Lipase can be cleaved off from anchor to yield functionally active protein in the supernatant.	(*71*)
FLO1/ *S. cerevisiae*	flocculation domain	Mutated lipase RML/ *Rhizomucor miehei*	RML variants with increased activity in organic solvent were created by site-directed mutagenesis.	(*72*)
Yarrowia lipolytica				
FLO1/ *S. cerevisiae*	flocculation domain	lipRS/ *Rhizopus stolonifer*	Whole cell lipase for oily wastewater treatment.	(*73*)

The first immobilization of a lipase on the surface of Gram-positive bacteria was demonstrated in 1996 (*48*). The anchored lipase was originally isolated from *Staphylococcus hyicus*. The C-terminal region of *Staphylococcus aureus* fibronectin-binding protein B (FnBPB, Table 3) with an LPETG motif was used to link the lipase covalently to the PG layer of *S. carnosus* (Fig. 1). This cell-surface-displayed lipase retained more than 80 % of the specific activity of unmodified *S. hyicus* lipase secreted by *S. carnosus* cells. The length of the spacer region between the cell-wall sorting signal and the C-terminus of the lipase significantly influenced enzyme activity (Table 1). It was proposed that the spacer must exceed a critical length to allow efficient folding of the enzyme outside the peptidoglycan layer of the cell wall (*48*). In addition, fused protein released by lysostaphin treatment exhibited the same specific activity as that of the unmodified lipase. Thus, the results demonstrated that C-terminal region of FnBPB can be used for effective immobilization of enzymes on the cell wall of *S. carnosus*.

The first example of extracellular lipase (LipB) displayed on the cell wall of *B. subtilis* was reported in 1999 (*45*). The major vegetative autolysin CwlB (LytC), non-covalently attached to the PG of *B. subtilis* envelope was used to anchor LipB. The C-terminus required for the CwlB catalytic activity was removed and LipB was fused to the remaining cell-wall-binding domain (CWB_b, Table 3). Fused LipB lipase non-covalently linked to the *B. subtilis* cell wall retained its activity. However, due to the observed problems with lipase stability and yield (Table 1), the accumulation of recombinant CWB_b-LipB was examined in several *B. subtilis* protease-deficient hosts (*17*). The strains lacking two major extracellular proteases, NprE and AprA, did not show a significant increase in the CWB_b-LipB accumulation. On the contrary, mutation in the cell-wall-binding protease, WprA resulted in a greater accumulation of recombinant lipase but only in the stationary phase. Mutation in the SigD, a minor alternative sigma factor that exhibits pleiotropic effects especially for various surface proteins, led to considerable increase in the accumulation of CWB_b-LipB throughout the *B. subtilis* growth. The best result was obtained with the double mutant, *wprAsigD*, in which the recombinant lipase comprised 36 % of the total cell surface proteins. The same system was used to expose a functional *Aspergillus oryzae* lipase, CutL, on the surface of *B. subtilis wprAsigD* (*46*). The recombinant lipase CWB_b-CutL accumulated up to 10 % of the total proteins extracted from the bacterial cell surface.

Additional CWB domain of the peptidoglycan hydrolase CwlC (CWB_c, Table 3) was explored as a possible anchor (*47*). Since extracellular lipases LipB from *B. subtilis* and CutL from *A. oryzae* exhibited different substrate specificities, they were simultaneously displayed on the cell surface of *B. subtilis*. CutL was fused to CWB_b as described above, while LipB was fused to the N-terminus of the CWB_c. Chimeric genes were co-transcribed from the *tet* promoter and both lipases accumulated efficiently on the cell surface (Table 1). These results demonstrated that CWB domains could be useful for anchoring hydrolytic enzymes on the cell surface of *B. subtilis*. Additionally, since CWB domains are non-covalently linked to the cell wall, fused proteins can be extracted with a high concentration of LiCl (*17*,*93*,*94*).

Gram-positive bacteria are not used for anchoring lipolytic enzymes as frequently as Gram-negative. However, the rigid structure of their cell walls provides for better viability when cells are subjected to mechanical stress. This property and the presence of only one membrane barrier for passenger transport represent advantages when choosing Gram-positive bacteria as hosts for the whole-cell biocatalysts.

Gram-negative bacteria

Different carrier proteins have been successfully used to develop display systems in Gram-negative bacteria (*1*, *3*,*6*,*12*). These anchoring proteins differ in their structure. Integral outer membrane proteins (OMPs) generally consist of antiparallel amphipathic β-strands that fold into cylindrical β-barrels with a hydrophilic interior and hydrophobic residues pointing outward to face the membrane lipids. Outer membrane also contains lipoproteins which are anchored *via* an N-terminal N-acyl-diacylglycerylcysteine (*95*). All reported anchoring mechanisms used to display lipolytic enzymes on the surface of Gram-negative bacteria are listed in Table 3 and shown in Fig. 1. Typical carrier proteins could be ascribed to three main groups: autotransporters, lipoproteins and outer membrane proteins, and all were used to anchor different passengers in *E. coli*, the most exploited bacterial host for displaying hydrolytic enzymes (Table 1).

Table 3. Microbial cell surface display systems

Anchor	Anchor origin	Comments	Ref.
		Bacterial systems	
Autotransporters			
AIDA-I	*E. coli*	AIDA-I was used for effective display of numerous enzymes including β-lactamase, sorbitol dehydrogenase and organophosphorus hydrolase on the surface of *E. coli*. The β-barrel and the linker region of AIDA-I were employed in combination with signal peptides of different origins (CTB, PelB, OmpA or AIDA-I) in various display studies.	(*1,3,74*)
EstA	*P. aeruginosa*	EstA possesses a C-terminal β-barrel domain that allows translocation of the N-terminal passenger with the esterase activity. EstA can be correctly localized in the outer membrane of *E. coli*. This autotransporter was used for surface display of various recombinant enzymes.	(*1,3,75*)
EstA	*P. putida*	*P. putida* EstA shares the identical amino acid sequence as *P. aeruginosa* EstA and can be considered as being the same autotransporter.	(*1,3*)
Lipoproteins			
INP	*P. syringae*	INP, a glycosyl phosphatidylinositol-anchored outer membrane lipoprotein, is frequently used to develop whole-cell biocatalysts (*E. coli*) since it allows anchoring of large passengers.	(*6*)
INPNC	*P. syringae*	INPNC, truncated version of INP, was more efficient in enzyme display (see Table 1).	(*76*)
Outer membrane proteins			
FadL	*E. coli*	FadL consists of 14 antiparallel β-strands which form a β-barrel structure with distinctive features. Arg384 located on the external loop provides good anchoring site, as demonstrated experimentally.	(*77*)
OmpC	*S. typhimurium*	OmpC from *S. typhimurium* and *E. coli* share 77 % identity. The L7 loop was used as a fusion site. In addition to lipase display, this system was used for display of polyhistidine peptides and green fluorescent protein (GFP) in *E. coli*.	(*78*)
OmpC	*E. coli*	OmpC is one of the most abundant outer membrane proteins in *E. coli* (up to 10^5 molecules per cell). L7 loop is convenient site for fusion.	(*79*)
OmpX	*E. coli*	OmpX (18.6 kDa) is channel protein with β-barrel domain. Besides lipase (49.9 kDa), this system was efficiently used to anchor endoxylanase (23.3 kDa) and alkaline phosphatase (49.4 kDa). Circularly permuted OmpX (CPX) with N- and C-termini on the external cell surface also provides a highly effective method for screening peptide libraries.	(*39,80, 81*)
OmpW	*E. coli*	OmpW belongs to homologous group of small outer membrane β-barrel proteins. It forms an eight-stranded β-barrel with an unusual hydrophobic channel.	(*82,83*)
OprF	*P. aeruginosa*	OprF functions as a nonspecific porin and has a structural role in maintaining cell shape and outer membrane integrity. In the first application, the efficient sandwich-fusion of a 4-aa epitope from malaria parasite to OprF was performed in *E. coli.*	(*83–85*)
P*gsA*	*B. subtilis*	PgsA is a component of PGA synthetase complex, predicted to function as a poly-γ-glutamate transporter. Enzyme complex PgsBCA is stably anchored into cell membrane by the action of PgsA. This system was also efficiently used to display large proteins on the cell surface of *Lactobacillus casei*.	(*86,87*)
Peptidoglycan attached proteins			
CWB_b	*B. subtilis*	CWB_b is a truncated version of the major cell wall-binding autolysin (CwlB). This autolysin comprises two domains: C-terminal or catalytic domain and N-terminal or cell wall-binding domain (CWB_b).	(*88*)
CWB_c	*B. subtilis*	C-terminal domain of CwlC hydrolase (CWB_c) is one of the smallest cell wall-binding domains.	(*89*)
FnBPB	*S. aureus*	C-terminal region of fibronectin-binding protein B (FnBPB) comprises the authentic cell wall-spanning region and cell wall-sorting signal. This system was used to display *E. coli* β-lactamase.	(*48*)
		Yeast systems	
GPI anchors			
α-agglutinin/ Sag1	*S. cerevisiae*	N-terminal half of Sag1 is homologous to the immunoglobulin superfamily and contains binding site for a-agglutinin, while C-terminal half contains GPI anchor. C-terminal GPI-anchoring signal is small and sufficient for the attachment of GPI, and it is removed from the sequence of the mature protein in the process of anchor attachment. GPI system has been successfully used to express and localize many different recombinant proteins on the yeast surface.	(*13, 49–52, 58–60, 65–67*)
Cwp2	*S. cerevisiae*	Cwp2 precursor is GPI-anchored, while mature protein is covalently linked to the cell wall. It is one of major constituents of the cell wall, plays a role in wall stabilization and it is involved in low pH resistance.	(*13,62*)

Tab. 3 – continued

Anchor	Anchor origin	Comments	Ref.
Sed1	*S. cerevisiae*	Sed1 is major stress-induced structural GPI-cell wall glycoprotein, associated with translating ribosomes and possibly plays a role in mitochondrial genome maintenance.	(*13*,*52*, *68*)
Gcw21	*P. pastoris*	Fragment of the GPI-anchored cell wall protein Gcw21p without an internal N-terminal signal peptide was amplified from the genomic DNA of *P. pastoris* GS115 and fused to the C-terminal sequence of passenger protein.	(*69*)
a-agglutinin			
Aga2	*S. cerevisiae*	Aga2 is adhesion subunit of a-agglutinin linked to anchorage subunit Aga1 *via* two disulphide bonds. C-terminal sequence of Aga2 acts as a ligand for α-agglutinin (Sag1p) during agglutination.	(*13*,*61*, *63*,*70*)
Pir proteins			
Pir 1–4	*S. cerevisiae*	All four proteins share high sequence homology and contain a characteristic N-terminal motif required for the covalent attachment of the protein, but only the complete Pir proteins in native conformation were found to be efficiently linked. Chimeric protein consisting of the whole Pir and the desired passenger protein has to be created to achieve effective incorporation into the cell wall.	(*13*)
Non-covalent attachment			
Flo1	*S. cerevisiae*	Flo1 is a lectin-like cell wall protein that contains the flocculation functional domain which adheres non-covalently to cell wall mannans. Flo1 cell-surface display system consists of the secretion signal and the 3' region of the *FLO1* gene encoding amino acids 1 to 1099 (FS) or 1 to 1417 (FL) respectively. Both systems (FS and FL) showed to be effective for immobilization of passenger proteins whose catalytic sites are near their C-termini.	(*53–57*, *64*,*67*, *71–73*)

Autotransporters (*1*,*3*,*96*) are OMPs that share a common general structure consisting of a signal peptide, passenger domain and membrane anchor domain (also named the translocator domain). The signal peptide enables transport of the precursor protein across the inner membrane, while the C-terminal domain forms a β-barrel within the outer membrane and subsequently translocates the passenger domain to the cell surface. Their natural passengers typically harbour enzymatic activities and exert virulence function.

Autotransporter AIDA-I (Table 3) is adhesin involved in diffuse adherence that mediates bacterial attachment to a broad variety of cells. It was used to anchor carboxylesterase (EstA) from *Burkholderia gladioli* to the surface of *E. coli* (*30*). Signal peptide of cholera toxin (CTB) was fused to the N-terminus of EstA to transport synthesized protein into the periplasmic space. For the transport across the outer membrane, truncated AIDA-I (linker region and the β-barrel anchoring domain) was fused to the C-terminus of EstA. This construct, EstA-AIDA-I enabled translocation, anchoring and functional esterase surface display (Fig. 1, TAT). The same autodisplay system was exploited for anchoring ApeE (GDSL esterase) from *Salmonella enterica* serovar Typhymurium (*31*). The natural ApeE contains a β-barrel domain within the C--terminus that anchors this enzyme in the outer membrane, while the catalytic domain at the N-terminus is directed towards periplasm. The C-terminal extension of ApeE was removed and the catalytic domain (including conserved blocks I to V) was fused to the transport domain of AIDA-I (Table 3). This resulted in the surface display of a highly active recombinant enzyme in *E. coli* (EsjA). In addition, differences in substrate preferences between ApeE and EsjA were reported indicating that the fusion could have had a considerable effect on the enzyme structure (Table 1) (*31*).

Several other autotransporters possess hydrolase passenger domains belonging to the GDSL family of lipolytic enzymes. Esterase EstA of *Pseudomonas aeruginosa* (Table 3), a typical example of such type, is very well studied. This bacterial phospholipase plays an important role as a virulence factor (*3*), its crystal structure has been solved recently (*97*) and its potential for biotechnological applications has been demonstrated (*3*). EstA was used to display lipases of *B. subtilis* (LipA) and *Serratia marcescens* on the cell surface of *E. coli* (*32*). Lipases were fused to the N-terminus of inactivated esterase (Fig. 1, AT). This system efficiently translocated functionally active hydrolases (Table 1) that varied in amino acid sequence length, LipA (181aa) and *S. marcescens* lipase (613 aa). The same autodisplay system was used to deliver lipase-specific chaperone, LipH from *P. aeruginosa* (*33*), to the surface of *E. coli* cells. LipH is a member of lipase-specific foldases (Lif) that are found anchored to the periplasmic side of bacterial inner membrane in a variety of Gram-negative bacteria. Their biological role is to convert cognate lipases into their active conformations. The functionality of surface-exposed LipH was demonstrated by applying an *in vitro* refolding assay. This foldase allowed renaturation of chemically denatured lipase with efficiency comparable to that of the soluble LipH, thus indicating that anchoring of foldase to the outer membrane did not negatively influence its functionality (Table 1 and Fig. 1, AT). Functional display of industrially important *Pseudomonas* and *Burkholderia* lipases using only translocator/anchor domain of EstA from *P. putida* (Table 3) was obtained on the cell surface of *E. coli* (*15*). Lipases from *P. aeruginosa* (PAL) and *B. cepacia* (BCL) required specific Lif chaperone for proper folding. Although truncated EstA efficiently exposed these enzymes on the cell surface, the whole cells showed significant lipase activity only when lipases were coexpressed with their cognate Lif proteins. The mechanism by which Lif

proteins, known to be anchored to the periplasmic side of the inner membrane, facilitate folding of surface displayed lipases is not well understood. The authors propose that Lif proteins mediate the conversion of an unfolded lipase to a partially folded form and that this process takes place simultaneously with translocation through the outer membrane (*15*). In the same work, extracellular lipase from *P. fluorescens* (PFL) was also anchored to the outer membrane by the same autotransporter. PFL lipase did not require specific chaperone; however, it was fused to OmpA-signal sequence. This sequence stimulated PFL expression level that led to the increase in the whole-cell lipase activity.

The other group of carrier proteins used to display lipases belongs to lipoproteins. Among these, the ice-nucleation protein (INP), an outer membrane lipoprotein found in several Gram-negative bacteria, was commonly used for the surface exposure of enzymes (Table 3). INP is embedded by its N-terminus into phospholipid moiety of the outer membrane (Fig. 1, INP). Internal region of INP (81 %) consists of a series of hierarchically organized repeated amino acid sequences of 8, 16, and 48 residues which act as templates for ice crystal formation (Table 3). The specific C-terminal region is highly hydrophilic and displayed on the cell surface (*76*). This protein is frequently used as anchoring system because it can easily translocate very large proteins as well as proteins containing disulphide bonds and multiple cofactors. In addition, INP is stably expressed in the stationary phase and the length of internal repeating domain is adjustable (Fig. 1, INPNC) (*6*,*12*). The C-terminus of INP from *Pseudomonas syringae* successfully anchored various proteins, including carboxymethylcellulase (*76*), transglucosidase (*98*), organophosphorus hydrolase (*99*), chitinase (*100*), cytochrome P450 (*101*) and lipolytic enzymes described here in more detail.

The extracellular lipase TliA from *P. fluorescens* was displayed on the surface of *E. coli* using the INP and the INP-deleted in the internal repeating domain (INPNC) as anchors (Table 1) (*34*). The surface exposure of TliA was verified by FACS and halo-forming activity on tributyrin emulsion agar plate. INPNC-TliA fusion proteins displayed twofold higher activity than INP-TliA. Lipase-displaying cells were efficient in hydrolyzing artificial (*p*-nitrophenyl palmitate) and natural (olive oil) substrates. Besides, the procedure for visual screening of error-prone PCR-generated enzyme library on agar plates was optimized. Selected clones exhibited up to 29-fold increase in the whole-cell activity, indicating INP potential for the development of the whole-cell biocatalysts. Fusion proteins, INP-TliA and INPNC-TliA, were also displayed on the surface of a solvent-resistant *P. putida* (*44*) (Table 1). The selected host proved to be efficient for a variety of whole-cell bioconversions in the presence of organic solvents. Cells were able to hydrolyze olive oil, synthesize triacylglycerol in isooctane, and catalyze chiral resolution of racemic 4-nitrophenyl 2-phenylpropionate in a two-phase aqueous-organic solvent system. Moreover, the INPNC-TliA exhibited higher stability than free TliA, suggesting that surface immobilization could be a method of choice for stabilizing enzymes against heat and organic solvents. In another study, INPNC was used to display mosquito carboxylesterase B1 (CaE B1) on the *E. coli* surface (*35*), which had demonstrated a high-rate degradation of some organophosphorus pesticides. More than 50 % of the enzymatically active CaE B1 fused to the C-terminus of truncated INPNC was exported across the membrane and anchored onto the cell surface. Degradation of pesticides was significantly improved by whole-cell biocatalysts compared to the cells expressing CaE B1 intracellularly. In addition, these cells also exhibited superior stability over prolonged period of time (two weeks).

Numerous β-barrel proteins found in the outer membrane of Gram-negative bacteria, commonly called outer membrane proteins (OMPs), belong to the third group of anchoring proteins. These proteins perform many different cellular functions including acting as porins, transporters, enzymes, and receptors. In Gram-negative bacteria, OMPs are transported across the inner membrane into the periplasm by the SecYEG translocon. With the help of chaperones, nascent OMPs are guided across the periplasm and peptidoglycan layer to the outer membrane. BAM-complex (β-barrel assembly machinery) assists in folding and insertion of OMPs into the outer membrane (*96*). The β-barrels are composed of antiparallel β-strand pairs connected by short loops at the periplasmic side and by long loops at the external side. The external loops are more tolerant to substitutions, insertions and deletions and therefore can be used as fusion sites for displaying heterologous proteins (*12*). However, insertional fusion only tolerates small peptides without significant loss of stability, while successful display of fairly large passengers requires fusion to the truncated C-terminus (Fig. 1, OMP). OMPs including OmpA, OmpC, FadL, LamB and OprF (Table 3) have been widely used for surface display of various proteins because they provide efficient secretory signals and a strong anchoring structure with many potential passenger fusion sites (*6*). It is worth noting that previously described autotransporters (AIDA-I and EstA) also belong to β-barrel proteins. This distinct subgroup possesses a β-barrel transmembrane domain and an enzymatically active passenger domain.

The first report of a bacterial lipase display on the *E. coli* cell surface employing an outer membrane protein (FadL) was published in 2004 (Table 1) (*36*). FadL is an OMP involved in binding and transportation of long-chain fatty acids and in binding of bacteriophage T2 in *E. coli*. The structure of this protein and its distinctive features have been described in detail previously (*77*). FadL is a monomer, with a long (approx. 50 Å) barrel composed of 14 antiparallel β strands, a number that has not been observed in other OM proteins (Table 3). The truncated *fadL* gene encoding 384 amino acids from the N-terminus was used to fuse a thermostable *Bacillus* sp. TG43 lipase (*36*). The lipase fused to Arg^{384} was exposed to the external face of the outer membrane without disturbing the β-barrel transmembrane domain. Cell surface-displayed lipase exhibited very good stability at high temperatures and retained over 90 % of the full activity after incubation at 50 °C for a week. It was also demonstrated that *E. coli* displaying lipase could be used as a whole-cell catalyst for kinetic resolution of racemic substrates. The results showed better performance of the an-

chored lipase in comparison with crude lipase or cross-linked lipase crystal, pointing out FadL as a useful anchoring motif for whole-cell biocatalysis.

C-terminally truncated *Salmonella typhimurium* OmpC (Table 3) was used to display *P. fluorescens* TliA lipase on the surface of *E. coli* (*37*). The use of strong T7 promoter inhibited successful display of lipase on the cell surface, possibly indicating the jamming of the cellular secretory machinery. Efficient anchoring of the lipase was obtained by decreasing expression efficiency, *i.e.* using the *lac* promoter. Lipase display was confirmed by immunofluorescence microscopy and the whole cell activity. It was found that the enzyme maintained most of its activity and selectivity during 10 repeated reactions over 120 hours. Overall results suggest that cell surface-immobilized lipase exhibited high enantioselectivity, broad substrate specificity and reusability, and that this strategy may be useful for displaying other enzymes as well. Applying the same strategy (*38*), an efficient display of TliA lipase was achieved using *E. coli* OmpC as an anchoring motif (Table 3). The truncated *ompC* gene encoding 288 amino acids from the N-terminus was fused to *tliA* gene encoding TliA of 426 amino acids. Based on the transcriptome profiling and knock-out experiments, the authors demonstrated that GadBC coexpression improved significantly the display of the OmpC-TliA fusion protein (Table 1). This effect was attributed to the fact that GadBC proteins are involved in stress responses that could be triggered by increased protein export during cell surface display of lipase (*38*).

The outer membrane protein X (OmpX) was selected as a potential anchoring motif based on the result of the proteome analysis (*39*). This protein is small, monomeric and highly expressed (Table 3). OmpX possesses four extracellular loops protruding from the cell surface (*80,81*). Two different positions in the external loops (Lys^{122} in loop 3 and Val^{160} in loop 4) were examined for efficient TliA surface display (*39*). Lipase fused to Lys^{122} (loop 3) showed slightly higher activity than the one fused to loop 4. In accordance with this, another enzyme, endoxylanase, also displayed better activity when fused to Lys^{122}. This demonstrated that a relatively short anchoring motif is more suitable for efficient translocation and anchoring of passengers to the outer membrane. However, display efficiency of the smaller enzyme (endoxylanase, 23 kDa) was higher than that of lipase (50 kDa). Another report also demonstrated that OmpX-fused display system could efficiently accommodate and display small peptides on the cell surface (*80*).

Additional small and highly expressed OMP, OmpW, was successfully used to express functional TliA on the surface of *E. coli* (*40*) (Table 3). Biological role of OmpW is not well known; it acts as a colicin S4 receptor and may be involved in protecting bacteria against various environmental stresses. In contrast to OmpX and other OMPs, OmpW is not randomly distributed on the entire cell surface but it localizes at the poles (*39*). Based on the available structural data, Lee *et al.* (*40*) selected two fusion sites in OmpW to display TliA. Both Gly^{139} and Ala^{191} were suitable for lipase surface display and none of the recombinant cells exhibited retarded growth. However, $OmpW^{Gly139}$-TliA fusion resulted in higher lipase activity. The authors demonstrated that the displayed lipase can be employed in enantioselective hydrolysis and transesterification reactions in aqueous and organic media over prolonged period (5 days) at a rather high reaction temperature (47 °C). Interestingly, Yim *et al.* (*39*) reported pronounced decrease of the outer membrane integrity and severe cell lysis when using OmpW for TliA display. Discrepancies in these results could possibly be ascribed to the selection of different fusion points. However, it is not possible to reach the final conclusion since Yim *et al.* (*39*) did not report details of their cloning sites.

A major OMP from *Pseudomonas aeruginosa*, OprF (Table 3) was also used to display TliA on the cell surfaces of *E. coli* (*41*) and *P. putida* (*43*). The structure of OprF has been proposed to consist of three domains, the N-terminus forming a β-barrel, a loop or hinge region, and a C-terminus associated with the peptidoglycan layer. Lee *et al.* (*41*) analyzed four potential fusion sites (Lys^{164}, Val^{188}, Ala^{196} and Arg^{213}) for TliA display in *E. coli*. Growth defects were observed when passenger was fused to Ala^{196} and Arg^{213}, most likely due to the membrane instability observed frequently during cell surface display. In contrast, host cells (*E. coli* or *P. putida*) did not exhibit growth defects when Lys^{164} and Val^{188} were used as fusion sites (*41,43*). Interestingly, while Val^{188} was found to be the optimal fusion site for displaying lipase in *E. coli*, the highest whole-cell lipase activity in *P. putida* was obtained when TliA was fused to Lys^{164} (Table 1). In addition, the lipase activity measured in *P. putida* was more than fivefold higher than in *E. coli* displaying TliA. The authors suggested that this could have been ascribed to the fact that both OprF and TliA proteins originated from *Pseudomonas* strains. Furthermore, cell surface-displayed lipase in *E. coli* showed the highest activity at 37 °C and at pH=8.0, while in *P. putida* the highest activity was observed at 47 °C and at pH=9.0. This result was similar to that obtained with the free enzyme. Most importantly, lipase displayed on the surface of *P. putida* showed much higher stability in organic solvents, retaining over 90 % of its initial activity at 47 °C for a week. In comparison, lipase displayed on the surface of *E. coli* retained approx. 80 % of its initial activity after a one-week exposure at 37 °C. Enantioselective resolution of 1-phenyl ethanol in hexane was also much better with TliA displayed on the surface of *P. putida* because the reaction could be carried out at 47 °C without inactivation of the whole-cell enzyme. Based on these results, it can be expected that *Pseudomonas* cell surface display system (OprF) will be used in a wide range of biocatalytic applications (*43*).

PgsA from *B. subtilis* is a component of poly-γ-glutamate (PGA) synthetase complex (Table 3) (*102*). Transmembrane domain of PgsA is located at the N-terminus while the C-terminus is predicted to be exposed at the outer side of the cell wall of Gram-positive bacteria. Interestingly, Narita *et al.* (*42*) used PgsA as an anchor protein to display enzymes on the surface of a Gram-negative bacterium, *E. coli*. Industrially important lipase B (CALB) from *Candida antarctica* was successfully displayed on the cell surface. This 34-kDa enzyme was produced and accumulated gradually during *E. coli* growth, reaching maximum activity in the stationary phase (36 h). On the contrary, the authors could not express the free CALB

in *E. coli*. Functional expression of CALB fused to PgsA suggested that this anchor protein facilitated proper folding of CALB. *E. coli* displaying lipase was used for enantioselective transesterification reaction. The yield and enantiomeric excess were similar to the results obtained previously with CALB immobilized on the yeast cells (*57*), demonstrating that *E. coli* cells have a potential as whole-cell biocatalysts for the production of various chemicals. In addition, PgsA anchoring system also proved to be efficient in functional anchoring of a very large enzyme, α-amylase (AmyA, 77 kDa) from *Streptococcus bovis* on the surface of *E. coli* (*42*).

High-throughput screening of enzymes displayed on the bacterial surface

Efficient screening of large clone libraries created by directed evolution is crucial for a successful selection of functionally improved enzyme variants. Here we describe more recent approach that employs biotin tyramide ester as a substrate for the enzyme of interest displayed on the cell surface, subsequently decorated by covalent conjugation with horseradish peroxidase (HRP). Only cells that express active enzymes can hydrolyze an ester substrate to release biotin tyramide that, in turn, can be activated by HRP. This radical then spontaneously couples to random tyrosine residues found in the cell surface proteins. Therefore, only bacteria with the surface-exposed esterase activity could be selectively labelled with biotin. Such cells could be easily isolated from a very large background of inactive cells by using streptavidin-coated magnetic beads (*8*). In recent years, immunofluorescence staining and FACS analysis has become a powerful technique to analyze efficiency of protein cell surface display (*32*) and a high-throughput screening tool for identification and isolation of enzyme variants from large libraries (*6*). FACS technology allows fast evaluation of mutant proteins from PCR-generated libraries (10^8 cells) within few hours (*7*). Therefore, coupling of *E. coli* display systems with FACS provides an invaluable tool for screening lipolytic enzyme libraries. Active cells with deposited biotin label can be identified and isolated by FACS after labelling with fluorophore-carrying streptavidin. Sensitivity of this method was tested by using error-prone PCR-generated LipA variants exposed on the cell surface of *E. coli* (*8*). Extent of biotin tyramide deposition corresponded to esterase activity, *i.e.* the cells that had fewer biotin molecules deposited on their surface coupled less streptavidin-conjugated fluorophore. Based on this result, a high-throughput screening technique was applied for identification and isolation of enantioselective EstA variants obtained by error-prone PCR (*7*).

Architecture of the Fungal Cell Surface and Systems Used to Display Lipolytic Enzymes

Unlike bacterial peptidoglycan, fungal cell walls have an asymmetrically layered structure with an inner layer composed of β-1,3-glucan, a branched polymer containing on average 1500 glucose monomers. It forms strong helical structures capped by short chitin chains at the non-reducing ends, providing the mechanical stabilisation to the cell (*103,104*). The outer layer of the wall is composed of mannoproteins, most of which are both O- and N-glycosylated. The number of *Saccharomyces cerevisiae*, and most probably other yeast species and genera, cell wall mannoproteins is quite high. More than 30 different proteins have been extracted from the wall and the *in silico* survey indicates that the number could be as much as twice higher (*105*).

There are at least three and most probably four different ways by which yeast cell wall proteins are linked to β-1,3-glucan. One group of proteins is simply non-covalently adsorbed to β-1,3-glucan chains. Other proteins are covalently bonded in one of the two ways. Some proteins are linked to β-1,3-glucan through glycosylphosphatidylinositol (GPI) anchors and β-1,6-glucan, while the other group, so called Pir protein family, is bound through ester linkages formed between particular glutamates contained in characteristic repeating units and glucose (Fig. 2). Recently, it has been found that Scw4 and Scw10, two proteins previously identified among the non-covalently attached cell wall proteins, are also partly covalently linked to β-1,3-glucan by a novel, so far unexplained linkage. The reason for the existence of different attachment mechanisms and the way they are related to the function(s) of the proteins has still not been revealed, to a large extent due to the lack of general understanding of their physiological roles.

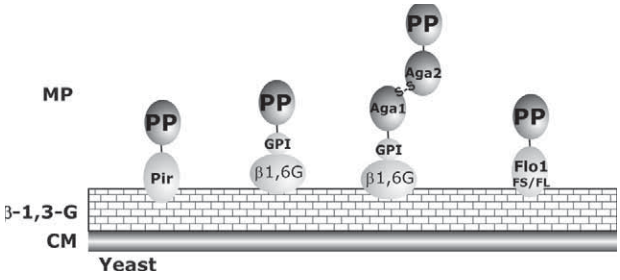

Fig. 2. Effective systems for the display of lipolytic enzymes on the cell surface of yeasts. CM=cytoplasmic membrane, β-1,3-G=β-1,3-glucan, MP=mannoproteins, β-1,6G=β-1,6-glucan, GPI=glycosylphosphatidylinositol, PP=passenger protein

Layered structure of the yeast cell wall and the external localization of mannoproteins motivated the development of systems for heterologous expression of proteins in a way that they mimic autochthonous cell wall proteins. Therefore, the strategy for surface display in yeasts does not differ markedly from the one used in bacteria. Clearly, as in bacteria, the achievement of this goal depends on our knowledge of the mechanisms and internal signals leading to incorporation of wall-resident proteins.

Non-covalent attachment of proteins in the cell wall

In this chapter we shall only briefly mention the class of non-covalently attached cell wall proteins, mainly because there seem to be no particular signals leading to their incorporation into the cell wall. Rather, they are transported through the plasma membrane and released into the periplasmic space where β-1,3-glucan adsorbs

them by forming hydrogen bonds in the extent depending on the hydrogen-bonding potential of individual proteins. As a result, a number of yeast cell wall proteins can be extracted from the wall by sodium dodecyl sulphate (SDS), mercaptoethanol, dithiothreitol, or a combination of these reagents, often at increased temperature (*106–108*). Clearly, this way of cell wall protein localization has little importance for surface display since the non-covalently attached proteins are released to the surrounding medium to a great extent. The only exception is a display system involving the Flo1 from *S. cerevisiae* (Fig. 2, Table 3). Flo1 is a lectin-like cell wall protein which contains the flocculation functional domain located near the N-terminus. This part of the protein adheres non-covalently to cell wall mannans of other cells (*109*). Matsumoto *et al*. (*56*) developed a new cell-surface display system employing flocculation functional domain with insertion sites for the passenger proteins. This domain consists of the secretion signal and the 3' region of the *FLO1* gene encoding either amino acids 1 to 1099 (FS) or 1 to 1417 (FL). The N-terminus of the passenger protein is fused to the Flo1 flocculation domain and the recombinant protein is expressed under the control of the appropriate inducible promoter. Both systems (FS and FL) showed to be effective for immobilization of passenger proteins whose catalytic sites are near their C-termini.

The most exploited fungal lipase for biotechnological purposes is lipase B from *Candida antarctica* (CALB). CALB is composed of a prepro-region (a signal peptide of 18 and a propeptide of 7 amino acid residues) and a mature region of 317 amino acid residues. It belongs to the hydrolase family with a Ser–His–Asp catalytic triad in its active site (*110*). CALB exhibits high substrate selectivity (regioselectivity and enantioselectivity) so it is one of the lipases frequently used in the organic synthesis. However, its bulk industrial application is limited by the high cost of commercially available immobilized CALB preparations. CALB–displaying whole-cell yeast biocatalysts have been constructed for use in many different processes (production of flavour and fragrance esters, polyesters, biopolymers of lactic acid, biodiesel, *etc.*), and extensive research has focused on the application of genetic engineering techniques resulting in increased whole-cell biocatalytic activity and stability. Cell surface display systems in *S. cerevisiae*, and lately also in *P. pastoris*, have been established, using most frequently fragments of *S. cerevisiae* GPI-anchored cell wall proteins α-agglutinin or Flo1 as the anchor for immobilization of heterologous proteins (Fig. 2).

Tanino *et al*. (*53*) isolated lipase B from *C. antarctica* CBS 6678 that exhibited high hydrolytic and ester synthesis activities, and constructed CALB-displaying yeast whole-cell biocatalysts using the Flo1 short (FS) anchor system. A comparison of the lipase hydrolytic activities of yeast whole cells displaying only mature CALB and those displaying CALB with a Pro region (ProCALB) revealed that mature CALB is preferable for yeast cell surface display.

Tanino *et al*. (*54*) constructed a yeast whole-cell biocatalyst by integration of four CALB cell-surface display expression cassettes into the genome. The authors used Flo1 flocculation domain as anchor, in a- and α-type haploid cells of *S. cerevisiae*. Diploids gained by mating contained eight copies of the CALB cell-surface expression cassette and reached ninefold higher lipase activity compared to the haploid CALB displaying yeast using a multi-copy plasmid (*53*). Moreover, this whole-cell biocatalyst could repeatedly catalyze the polyester (polybutylene adipate) synthesis reaction with reduced by-product production.

The hydrolytic activity of the whole-cell biocatalyst bearing CALB immobilized by α-agglutinin system (see later, *50*) was determined to be 17 U per g of dry cells. The lipase hydrolytic activity of the yeast whole cells displaying CALB immobilized by Flo1 short anchor system (FS) was 20.4 U per g of dry cells (*53*). Diploid yeast cells containing genome integrated eight copies of the CALB cell-surface expression cassette (using FS) reached the highest hydrolytic activity of 117 U per g of dry cells, while in both a- and α-type yeast cells, with the two copies of the CALB cell-surface display expression cassette introduced, reached 43.6 and 32.2 U per g of dry cells, respectively (*54*). According to this, Flo1 system showed somewhat better results than α-agglutinin system for immobilization of CALB, but the best results were gained with genome-integrated cell-surface expression cassette using FS. Activity was further increased with increased number of genome-integrated copies of cell-surface expression cassette (*49*).

The *Rhizopus oryzae* lipase (ROL) possesses high structural and sequential similarities to other fungal lipases, and has been studied extensively. ROL is generally considered as safe for human oral use so it could be used for food-producing processes, as well as effective biocatalyst in industrial bioconversion processes (resolution of enantiomers, enantioselective transesterification and stereospecific hydrolysis) in pharmaceutical and chemical industry (*111*). Matsumoto *et al*. (*56*) fused the N-terminus of ROL including a pro-sequence (ProROL) to the Flo1 flocculation functional domain (FS or FL, respectively) and studied the applicability of cells displaying active ProROL for biodiesel production in a solvent-free and water-containing system. Results showed that the displayed FS-ProROL had the same accessibility to substrates as free, soluble enzymes and no treatment was needed to enhance methanolysis reaction. Investigation of the applicability of this whole-cell biocatalyst to non-aqueous enantioselective transesterification (*57*) showed that ProROL-displaying yeast whole-cell biocatalyst could catalyze enantioselective transesterification in non-aqueous organic solvent. Nakamura *et al.* (*55*) displayed ROL on the cell surface of *S. cerevisiae via* the Flo1 N-terminal region. The ROL-displaying yeast catalyzed optical resolution of the (*R,S*)-1-benzyloxy-3-chloro-2-propyl monosuccinate and showed stable activity through at least eight reaction cycles.

The lipase genes of *Yarrowia lipolytica*, *LIP7* and *LIP8*, fused to *FLO1* flocculation domain sequence from *S. cerevisiae* at their N-termini, were expressed in *P. pastoris* (*71*). The surface-displayed lipases showed slightly higher stability against pH variation and DMSO treatment than the secreted proteins, but the thermal stability of the immobilized enzymes was reduced. These lipases can

be cleaved off from their anchor by enterokinase treatment to yield functionally active enzymes in the supernatant.

Jiang *et al.* (*64*) expressed and displayed lipase LipB52, isolated from *Pseudomonas fluorescens* B52, on *P. pastoris* and *S. cerevisiae* cell surface respectively, utilizing flocculation functional domain of Flo1 protein anchoring system. Results showed that LipB52 displayed on *P. pastoris* and *S. cerevisiae* exhibited similar substrate specificity, hydrolysis activity, transesterification activity, optimal temperature and pH. However, the LipB52 displayed on the *P. pastoris* exhibited better stability than the one displayed on *S. cerevisiae* cell surface and about five times higher concentration of cells. According to that, lipase displayed on *P. pastoris* showed to be more suitable for use as a whole-cell biocatalyst than that displayed on *S. cerevisiae* cell surface.

Yarrowia lipolytica can survive in oily wastewater for a long time, so it can be directly added into activated sludge used for oily wastewater treatment. Since the lipase activity secreted by *Y. lipolytica* is too low for effective oil degradation, the heterologous lipase lipRS from the *Rhizopus stolonifer* has been displayed on *Y. lipolytica* cell surface using the flocculation functional domain of Flo1 from *S. cerevisiae* as the protein anchor (*73*). The lipase-displaying yeast proved effective for whole-cell oily wastewater treatment.

Finally, *Rhizomucor miehei* lipase (RML) was efficiently displayed at the yeast surface using both the Flo1 and the α-agglutinin (see later) systems. To increase the activity of RML in organic solvent, Han *et al*. (*72*) created enzyme variants by site-directed mutagenesis. The obtained proteins were surface-displayed on *P. pastoris* by fusion to Flo1 as the anchor protein.

Covalent attachment of proteins to β-1,3-glucan via GPI anchors

To achieve a successful, stable surface display of a homologous or a heterologous protein, a method for its covalent incorporation into the wall had to be developed. This implied that comprehension of reactions leading to covalent linkages between cell wall autochthonous proteins and glucan was required. When cell walls from which non-covalently attached proteins had previously been removed were treated with different glucanase preparations, a set of proteins was released enabling their structural analysis (*107,112*). Most of them were found to carry a remnant of the glycosylphosphatidylinositol (GPI) anchor at the C-terminus (*113,114*). GPI anchors are assembled by a sequence of reactions occurring at the luminal side of the ER and then transferred to proteins containing distinct GPI-anchoring signals. Such signals are composed of C-terminally located amino acid sequences consisting of about 10–15 hydrophobic amino acids, followed by about 10 amino acids of moderate polarity (*115–117*). The transfer is accomplished by the proteolytic removal of the signal amino acids with the concomitant transamidation between the carboxyl group of the new C-terminal amino acid, and the ethanolamine phosphate of the GPI anchor. It has been found that there are several structural preferences for this reaction so that the attachment amino acid is typically G, A, S, N or D and that there is usually G, A or S at the position +2 from the attachment site. Membrane GPI-anchored proteins are transported along the secretory pathway eventually leading to their exposure at the outer side of the plasma membrane. Up to this level, the process is not significantly different from the one in higher eukaryotes. In yeasts, however, another reaction takes place consisting of a postulated transglycosidation transferring part of the GPI anchor carrying the passenger to β-1,6--glucan (*118,119*). The enzyme conducting this reaction has not yet been found. Besides, it is worth noting that not all GPI-anchored proteins undergo this reaction. Several GPI-anchored proteins, usually having a particular dibasic motif (STOP transfer) next to the GPI attachment site, remain anchored in the membrane (*120*). Some proteins even have dual localization depending on their site in the cell wall. Although *in silico* survey revealed more than 50 genes in *S. cerevisiae* putatively coding for GPI--anchored proteins (*105*), only about half of them have actually been found in the cell wall either because some of them were present in low amounts, or due to specific transcription regulations of their genes (*121*). Although for many GPI-anchored proteins their physiological role remains obscure, members of this class studied in more detail indicate that there is no universal role of these proteins (for recent reviews see *122–125*). GPI proteins used as sources of GPI anchoring signals for surface display of heterologous proteins are α-agglutinin, Sed1, and Cwp2 from *S. cerevisiae* and Gcw21 from *P. pastoris* (Table 3).

Structural studies have revealed that the C-terminal GPI-anchoring signal alone is sufficient for the attachment of GPI to any protein directed to ER, *i.e.* to the secretory pathway. Since the transglycosylation reaction transferring the GPI protein to β-1,6-glucan seems to be dependent only on the lack of a dibasic 'stop-transfer' motif, it is most likely that the final destination of such an engineered passenger protein would be the external mannan layer of the wall. Therefore, GPI anchoring signal seems to be a handy genetic element for protein surface display since it is small and therefore not likely to influence the protein conformation. Besides, it is removed from the sequence of the mature protein in the process of anchor attachment. Indeed, there have been several successful attempts to express and localize different proteins in this way. Here, we shall focus on lipolytic enzymes only.

Kato *et al*. (*51*) constructed a mutated version of the already mentioned *C. antarctica* lipase B (mCALB) on the basis of the primary sequences of CALBs from *C. antarctica* CBS 6678 strain and from *C. antarctica* LF 058 strain, in which the first conserved glycine in the catalytic motif GXSXG was replaced by a threonine to give TWSQG. mCALB displayed on yeast cell surface by α-agglutinin GPI-anchoring signal showed a relatively high thermal stability and high preference for short chain fatty acids, which is an advantage in some applications like in the production of flavours.

Inaba *et al.* (*50*) constructed the whole-cell biocatalyst displaying CALB on the yeast cell surface using α--agglutinin anchor system. The lyophilized CALB-displaying yeasts showed their original hydrolytic activity

and were also utilized for ester synthesis using ethanol and L-lactic or D-lactic acid as substrates in water-saturated heptane. CALB-displaying yeasts successfully catalyzed ethyl lactate synthesis, suggesting that they can supply the enantiomeric lactic esters for preparation of improved biopolymers of lactic acid (*50*).

To improve the CALB activity on the yeast surface, Han *et al.* (*49*) introduced an artificially synthesized celAL linker (based on *Neocallimastix patriciarum* cellulase A (celA) with a FLAG peptide at the C-terminus) between the CALB and α-agglutinin anchor. This linker is rich in Asn residues, and contains only a few Ser residues and one potential N-glycosylation site, so it is not likely to be heavily glycosylated. *S. cerevisiae* cells displaying the hybrid protein were successfully employed to catalyze the esterification of hexanoic acid and ethanol to ethyl hexanoate (a typical fragrance compound of Chinese liquor and Japanese sake) in *n*-heptane, and showed to be promising in industrial production due to good operational and storage stability.

To develop a high efficiency yeast display system for CALB, Sun *et al*. (*52*) fused two copies of CALB gene with *S. cerevisiae* genes coding for GPI-bound Sed1 and the C-terminal half of Sag1 (α-agglutinin), linked by a 2A peptide of foot-and-mouth disease virus (FMDV) in a single open reading frame. FMDV encodes all its proteins as a single open reading frame in which individual genes are separated by 2A peptides. This sequence drives programmed translation in which the protein synthesis is terminated after reaching one codon before the last and then continues with the synthesis of the next protein starting with the last codon of 2A. Therefore, this genetic system provides a handy tool for coexpression of genes using a single promoter. The obtained results showed that the fusion product was translated to give two independent proteins displayed on the cell surface: CALB-Sed1 and CALB-Sag1. The heterogeneous co-displaying yeast system mediated by FMDV 2A peptide resulted in the improved efficiency of enzyme-displaying yeast cells.

Some of the disadvantages of heterologous protein production in *S. cerevisiae* are sometimes low production yields even with strong promoters, intracellular or periplasmic localization of proteins larger than 30 kDa and hyperglycosylation of proteins that may affect the protein characteristics (*126*). Furthermore, *S. cerevisiae* uses fermentation metabolism, so in the high-cell-density culture the concentration of ethanol readily reaches toxic levels and limits the cell growth and production of heterologous protein. Opposite to *Saccharomyces* strains, *P. pastoris* using respiration metabolism can grow at high cell densities without significant accumulation of ethanol and can produce higher concentration of heterologous proteins (*126*). Su *et al*. (*67*) compared the activity and stability of CALB displayed on the cell surface of *P. pastoris* and *S. cerevisiae* using α-agglutinin GPI signal and the FS sequence as anchor, respectively. The activity of CALB displayed on *P. pastoris* was tenfold higher than that displayed on *S. cerevisiae*. Although the hydrolytic activities of lipases displayed on yeast cell surface were similar (200 U per g of dry cells for α-agglutinin and 270 U per g of dry cells for FS system), the synthetic activity and catalytic efficiency of CALB fused with α-agglutinin showed to be threefold higher than that of the FS fusion protein when applied to the synthesis of ethyl caproate and other short-chain flavour esters. CALB-displaying *P. pastoris* cells using α-agglutinin anchored system were shown to be a robust biocatalyst for large-scale industrial production of flavour esters (*66*). Using the optimal conditions, the synthesis of 12 short-chain flavour esters, obtained from substrates of different structures, was scaled up in a stirred batch reactor. The CALB-displaying *P. pastoris* cells showed good thermal stability and tolerance of high substrate concentrations and solvent. The biocatalyst was used for 10 consecutive batches of esterification, with less than 10 % loss of activity.

In addition to α-agglutinin display system, some other yeast cell wall proteins were used as the anchor for immobilization of CALB at the surface of *P. pastoris*. Su *et al.* (*68*) constructed heterologous protein CALB-Sed1, using *S. cerevisiae* GPI-anchored cell wall protein Sed1 as the anchor, and expressed it under the control of the alcohol oxidase 1 promoter (pAOX1). The fusion protein CALB-Sed1 was highly glycosylated and exhibited a higher thermostability than free CALB. Jin *et al*. (*69*) displayed CALB on the *P. pastoris* surface using a fragment of *P. pastoris* GS115 GPI-anchored cell wall protein Gcw21 as the anchor. Lipase-displaying whole cells exhibited good performance and storage stability, giving high yields of methyl ester from various feedstock including waste oils.

Washida *et al*. (*58*) fused the gene encoding ROL with the genes encoding the pre-α-factor leader sequence (secretion signal), and the C-terminal half of α-agglutinin including the GPI anchor attachment signal. The constructed gene was overexpressed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. Linker peptides (Gly/Ser repeat sequence) of different length (2 to 17 amino acids) were inserted at the C-terminal portion of ROL in order to preserve the conformation of the active site that is placed near the C-terminus. The ROL displayed on the yeast cell wall exhibited activity towards soluble 2,3-dimercaptopropan-1-ol tributyl ester (BALB) and insoluble triolein that was equal or even higher than the activity of secreted ROL. The activity towards triolein was the highest in constructs with linker peptides containing 17 amino acids, while constructs containing 14 amino acids long linker peptide showed the highest activity towards BALB.

In order to improve the ROL activity on the yeast surface, Shiraga *et al.* (*59*) used error-prone PCR technique to obtain enzyme variants with two or three nucleotide substitutions on average per coding sequence. The mutated ROL fragments were fused to a sequence encoding the C-terminal half of yeast α-agglutinin and expressed in *S. cerevisiae*. Results showed that five out of the 13 mutants selected had three- to sixfold higher activities than the wild type ROL-displaying yeast, while the selectivity for chain length was not changed. In heptane, the activity of the displayed ROL was 33-fold higher than the activity in aqueous solvents and $4.4 \cdot 10^4$-fold higher than the activity of the free ROL in organic solvents. Furthermore, Chen *et al.* (*60*) optimized and synthesized the cDNA sequence of ROL according to the codon bias of *S. cerevisiae*, and displayed it on the *S. cerevisiae*

cell surface using α-agglutinin as an anchor. Yeast cells displaying codon-optimized ROL showed 12.8-fold higher activity than wild type ROL-displaying yeasts.

Lipase Lip2 from *Yarrowia lipolytica* was displayed on the cell surface of *S. cerevisiae* using Cwp2 as an anchor protein (*62*). The displayed lipase did not lose any activity after being treated with 0.1 % Triton X-100 and 0.1 % Tween 80 for 30 min, and it retained 92 % of its original activity after incubation in 10 % DMSO for 30 min. It also exhibited better thermostability than free Lip2. Finally, Zhang *et al.* (*65*) displayed *Rhizomucor miehei* lipase (RML) on the surface of *S. cerevisiae*. Cell surface displaying RML-α-agglutinin lipase showed the highest activity at 45 °C and at pH=8.0.

A particular way of covalent immobilization of heterologous proteins in the yeast cell wall employs yeast a-agglutinin. *S. cerevisiae* a-agglutinin is a cell wall protein consisting of two subunits. The bigger, Aga1, is anchored to the cell wall *via* GPI anchor, while the smaller, Aga2, is linked to Aga1 through disulphide bridges. Thus, fusing the passenger to Aga2 would eventually lead to its exposure at the cell surface (Fig. 2). Liu *et al.* (*61*) fused *LIP7* and *LIP8* with *AGA2* in order to immobilize corresponding lipases at the *S. cerevisiae* surface (Table 3). Deletion of the putative signal sequences of Lip7 and Lip8 resulted in decreased activities of immobilized enzymes. The activities towards *p*-nitrophenyl caprylate were shown to be much higher than those using Flo1 as anchor protein in *P. pastoris* (*71*). The displayed lipases exhibit a preference for medium-chain fatty acids and a high thermal stability.

Geotrichum sp. lipase (GSL) was also displayed on *P. pastoris* using a-agglutinin (Aga2 subunit) anchor system from *S. cerevisiae* (*70*). The GSL-displaying yeast whole cells were used to enrich eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the fish oil by selective hydrolysis.

Aspergillus niger lipase (ANL), a widely used hydrolase, was displayed on the surface of *S. cerevisiae* using a-agglutinin (Aga2 subunit) as an anchor protein (*63*). The cells showed good thermostability, retaining almost 80 % of the full activity after incubation at 60 °C for 1 h, and >80 % of the full activity at 50 °C for 6 h. The displayed lipase showed a preference for medium-chain fatty acid esters, the optimal temperature of 45 °C, and the pH=7.0.

Covalent attachment of Pir proteins

A treatment of yeast cell walls with mild alkali results in the extraction of four proteins encoded by the *PIR* gene family (Proteins with Internal Repeats) (*107*). All four proteins share high sequence homology and contain a characteristic N-terminal motif of 12 amino acids (SQIGDGQVQATS), which occurs once in Pir4 but repeats between seven and eleven times in other Pir family members (Pir1–3). Deletion analysis of Pir4 revealed that the characteristic Pir motif was required for the covalent attachment of the protein in the wall since the protein lacking these 12 amino acids was secreted into the medium. Mutagenesis of individual amino acids showed that the substitution of any of the three glutamines, or the aspartic acid resulted in the lack of protein binding to the wall (*127*). Glutamine residue in the middle of the motif (Q_{74}) was identified as the actual attachment site and the putative reaction in which deamination of Q_{74} with the concomitant formation of the alkali-labile ester bond with one of the hydroxyl groups in β-1,3-glucan took place (*127*).

Unlike GPI anchors, Pir repetitive sequences are not sole requirements for the incorporation of proteins in the cell wall and their addition to the N-terminus of another protein would not lead to its covalent attachment to glucan. Actually, only the complete Pir proteins in native conformation were found to be efficiently linked (*128*). Therefore, to achieve a successful surface display of a protein through its N-terminus, a chimeric protein consisting of the whole Pir and the desired passenger protein has to be created (Fig. 2). This approach has led to successful immobilization of a number of proteins to *S. cerevisiae* and *P. pastoris* cell walls but to our knowledge no lipolytic enzymes have been immobilized to the cell surface this way (Table 3).

All lipolytic enzymes displayed at the yeast cell surfaces are listed in Table 2.

Conclusion

Conventional methods for immobilization of proteins on solid carriers frequently result in undesired changes in conformation, resulting in altered biochemical characteristics of immobilized proteins due to often unsuitable chemical treatments during the immobilization process. Activity, as well as stability of an immobilized protein is often decreased, diminishing the advantages of the protein immobilization. On the other hand, binding of proteins through non-covalent interactions is less threatening to the protein conformation, but proteins immobilized in this way are easily dissociated and subsequently lost during application. These disadvantages can be circumvented when proteins are immobilized at the microbial cell surface by the cell itself, using standard reactions for incorporation of autochthonous cell surface proteins. In this way, protein conformation is usually preserved and the stability of surface-displayed protein is often higher than the one of the soluble form. The variety of reactions by which proteins can be displayed at the cell surface of a microorganism enables finding an appropriate way for each protein of interest. Besides, there are examples showing that more than one protein can be displayed at the surface of a cell, indicating a possibility that strains capable of carrying out complex chemical transformation consisting of more reactions, or requiring whole biochemical pathways will soon be constructed. Alternatively, mixed cultures consisting of microbial strains displaying different enzymes at their surfaces could be used in such complex situations.

In each case, an ever increasing number of papers dealing with this topic clearly show how basic knowledge gained on cell biology and biochemistry of the microbial cell walls can be successfully employed for biotechnological applications. Considering the practical importance of lipolytic enzymes, it is of no surprise that they are a frequent example of protein display at microbial cell surfaces.

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