Orthogonal Surface Tags for Whole-Cell Biocatalysis

*Theo Peschke, Kersten S. Rabe, and Christof M. Niemeyer**

Abstract: *We herein describe the engineering of E. coli strains that display orthogonal tags for immobilization on their surface and overexpress a functional heterologous "protein content" in their cytosol at the same time. Using the outer membrane protein Lpp ompA, cell surface display of the streptavidin binding peptide, the SpyTag/SpyCatcher system, or a HaloTag variant allowed us to generate bacterial strains that can selectively bind to solid substrates, as demonstrated with magnetic microbeads. The simultaneous cytosolic expres sion of functional content was demonstrated for fluorescent proteins or stereoselective ketoreductase enzymes. The latter strains gave high selectivities for specific immobilization onto complementary surfaces and also in the whole cell stereospe cific transformation of a prochiral C_S symmetric nitrodiketone.*

The selective immobilization of bacterial cells on solid surfaces is of paramount interest for a broad range of applications in biocatalysis,[1,2] biosensing,[3] or wastewater purification.[4] Numerous methods have been explored for bacterial cell immobilization,[1,5] which are based on either encapsulation into hydrogels and polymers[6] or adsorption onto porous and inert support materials.[7] Whereas the former approach primarily aims to protect bacteria from hostile environments, the latter is employed when efficient mass transport between solutes and immobilized cells is required in heterogeneous reactions. The immobilization of microbial cells is highly advantageous for continuous bio reactor processes because it enables a constant growth milieu as well as stabilized conditions, high cell densities, and reusability for efficient and economic production of fuels and chemicals.[8,9] As simple chemical immobilization through covalent coupling or non covalent adsorption can lead to cell damage and reduced biological activities or unstable cell attachment, respectively, directional methods are currently explored. These methods usually depend on the prior surface immobilization of specific binders for a bacterial strain, such as antibodies[10] or genetically engineered cell wall binding domains,[11] or require the attachment of a synthetic tag to the bacterial cell wall, such as oligonucleotides.[12]

The so called cell surface display technique provides an elegant means for the directional attachment and presenta tion of peptides and proteins on microbial cell walls by means of genetic fusion to generic membrane anchor motifs of the host, such as the outer membrane protein Lpp ompA.[13] Whereas cell surface display is extensively used for the directed evolution of polypeptide binders for vaccination[14,15] and biocatalysis,[16,17] only few reports concern its application for the specific immobilization of host cells on solid sub strates. The prior work includes cell surface anchored cellu lase or cellulose binding domains,[18] chitin binding domains,[19] peptide tagged amyloid proteins,[20] "sticky" cat echolamine moieties,[21] or cucurbituril binding peptides,[22] thereby nicely illustrating the utility of this approach. How ever, to the best of our knowledge, cell surface display has not yet been used for orthogonal tagging and selective immobi lization of recombinant bacteria that contain enzymes for whole cell biocatalysis. We herein describe the engineering of *E. coli* strains that display orthogonal tags for immobilization on their surface and overexpress functional heterologous proteins in their cytosol at the same time (Figure 1).

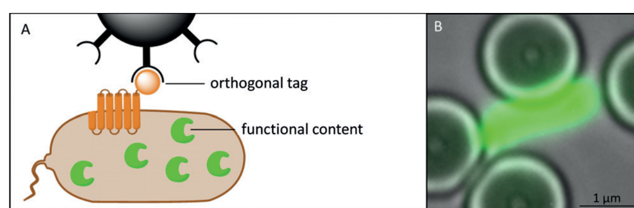

Figure 1. A) Schematic representation of an *E. coli* cell displaying orthogonal tags for immobilization on the cell surface and over expressing functional heterologous proteins in its cytosol at the same time. B) The microscopy image shows a bacterial cell that expresses YFP (green rod) and displays the SBP tag on its surface to facilitate binding to streptavidin coated magnetic microparticles.

To experimentally investigate our concept, we chose three different orthogonal tagging systems that are well established in bioconjugation chemistry but have not yet been exploited for whole cell immobilization. The 39 amino acid long streptavidin binding peptide (SBP) tag[23] binds to the protein streptavidin (STV) with high affinity and is commonly applied for chromatographic purification of recombinant proteins.[24] The SpyTag/SpyCatcher system consists of the 113 amino acid long SpyCatcher (SC) protein, which generates a covalent isopeptide bond between one of its lysine residues and an aspartate residue of the 13 amino acid long SpyTag (ST) peptide.[25] The self labeling Halo based oligonucleotide binder (HOB) tag protein (293 amino acids) forms a covalent bond with small molecule chlorohexane (CH) ligands in a similar fashion as the regular HaloTag protein, which is commonly used for imaging in cell biology.[26] HOB was genetically engineered to bind to CH ligands attached to DNA oligonucleotides and DNA nanostructures with a sig nificantly higher efficiency than Halo.[27]

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Plasmids encoding for a fusion of the bacterial membrane protein Lpp ompA that is connected to the SBP, ST, SC, or HOB polypeptide tags via a flexible GGGGS linker were cloned into the pTF16 backbone of a plasmid that carries an chloramphenicol resistance and a p15A ori and for which the protein expression is tightly controlled with an arabinose dependent promotor. Full details are given in the Supporting Information. Transformation of *E. coli* BL21(DE3) with the engineered plasmids led to the formation of recombinant bacteria displaying either the SBP, ST, SC, or HOB tag on their surface, which are denoted as *E.coli* SBP, *E.coli* ST, *E.coli* SC, or *E.coli* HOB, respectively, in the following.

We initially investigated the accessibility and binding selectivity of the displayed tags. To this end, a selection of fluorescent probes, namely Cy3 labeled STV, SC eGFP, and ST eGFP fusion protein or a CH derivatized 22 mer oligo nucleotide labeled with Cy5 (Cy5 F5 CH), were added to a suspension of tag presenting cells. After incubation for one hour, the cells were spun down, and the supernatant was removed. After washing, the cells were analyzed by fluores cence microscopy (Figure 2A), and quantitative data were obtained with a fluorometric microplate reader (Figure 2B). The results clearly indicated that all four tags on the cell surfaces were accessible and bound their interaction partners in a highly specific manner.

We then tested whether the surface displayed tags can be harnessed for cell immobilization on magnetic microbeads. To this end, we used microbeads coated with STV, CH ligands, or SC protein, in the following denoted as MB STVs, MB CHs, and MB SCs, respectively. MB STVs were commercially available, MB CHs were prepared from MB STVs using a biotin CH linker, and MB SCs were generated by covalent immobilization of purified SC protein onto amino reactive, epoxide coated microparticles. The functionality of the three different microbeads was confirmed by competitive binding studies using purified, complementary tagged fluorescent proteins (SBP , Halo , and ST tagged eGFP and mKate; see the Supporting Information, Figure S3). The results clearly indicated that the beads were capable of specific immobiliza tion of the complementary probes.

We then generated a number of tag presenting *E. coli* strains that also contained plasmids for overexpression of a functional heterologous protein content in their cytosol. These plasmids were based on the pET plasmid backbone, which renders them completely orthogonal to the pFT16 based immobilization plasmids as they carry an ampicillin resistance, a colE1 ori, and a T7 promotor, which is selectively inducible by IPTG.

We tested the yellow fluorescent protein (YFP) as the first model content by creating the strains YFP@*E.coli* SBP, YFP@*E.coli* ST, and YFP@*E.coli* HOB. Binding studies were carried out by incubating the cells with either the MB STV, MB SC, or MB CH beads (described above). After incubation, the beads were isolated by magnetic separation and then analyzed by fluorescence microscopy (Figure 3). It is clearly evident from the images that the three different *E. coli* strains bound only to the beads that contained the comple mentary affinity tag. Furthermore, as both the beads and the bacteria presented multiple copies of the interaction partners

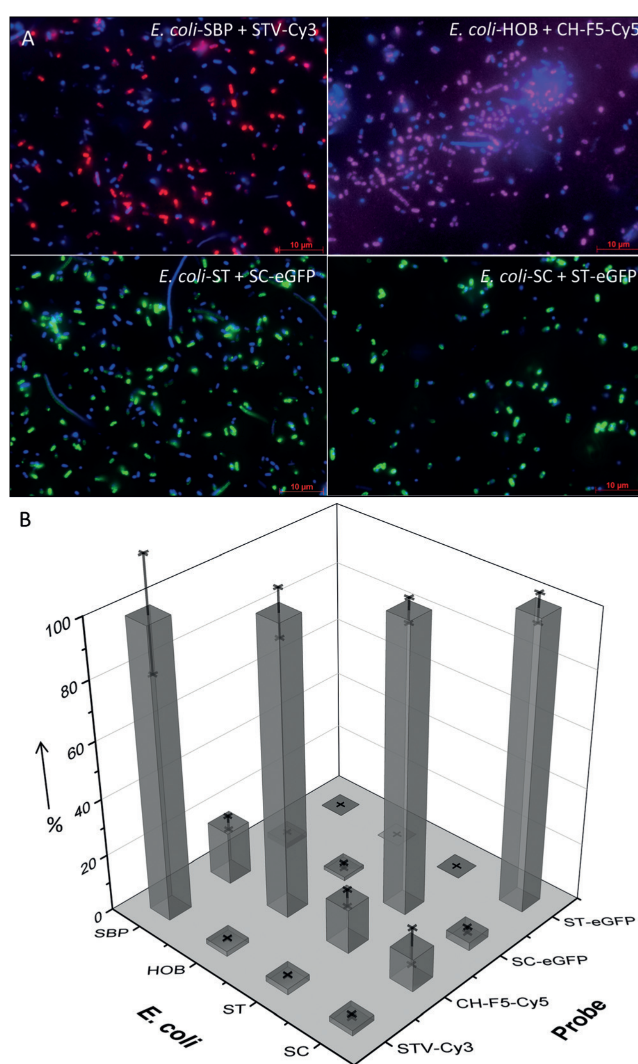


Figure 2. Selective binding of fluorescent biomolecular probes by *E. coli* cells presenting the corresponding affinity tag on their surface. Each of the four different strains was incubated with the four different probes. After removal of unbound probe, the remaining fluorescence of the cells was determined. A) Representative fluorescence micro graphs of *E. coli* strains labeled with their specific complementary probe (blue: DAPI stained bacteria, red: STV Cy3, purple: CH F5 Cy5, green: SC/ST eGFP). Scale bars: 10 μm. B) Quantitative fluorometric data. The bars indicate the relative fluorescence units (RFUs) per amount of cells normalized to the highest fluorescence signal within each set of probes. For characterization of the probes and additional fluorescence micrographs, see Figures S1 and S2, respectively.

on their surface, polyvalent binding led to the formation of cell bead aggregates (Figure S4).

As immobilized microbial cells are valuable biocatalysts for the production of chiral compounds,[2] we investigated the use of tag presenting *E. coli* strains that also contained plasmids for the overexpression of stereoselective enzymes in whole cell biocatalysis. In a previous study, we had identified several stereoselective ketoreductases (KREDs) for the reduction of the prochiral C_S symmetric nitrodiketone (NDK) **1** (Scheme 1).[28] Depending on the KRED, substrate **1** can be reduced on either one or both of the two carbonyl

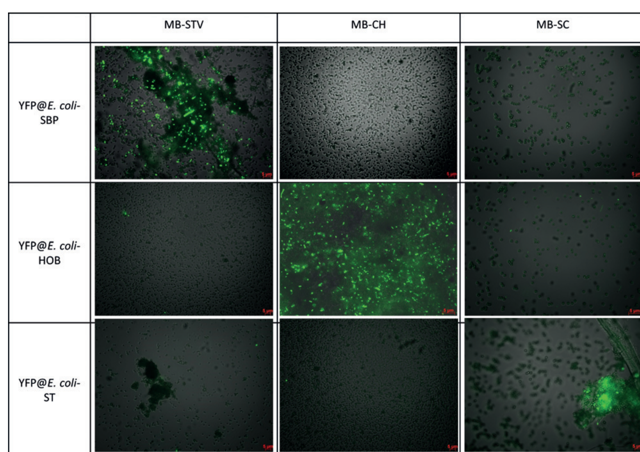


Figure 3. Cells that present an affinity tag on their surface and express the fluorescent protein YFP as a "functional content" in their cytosol at the same time can be selectively immobilized on beads bearing the corresponding interaction partner. The three different cells are harbor ing two orthogonal plasmids, which encode for a fusion protein of the membrane protein Lpp ompA and the respective tag (SBP, HOB, or ST) as well as for the YFP content (visible as green features). Selective binding to the beads leads to the formation of cell bead aggregates. Scale bars: 5 µm.

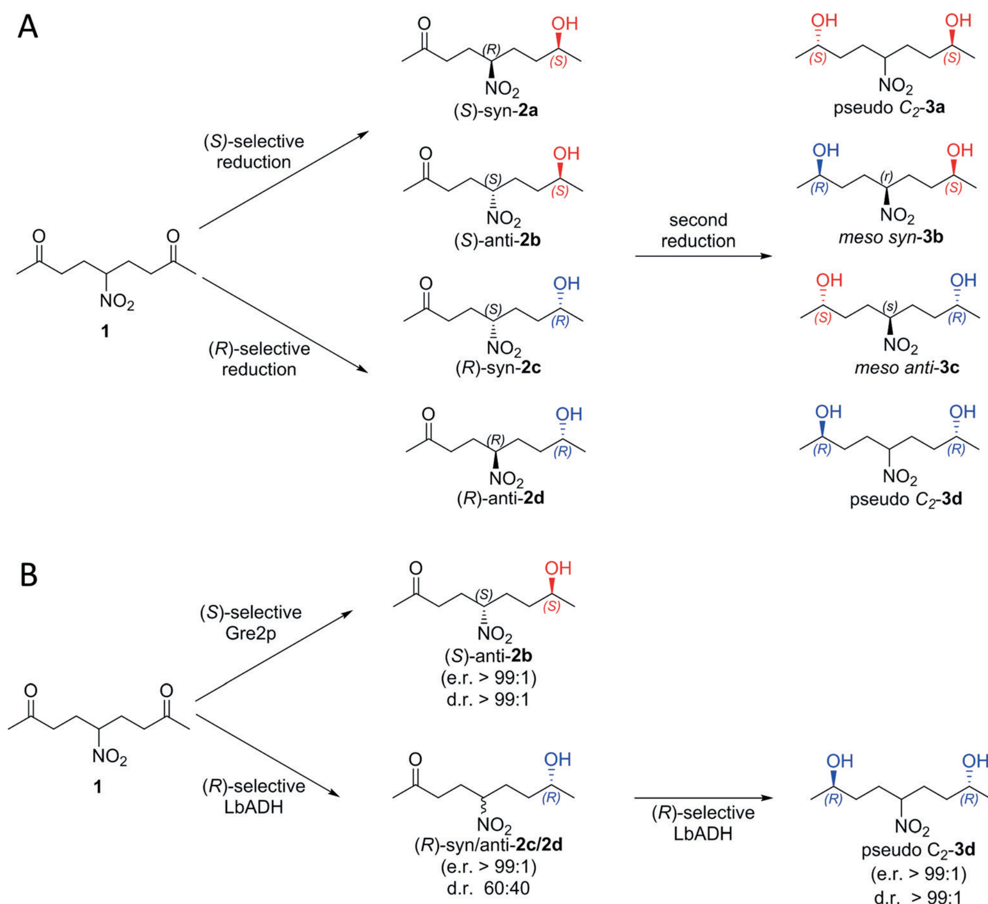
Table 1: Biocatalytic activity of self immobilizing strains.

	MB	LbADH@*E.coli*			Gre2p@*E.coli*			Products [%]		
	STV	SBP	HOB	ST	SBP	HOB	ST	*R*[a]	*S*[b]	SD[c]
1		x						> 99	< 1	< 1
2			x					> 99	< 1	< 1
3				x				> 99	< 1	< 1
4					x			< 1	> 99	< 1
5						x		< 1	> 99	< 1
6							x	< 1	> 99	< 1
7	x		x		x			4	96	4
8	x		x	x	x			6	94	1

[a] Overall amount of *R* configured hydroxyketones **2c**/**2d** and diol **3d** as determined by HPLC analysis on chiral stationary phase. [b] Only the *S* configured hydroxyketone **2b** was produced under the given reaction conditions. [c] Standard deviation, obtained from at least two inde pendent experiments.

functional groups, and all possible isomeric hydroxy ketone and diol products can be separated and iden tified by chiral HPLC anal ysis.[28] Therefore, the trans formation of **1** was ideally suited to study the activity of our self immobilizing biocatalysts.

We prepared *E. coli* strains in which the sur face displayed SBP, HOB, or ST tags were combined with either the *R* selective alcohol dehydrogenase from *Lactobacillus brevis* ATCC 14869 (LbADH) or the *S* selective alcohol dehydrogenase from *Sac charomyces cerevisiae* YJM193 (Gre2p), leading to the strains LbADH@*E. coli* SBP, LbADH@*E.coli* HOB, LbADH@*E.coli* ST, Gre2p@*E.coli* SBP, Gre2 p@*E.coli* HOB, and Gre2 p@*E.coli* ST, respectively (Table 1). To test whether the tag display affected the biocatalytic properties, we first determined the whole cell enzymatic activity of the KRED content by chiral HPLC analysis (Table 1, entries 1 6 and Table S2). In the case of cells expressing the enzyme LbADH, NDK **1** was converted into the *R* configured hydroxyketones **2c**/**2d** and diol **3d** with a diastereoselectivity of > 99%, as determined by chiral HPLC analysis (entries 1 3). In contrast, cells expressing the enzyme Gre2p produced exclusively the *S*



Scheme 1. A) Sequential biocatalytic reduction of 5 nitrononane 2,8 dione (NDK; **1**) enables the stereoselec tive synthesis of the stereoisomeric hydroxyketones **2** and diols **3**. B) The two model enzymes used in this study, Gre2p and LbADH, lead to selective formation of hydroxyketones **2b** and **2c**/**2d**, respectively. Whereas Gre2p hardly accepts hydroxyketones as substrates, LbADH can reduce the hydroxyketones **2c**/**2d** to produce the *pseudo* C_2 symmetric diol **3d**.[28] All stereoisomers can be quantified by HPLC analysis on a chiral stationary phase (Figure S5).

configured hydroxyketone **2b** with an excellent selectivity of >99% (entries 4 6), as expected from our previous study.[28]

Owing to the high stereoselectivities, the "catalytic content" was also used as a marker for the selective self immobilization of the bacteria onto the magnetic microbeads. To this end, combinations of tag displaying KRED@*E.coli* strains were allowed to competitively bind onto MB STV. The attached cells were harvested by magnetic separation, regrown, and then used for the transformation of NDK **1**. HPLC analysis on a chiral stationary phase clearly showed that the competitive binding of LbADH@*E.coli* HOB and Gre2p@*E.coli* SBP led to almost exclusive formation of the *S* configured hydroxyketone **2b** (Table 1, entry 7), which is in agreement with the expected selective binding of Gre2p@*E.coli* SBP onto MB STV. Even in a highly demand ing test, in which Gre2p@*E.coli* SBP, as the cells with a significantly lower enzymatic activity (see Table S2), were competing against two strains harboring the more active LbADH (LbADH@*E.coli* HOB, LbADH@*E.coli* ST; Table 1, entry 8), specific immobilization led to almost exclusive formation of the *S* configured hydroxyketone.

Furthermore, 500 fold scale up of the reaction with microbead immobilized Gre2p@*E.coli* SBP (250 mg NDK **1** in 500 mL LB Medium) followed by preparative extraction with ethyl acetate yielded the *S* configured hydroxyketone (71%). This result demonstrates that our novel cell surface display method does not compromise product isolation and purity.

In conclusion, we have reported on the engineering of *E. coli* strains that display orthogonal tags for immobilization on their surface and overexpress functional heterologous protein content in their cytosol at the same time. Based on the herein demonstrated applicability for stereoselective whole cell biocatalysis, we believe that this innovative approach will be important for the development of sustainable biotechno logical processes, which could strongly benefit from living self immobilizing biocatalysts. Furthermore, as suggested for DNA directed assembly schemes,[12] the affinity tags dis played on the cell surface could also be used to create patterns on surfaces (Figure S6) or clusters of bacteria communicating with each other in novel approaches for synthetic biology and biotechnology.

Acknowledgements

This work was supported by the Helmholtz program "Bio Interfaces in Technology and Medicine". We thank Alessan dro Angelin, Jens Bauer, Anke Dech, Ruben Garrecht, Marc Skoupi, Ann Kathrin Schneider, and Cornelia Ziegler for experimental help and Ishtiaq Ahmed for the synthesis of the nitrodiketone.

Conflict of interest

The authors declare no conflict of interest.

Keywords: bacteria · biocatalysis · cell surface display · immobilization techniques · stereoselective reactions

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