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Monitoring the Cellular Surface Display of Recombinant Proteins by Cysteine Labeling and Flow Cytometry

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Dedicated to Herbert H. Jose on the occasion of his 70th birthday.

A general method is described that allows one to follow the surface display of recombinant proteins in Escherichia coli *without having to use specific antibodies or enzymatic reactions. The method is based on cysteine-specific labeling through Michael addition to the double bond of maleimide and its derivatives, and takes advantage of the fact that naturally occurring surface proteins in* E. coli *contain no accessible cysteine residues. The method is easy to perform and could be simply applied to different analytic procedures including Western blot, spectral photometry, and flow cytometry. By using this new labeling method, single cells bearing a distinct protein at the surface could be selected by fluorescence-activated cell sorting. The data were obtained by using autodisplay, an efficient surface display system established for* E. coli, *but the method presented here represents rather a general solution for analyzing the surface display of recombinant proteins, independent of the cellular system used.*

KEYWORDS:

autodisplay · fluorescence · maleimide · Michael addition · protein engineering

Introduction

The display of a protein with a distinct function in large numbers on the surface of a living cell is a challenging exercise with increasing impact in many areas of biochemistry and biotechnology.[1] By displaying enzymes, whole-cell biofactories are obtained that can be accessed by substrates without restriction and that, in most cases, are easier to handle, immobilize, and stabilize in industrial processes than the free enzyme molecule.[2] Moreover, a pure biocatalyst can be maintained by a simple centrifugation step.[3] The application of cellular surface display has another significant advantage when used for creating and screening peptide or protein libraries in order to perform directed molecular evolution.[4] By selecting the correct structure expressed at the surface, the cell with the corresponding gene, which serves as an intrinsic label, is co-selected and can be used in further studies and applications. More recently, cellular surface display has led to promising progress in the fields of oral vaccines[5] and in the development of environmental bioadsorbents.[6]

From this perspective it is clear that systems for the surface display of a broad spectrum of molecules are needed, along with tools that allow the analysis and evaluation of these systems in terms of efficiency. As far as possible, these techniques must be compatible with automation processes in order to obtain high throughput. In all cases up to now, however, the detection of surface display has depended on a certain quality of the molecule to be displayed, either an affinity to an antibody, as in most cases, or a specific catalytic function. This fact has limited the promising strategy of cellular surface display to a restricted number of candidate molecules.

Here, we present for the first time a general method that allows verification and quantification of cellular surface display, independent of the displayed molecule's antigenic or catalytic properties. This strategy takes advantage of two properties. First, natural cell envelope proteins in *Escherichia coli* contain practically no cysteine residues accessible at the surface.[7] Second, there is a specific labeling procedure for cysteine residues based on Michael addition to the double bond of maleimide and its derivatives.[8, 9] In the present study, an artificially introduced cysteine and natural cysteine residues occurring in recombinant proteins displayed at the surface of *E. coli* could be used for labeling. The labeling allowed monitoring of the surface display with different methods, such as flow cytometry. Moreover, single cells with cysteine-containing proteins at the surface could be selected by fluorescence-activated cell sorting (FACS). The

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solution presented here represents a substantial improvement in the application of cellular surface display, for instance for use in automation processes.

Results

Construction of a passenger protein with a single cysteine residue

The surface display of a recombinant protein, a so-called passenger by autodisplay (an established surface display system in *E. coli*[10, 11] based on the autotransporter secretion pathway[12]) requires its integration into a polyprotein precursor of a defined structure (Figure 1 A). For this purpose, an artificial gene has to be constructed by PCR that encodes an N-terminal signal peptide (SP), the passenger, a so-called linker peptide, and the C-terminal β core. With the aid of the SP the precursor is transported across the inner membrane, and outer-membrane translocation is facilitated by the β core, which forms a porin-like structure. To obtain full surface exposure of the passenger protein an additional linker peptide is necessary (Figure 1 A). Details of this unique surface display system have been described elsewhere by Maurer et al.[10] and Jose et al.[11] In the present study, we started with plasmid pJM1013[13] to construct

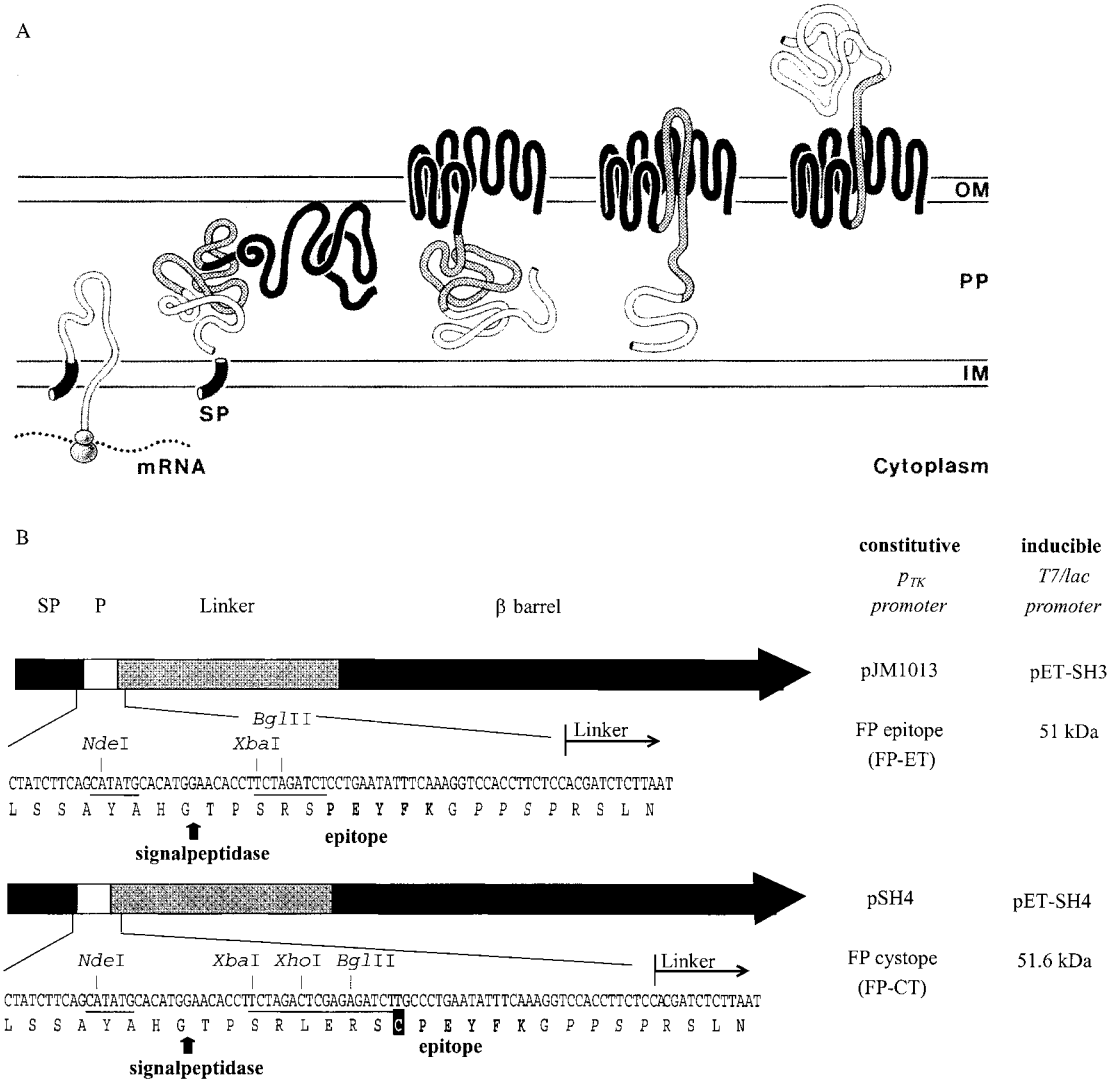


Figure 1. *A) Secretion mechanism of the autotransporter family of proteins. By using a typical signal peptide, a precursor protein is transported across the inner membrane. Once it arrives at the periplasm, the C-terminal part of the precursor folds as a porin-like structure, a so-called β barrel, within the outer membrane and the passenger is transmitted to the cell surface. For autodisplay, the signal peptide of the cholera toxin β subunit and the β barrel as well as the linker region of the* E. coli *adhesin AIDA-I[28] are used as described by Maurer et al.[10] SP = signal peptide; IM = inner membrane; PP = periplasm; OM = outer membrane. B) Structure of the autotransporter fusion proteins encoded by different plasmids used in this study. The environments of the passenger insertion site necessary to obtain surface translocation are given as sequences. Restriction endonuclease cleavage sites are underlined. The signal peptidase cleavage site is marked by an arrow. The site-specific cleavage site for IgA1 protease in the resulting fusion protein is written in italics and the linear epitope for a mouse monoclonal antibody (Dü142), which was used as an internal control, is written in bold. The single cysteine residue used for labeling and detection is indicated by a black box. p = plasmid, FP = fusion protein; ET = epitope-containing, CT = cysteine-containing "cystope"; FP-CT contains four amino acids in addition to those in FP-ET, therefore the molecular weight is slightly increased. The* CpoI *restriction site (not shown) used for cloning purposes is located 155 bp downstream of the underlined* BglII *restriction site. P = passenger.*

the gene for a passenger protein with a single cysteine residue. In our earlier studies we showed that pJM1013 encodes a precursor protein that facilitates efficient surface display of an epitope for the mouse monoclonal antibody Dü142 under the control of the constitutive promoter P_{TK}.[13] The codon for a single cysteine residue and an *Xho*I restriction site were inserted by using PCR mutagenesis, which resulted in plasmid pSH4 (Figure 1B). As a result of these insertions, the proteins encoded by pJM1013 (epitope-containing fusion protein, FP-ET) and pSH4 (cysteine- and epitope-containing fusion protein, FP-CT) differ slightly in size. Both plasmids were transformed into *E. coli* JK321, a well-adapted strain for the autodisplay of foreign passenger proteins,[14] and transport to the cell surface was analyzed.

Probing the surface display of the proteins FP-ET (expressed from plasmid pJM1013) and FP-CT (expressed from plasmid pSH4)

In previous experiments, differential cell fractionation was an adequate tool to find out whether a passenger is transported to the cell surface.[2, 3, 10, 11] As can be seen in Figure 2A, it was possible to detect a protein of the correct size in the outer-membrane fraction of *E. coli* JK321, which contains plasmid pSH4, as well as in JK321 with plasmid pJM1013. In both cases expression was almost as high as for the natural outer-membrane proteins OmpA and OmpF/C. A similar protein was neither detectable in the cytoplasm fraction nor in the inner membrane fraction (not shown). In a Western blot analysis that used the monoclonal antibody Dü142 for detection, both protein bands yielded a signal (Figure 2B), which identifies them as FP-CT (lane 1) and FP-ET (lane 3), respectively.

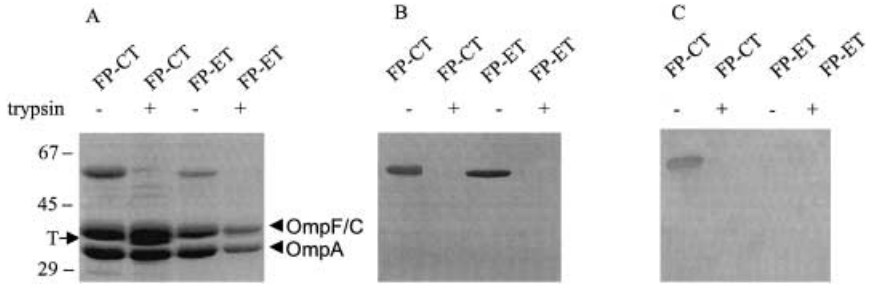


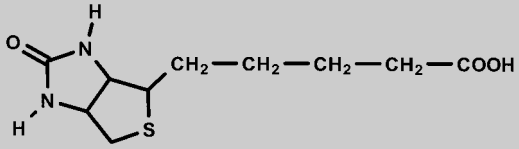
Figure 2. *Surface translocation and accessibility of FP-ET and FP-CT. Outer membranes were prepared from* E. coli *JK321 pJM1013 (FP-ET) and JK321 pSH4 (FP-CT) and subjected to SDS gel electrophoresis on a 12.5% PA gel followed by Coomassie brilliant blue staining (A). After being transferred onto a PVDF filter membrane, proteins were detected by the epitope-specific monoclonal mouse antibody Dü142 (B) or by biotin maleimide coupling followed by the addition of streptavidin – alkaline phosphatase conjugate (C) as described in the Experimental Section. Molecular weights of marker proteins are given in kDa on the left. Trypsin +/– : whole cells were/were not treated with trypsin before outer membranes were prepared. Natural outer membrane proteins OmpF/C and OmpA are marked by arrows. T = trypsin-resistant membrane-embedded autotransporter core.*

The next step was to show that the passenger domains of FP-CT and FP-ET are indeed exposed at the cell surface. For this purpose whole cells expressing pJM1013 and pSH4 were treated with trypsin before outer-membrane fractions were prepared. Trypsin is too large a molecule to pass through the outer membrane. Therefore, degradation of the passenger domain by trypsin added to whole cells clearly indicates its surface accessibility. As can be seen in Figure 2A and Figure 2B, external trypsin addition resulted in the degradation of FP-CT (lane 2) and FP-ET (lane 4). Integrity of the outer membrane was indicated by detection of an unaltered OmpA protein. OmpA has a C-terminal periplasmic extension, which is digested by trypsin when the outer membrane is leaky.[10] In Coomassie-stained sodium dodecyl sulfate (SDS) gels (Figure 2A), an additional protein band was detectable that corresponds to the membrane-embedded and thereby trypsin-resistant β core. An identical membrane-embedded trypsin-resistant core has already been described for other passenger proteins used in autodisplay.[10, 11] In summary, our results indicate that the passenger domains are transported to the cell surface of *E. coli* by both fusion proteins, FP-CT and FP-ET, and that these domains are freely accessible. As FP-CT contains a single cysteine residue, we developed a detection method based on maleimide coupling. After SDS-PAGE, protein bands were transferred onto a poly(vinylidene difluoride) (PVDF) membrane filter in the same manner as described before for Western blot experiments. Instead of using an antibody for detection, biotin-coupled maleimide was added (Table 1, for details see the Experimental Section). The idea was that maleimide would be covalently linked to the cysteine residue through Michael addition and after addition of streptavidin – alkaline phosphatase conjugate and a chromogenic substrate (X-phosphate) detectable staining would result. As can be seen in Figure 2C, FP-CT was indeed stained by this procedure, whereas FP-ET was not. This result indicates the feasibility of this new cysteine-dependent, antibody-independent filter detection assay. The fact that no other protein band was labeled supports the view that outer-membrane proteins in *E. coli* generally contain no cysteine residues, as was reported previously.[7]

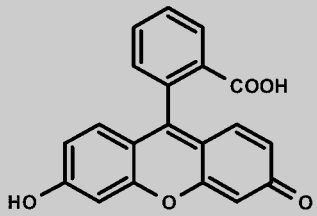
Photometric analysis of cells bearing a single cysteine residue

For this purpose, identical amounts (10^9 cells) of *E. coli* JK321 pSH4, which expresses FP-CT with a single cysteine residue, and control *E. coli* JK321 pJM1013, which expresses FP-ET without any cysteine residues, were incubated for 25 min with 500 μM biotin maleimide (for details, see the Experimental Section). After the addition of streptavidin – β-galactosidase conjugate, both cell samples were incubated for 5 min with *o*-nitrophenyl-β-D-galactoside, a chromogenic substrate for β-galactosidase. The release of 2-nitrophenol as a result of enzymatic activity was determined by the absorption observed at 405 nm. Cells expressing FP-CT with a single cysteine residue exhibited β-galactosidase activity of 153 Miller units,[15] whereas control cells

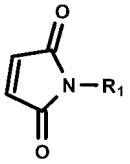
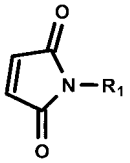
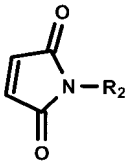
Table 1. *Maleimide coupling of cysteine at the cell surface and detection methods applied in this study.*



Biotin



Fluorescein

Target	Coupling		Enzyme linking	Method of detection
SH group		R_1 = Biotin	streptavidin – β-galactosidase	photometry
SH group		R_1 = Biotin	streptavidin – alkaline phosphatase	Western blot
SH group		R_2 = Fluorescein	none	flow cytometry

expressing FP-ET without cysteine yielded an activity of 23 Miller units (both results are the mean value of three independent experiments). This means that cells expressing FP-CT have β-galactosidase activity of 130 Miller units, when the results are adjusted according to the values obtained from control cells. This difference in activity is due to specific labeling of cysteine by biotin maleimide. The difference indicates that the labeling of a single cysteine residue expressed at the cell surface by autodisplay is sufficient for the analysis of such a low number of bacterial cells (10^9) by photometry. This result opens the door to the use of the method described in online monitoring of protein production, for example, for monitoring of cells within an industrial process. The technique can be applied in cases where the protein itself is the product of a process, for example, in order to obtain pure enzymes, and in cases where cells expressing high numbers of an enzyme or another functional protein are needed before they are submitted to synthetic purposes.

Inducible surface expression of a passenger protein with a single cysteine residue

In many industrial or synthetic applications it is a striking advantage to be able to control the expression of the recombinant protein of interest at the level of transcription. For example, this allows one to produce a high number of cells before starting the expression of the recombinant protein by the addition of a specific inducer. To obtain an inducible autodisplay system and to employ our cysteine-specific labeling method in monitoring the controlled induction of protein expression, fusion proteins FP-CT and FP-ET were expressed from an inducible T7/Lac promoter. For this purpose, the artificial precursor gene encoding the autotransporter domains with a passenger containing a single cysteine residue (FP-CT) was inserted into vector pET11d. This plasmid vector, which is commercially available, is specified for inducible protein expression. The new plasmid obtained by this strategy was named pET-SH4 (Figure 1 B). This plasmid allowed inducible expression of FP-CT from the T7/lac-promoter, which was pET11d derived. The gene encoding FP-ET was treated similarly, to give plasmid pET-SH3 (Figure 1 B), which was used as a control. Both plasmids were transformed into *E. coli* BL21 (DE3), a strain commercially available and commonly used for the T7/lac promoter-mediated induction of expression. Protein expression was subsequently analyzed. As shown in Figure 3, a huge amount of FP-CT could be detected in the outer-membrane fraction of BL21(DE3) pET-SH4 cells after induction with isopropyl-β-D-thiogalactopyranoside (IPTG). The expression rate of FP-CT was even higher than that of the natural outer-membrane proteins OmpA and OmpF/C. The protein could be detected in Coomassie-stained SDS-gels (Figure 3 A), as well as by Western blotting with antibody

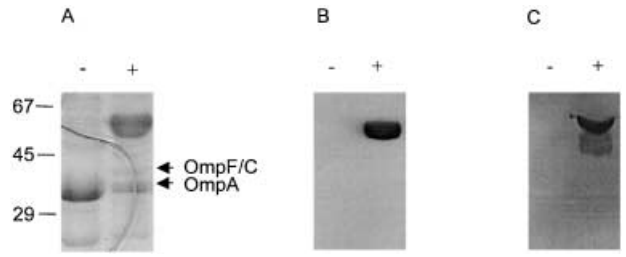


Figure 3. *Inducible surface display and detection by cysteine-specific labeling with biotin maleimide. Outer membranes were prepared from* E. coli *BL21 (DE3) pLysS pET-SH4 before (−), and 30 min after induction with IPTG (+) and were subjected to SDS gel electrophoresis on a 12.5% PA gel with subsequent Coomassie brilliant blue staining (A). After being transferred onto a PVDF membrane filter, proteins were detected either by the epitope-specific mouse monoclonal antibody Dü142 (B) or by biotin maleimide coupling followed by the addition of streptavidin – alkaline phosphatase conjugate (C) as described in the Experimental Section. Molecular weights of the marker proteins are indicated in kDa on the left. Natural outer membrane proteins OmpF/C and OmpA are marked by arrows.*

Dü142 (Figure 3B). After induction, a protein band corresponding to FP-CT that was not detectable in noninduced cells could also be detected by the cysteine-specific maleimide coupling method (Figure 3C). This result was in accordance with those obtained by Western blot and Coomassie staining of SDS-gels and indicates that T7/lac promoter-controlled expression of FP-CT was not leaky.

Flow cytometric analysis of cells bearing a protein with a single cysteine residue

Cells of *E. coli* BL21(DE3) pET-SH4 expressing FP-CT were labeled with fluorescein maleimide (Table 1). For this purpose, samples of a cell suspension with an optical density at 578 nm (OD_{578}) of 1.0 were incubated with 500 μM fluorescein maleimide for 15 min at room temperature, after induction by treatment for 30 min with IPTG. The reaction was stopped and excess labeling reagent removed by repeated washing with buffer, then cells were diluted to a final OD_{578} of 0.05 and subjected to flow cytometry on a FACSCalibur flow cytometer. *E. coli* BL21(DE3) pET-SH3 cells expressing FP-ET (no cysteine) were treated similarly prior to flow cytometry analysis and were used as a control. Figure 4 shows that the fluorescence intensity of BL21(DE3) expressing FP-CT (Figure 4B) was clearly distinguishable from the intrinsic background signal of control *E. coli* expressing FP-ET (Figure 4A). This result indicates that maleimide coupling of a single cysteine residue within a protein displayed on the *E. coli* surface is a valid labeling procedure for flow cytometry analysis.

The next step was to see whether this specific and sensitive labeling allows one to monitor the time course of expression of recombinant proteins on the surface of *E. coli* after induction. For this purpose, IPTG was added to samples of *E. coli* BL21(DE3) pET-SH4 cells to start expression of FP-CT, and after different periods of time samples were taken. These samples were labeled with fluorescein maleimide as described above and subsequently analyzed by flow cytometry. As can be seen in Figure 5, the mean value of fluorescence of 10000 cells analyzed for each sample increased continuously over a period of 90 min, whereas the mean fluorescence of control cells expressing FP-ET only slightly shifted. This result reflects the cumulative number of FP-CT molecules appearing on the surface of *E. coli* because of the induction of expression by IPTG. In summary, these results indicate that cysteine labeling by Michael addition to derivatives of maleimide is a valid and efficient method to monitor the appearance of recombinant proteins on the cell surface after induction of expression and is applicable in high-throughput methods such as flow cytometry.

Flow cytometric sorting of single cells bearing a cysteine-residue-containing protein at the surface

Cells of *E. coli* BL21(DE3) pET-SH4 expressing FP-CT (with a single cysteine residue) and *E. coli* BL21(DE3) pET-SH3 expressing FP-ET (without cysteine) were mixed in the ratio 1:1, labeled with fluorescein maleimide, and analyzed by flow cytometry. As can be seen in Figure 6C, this resulted in two cell populations that could be discriminated by fluorescence intensity within one sample. The relative fluorescence intensities of both were identical to those obtained when cells of BL21(DE3) pET-SH3 (FP-ET) and pET-SH4 (FP-CT) were labeled and analyzed separately (Figure 6A and Figure 6B, respectively). A sorting gate was defined within the mixed sample (Figure 6C) so that cells with a fluorescence corresponding to that of FP-CT-positive cells (Figure 6B) were selected by the sort module of the FACSCalibur cytometer. These cells were collected and immediately subjected to flow cytometry again, after an aliquot of cells was removed for

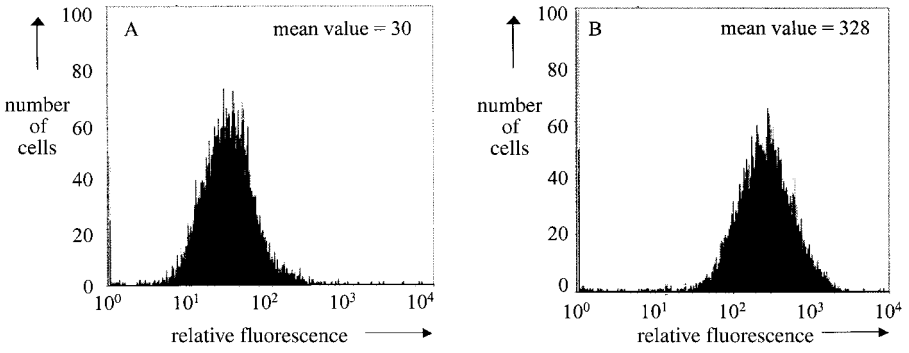


Figure 4. *Flow cytometric analysis of whole* E. coli *cells expressing FP-ET (A) and FP-CT (B).* E. coli *BL21(DE3) pLysS containing pET-SH3 (FP-ET), and BL21(DE3) pLysS containing pET-SH4 (FP-CT) were labeled with fluorescein maleimide 30 min after induction with IPTG and subjected to flow cytometry as described in the Experimental Section. For each sample, 10000 cells were analyzed and cellular fluorescence is described as a histogram plot, 30 min after induction with IPTG. The mean value of relative fluorescence of* E. coli *expressing FP-CT (328, B) is almost 11 times higher than that of cells expressing FP-ET (30, A).*

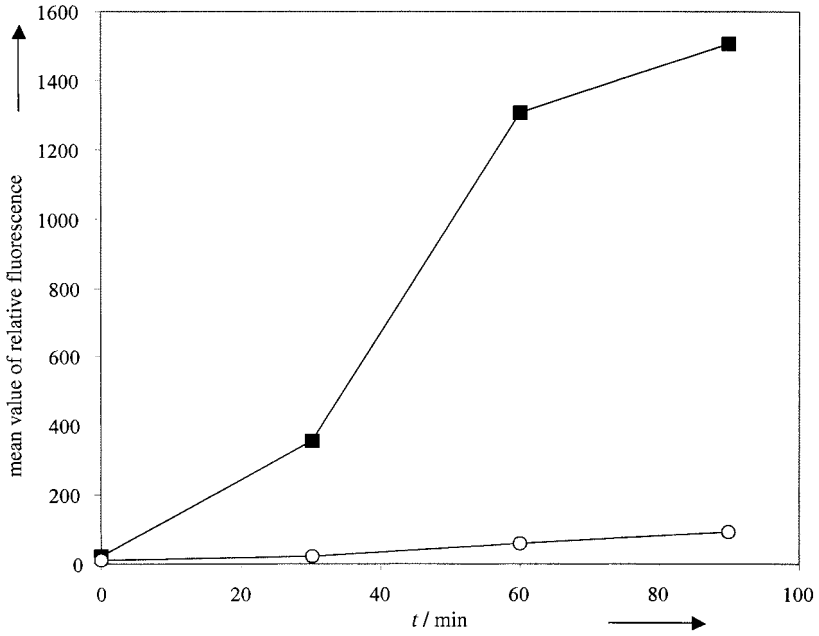


Figure 5. *Time course of induced surface display monitored by fluorescein maleimide labeling and flow cytometry. Cells expressing FP-CT (*E. coli *BL21(DE3) pLysS pET-SH4; black squares) and cells expressing FP-ET (*E. coli *BL21(DE3) pLysS pET-SH3; open circles) were induced with IPTG. At the indicated time points, samples were taken and labeled with fluorescein maleimide as described in the Experimental Section. For every sample the mean value of fluorescence was determined for 10 000 cells by flow cytometry and plotted versus time after induction. The experiment was repeated three times independently with similar results.*

growth and subsequent DNA analysis. Figure 6D shows the cellular fluorescence distribution of the sorted sample. As can be seen, after only one round of cell sorting, a population with high fluorescence corresponding to FP-CT cells was obtained from the mixture of positive and negative cells. To verify the identity of cells that were sorted by this procedure, the aliquot of removed cells (see above) was diluted and adequate dilutions were grown on agar plates to obtain single colonies. The number of single-cell clones obtained by this procedure was identical to that determined by flow cytometry, which indicates that cells survived the labeling and sorting protocol without loss. From ten single-cell clones obtained, plasmid DNA was prepared and subjected to restriction analysis. In this analysis we took advantage of the fact that pET-SH4 contains an *Xho*I restriction site which is absent in pET-SH3 (Figure 1). All single-cell clones contained plasmid pET-SH4. These results indicate that labeling of a single cysteine residue expressed within a protein at the cell surface allows selection of corresponding cells from a mixed population by fluorescence-activated cell sorting. The method enables a rapid and high-throughput selection of cells expressing a protein, for example, an enzyme of interest, from a mixture with nonexpressing cells in order to obtain an optimized cell population before application to an industrial or synthetic process.

For cell sorting 270 000 cells of the mixture were analyzed and a total of 86 000 cells were sorted out. As a result of the sorting procedure, the selected cells were considerably diluted. As a consequence, a much larger volume of this cell solution had to pass through the flow cytometer before 10 000 cells, the fixed number of cells for each experiment, were analyzed for fluorescence. This fact resulted in the appearance of a peak with low fluorescence (Figure 6D) that did not contain any growing cells and is therefore assumed to consist of buffer impurities.

Cysteine labeling of bovine adrenodoxin displayed at the *E. coli* surface

To see whether naturally occurring cysteine residues within passenger proteins displayed on the *E. coli* surface can be used for labeling, the ferredoxin from bovine adrenal cortex, adrenodoxin (Adx) was investigated. Adx contains five cysteine residues and an iron–sulfur cluster as the prosthetic group. Four of the five cysteine residues are involved in binding the [2Fe–2S] group. It has been shown in earlier studies that apo-Adx is efficiently transported to the *E. coli* cell surface by the autotransporter pathway.[2] The prosthetic group can be inserted after transport to yield active, electron-transferring holo-Adx.[11] For the present study we confined ourselves to the investigation of surface-displayed apo-Adx. For this purpose, the coding region of Adx was inserted into vector pET-SH3 and surface translocation was verified as described before. Cells of an overnight culture were used to inoculate fresh medium that was incubated until exponential cell growth was obtained. Adx expression was induced by treatment with IPTG for 30 min; cells were then harvested, adjusted to an OD_{578} value of 1.0, and labeled with fluorescein maleimide as described above. Figure 7B shows that this treatment resulted in a significantly increased fluorescence of cells in comparison to control pET-SH3 cells (Figure 7A). This result indicates that the labeling and detection methods presented here can be used for monitoring the expression of passenger proteins that contain inherent cysteine residues.

Discussion

In the presented study, we describe a new method for the specific labeling of recombinant proteins expressed at the surface of *E. coli* cells. The method is useful in monitoring the appearance of recombinant proteins at the cell surface and as a tool for flow-cytometer-based high-throughput analytical and selection purposes. Natural proteins of the cell envelope were clearly not labeled by this method. The method is based on the

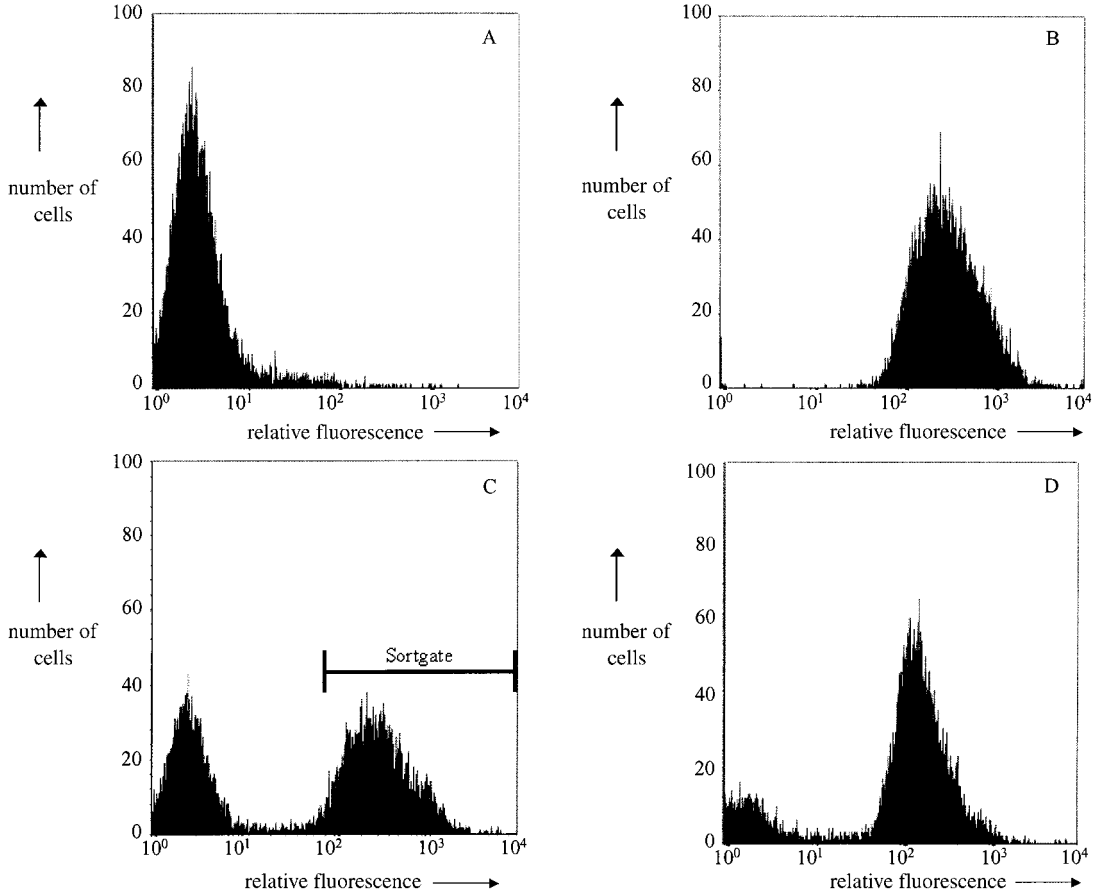


Figure 6. *Fluorescence-activated cell sorting (FACS) of* E. coli *cells expressing FP-CT after labeling with fluorescein maleimide. A) Mean fluorescence of cells expressing FP-ET (*E. coli *BL21(DE3) pLysS pET-SH3) labeled with fluorescein maleimide. B) Mean fluorescence of cells expressing FP-CT (*E. coli *BL21(DE3) pLysS pET-SH4) labeled with fluorescein maleimide. C) Mean fluorescence of a 1:1 mixture of cells expressing FP-ET and cells expressing FP-CT after labeling with fluorescein maleimide. The sortgate used for selection of highly fluorescent cells is indicated by a black bar. D) Mean fluorescence of cells after FACS by the sortgate described in (C). For each measurement, 10 000 cells were analyzed.*

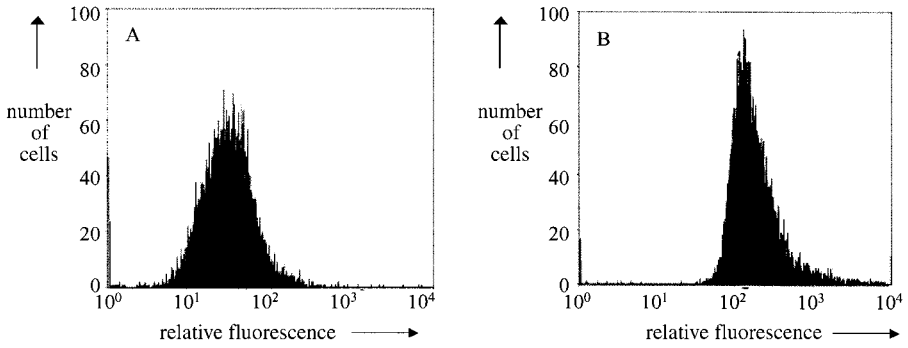


Figure 7. *Flow cytometric analysis of the surface display of bovine adrenodoxine (Adx), a naturally cysteine-containing protein.* E. coli *BL 21(DE3) cells containing plasmid pET-SH3 with the Adx-encoding DNA inserted by the* XbaI/BglII *restriction sites were labeled with fluorescein maleimide and analyzed by flow cytometry (B). The mean fluorescence of these cells (271) was significantly increased in comparison to the mean value of fluorescence of cells expressing FP-ET (39;* E. coli *BL 21(DE3) pET-SH3), which were applied as a control (A).*

Michael addition of cysteine to the double bond of different maleimide derivatives. Other specific labeling reactions for the sulfhydryl group of cysteine are known, for example, alkylation by holoacetyl derivatives or treatment with pyridyl disulfide derivatives,[9] and could be performed in a similar manner. The maleimide reaction with sulfhydryl groups has been successfully used previously in macromolecular prodrug concepts,[16] in prolonging the in vivo half-life of therapeutic antibody fragments,[17] and in investigations of the structure of bacterial receptors.[7, 18] In our experiments, two forms of maleimide derivatives were applied. Biotin maleimide was used for photometric detection and filter detection after Western blotting. Fluorescein maleimide was the label for fluorescence-activated cell sorting and flow cytometer analysis. A single cysteine residue within the protein displayed at the cell surface was sufficient to obtain efficient and detectable labeling by both maleimide derivatives. In addition to the single cysteine residue that was

inserted especially for this study within a linker region (Figure 1 B), passenger-inherent cysteine residues could also be used for labeling, as shown for bovine adrenodoxin (Adx, Figure 7).

A third possible strategy for monitoring the surface expression of a recombinant protein with autodisplay is to insert the coding region of a passenger protein without any cysteine residues, for example, into plasmid pET-SH4 at the *Xba*I, *Xho*I, or *Bgl*II restriction site (Figure 1 B). This insertion will result in co-secretion of the cysteine residue derived from pET-SH4, and surface translocation of the recombinant passenger can be detected by the co-secreted single cysteine residue. This process has been performed to analyze the surface display of bacterial esterase, for which a specific antibody was not available. Transport and surface accessibility were verified by cysteine labeling and subsequent Western-blot-like experiments.[19]

The study presented here is based on the idea that surface proteins of *E. coli* either contain no cysteine residues or none accessible to other molecules for reaction. Our results clearly support this previously reported hypothesis.[7] Control cells of *E. coli* expressing passenger proteins without cysteine were always negative regardless of the detection method applied (Figures 2 C, 5, 6 A); *E. coli* cells without any plasmid were likewise negative (not shown). Since the periplasm of *E. coli* contains a number of proteins with freely accessible and reactive cysteine residues,[20, 21] this result also indicates that the maleimide derivatives used in this study were not able to penetrate the outer membrane barrier. Clearly, the derivatives were neither hydrophobic enough to pass through the asymmetric lipid bilayer nor small enough to pass through the hydrophilic pores provided by the porins. This finding is important because it permits the use of the maleimide-dependent labeling and detection method to distinguish surface exposure from, for instance, periplasmic location of a protein containing one or several cysteine residues.

The advantages of the labeling and detection method described here are obvious. Only minimal amounts of cells are needed and these cells can be labeled within less than 1 h. In addition, flow cytometric analysis of labeled cells is performed within seconds and can be automated. The content of a recombinant protein within a small sample can therefore be determined quickly and automatically, if desired. The strategy can be useful in, for example, the online monitoring of whole-cell protein expression at the surface during industrial processes.

It is clear that the labeling method described here can be used for protein purification or purification of cells expressing a certain protein. Labeling with biotin maleimide allows whole cells expressing a single cysteine residue at the surface to be coupled to streptavidin-coated beads, streptavidin – agarose, or any other streptavidin-modified matrix, for example, within liquid chromatography columns. In this strategy, cells expressing a distinct protein with one or several cysteine residues at the surface could be enriched from a mixture of cells that do not express the protein. Moreover, cells expressing an enzyme, antibody, or other functional protein at the surface can be immobilized for use in continuous reactions. Similar methods would allow cells to be fixed onto membranes for use, for example, in surface plasmon resonance experiments. If the recombinant passenger protein with one or several cysteine residues is released into the supernatant by a site-specific protease, for example, the passenger protein can be purified in a single step by taking advantage of its affinity for streptavidin. For this purpose, a specific IgA1 protease cleavage site was inserted during vector construction (Figure 1 B). External added protease will release the passenger protein with the attached cysteine residue into the supernatant when vectors pJM1013, pSH4, pET-SH3, or pET-SH4 are used. There the protein could be bound to streptavidin immobilized on a matrix (for example, a column). If a second cleavage site for a site-specific protease, for example, factor X, were inserted in N-terminal orientation to the reporter cysteine residue, this site could be used to remove the passenger without cysteine from the streptavidin-coated column. The feasibility of this cysteine-based one-step protein purification strategy is currently under investigation.

The striking advantage of the labeling and detection/purification procedure described here is the fact that a single amino acid (a single cysteine residue) is sufficient. Inherent cysteine residues can be used for labeling and purification or, if the protein is devoid of cysteine, a single cysteine residue can be added. The latter option appears to interfere less with the protein's natural structure and folding behavior than the minimum 8 amino acids necessary in the "epitope tagging" strategy,[22] or the 4 – 6 amino acids required for the "His tagging" strategy.[6] In both strategies, the linear epitope of a monoclonal antibody is connected to the passenger protein and surface translocation can be followed with the aid of the monoclonal antibody in Western blots, indirect immunofluorescence, or ELISA-like experiments.[6, 22]

In summary, the labeling method described here is a substantial improvement in the detection of recombinant proteins at the surface of *E. coli*. It can also be helpful in high-throughput analysis and rapid purification of cells expressing a certain protein, as well as in the purification of the recombinant protein itself. The data presented here were obtained with autodisplay, a surface display system based on the autotransporter secretion mechanism.[10, 11] The method may also be applied in other surface display systems.[4, 23–25]

Experimental Section

Bacterial strains, plasmids, and culture conditions: *E. coli* strain JK321 (Δ*ompT proC leu-6 trpE38 entA zih12*:Tn*10 dsbA*:*kan*) which was used for constitutive expression of fusion proteins in autodisplay, has been described elsewhere.[14] *E. coli* strain BL21(DE3) pLysS (F^- *ompT* *hsd*S_B (r_B^- m_B^-) *gal dcm*)(DE3)pLysS(Cm^R), which was used for the inducible expression, was obtained from Stratagene (La Jolla, USA). The vector used for inducible expression was pET11d (Novagen, Madison, USA). *E. coli* TOP10 (F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara – leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) and the vector pCR2.1-TOPO, which were used for cloning of PCR products, were obtained from Invitrogen (Groningen, the Netherlands). Bacteria were routinely grown at 37 °C in Luria – Bertani (LB) broth containing ampicillin (100 mg per liter). For expression studies, ethylenediaminetetraace-

tate (EDTA) was added to a final concentration of 10 μM and β-mercaptoethanol was added to a final concentration of 10 mM.

Recombinant DNA techniques: Plasmid pJM1013 has been described and characterized in former studies.[13] This plasmid directs the epitope (PEYFK) of a mouse monoclonal antibody to the cell surface, where it can be released by a specific IgA1 protease cleavage site when the protease is externally added to the cells (Figure 1). Expression of the autotransporter fusion protein in pJM1013 is under the control of the strong constitutive promotor P_{TK}.[10] Construction of pSH4 containing a single cysteine residue was achieved by amplifying the autotransporter domains genes by PCR from plasmid pJM1013 by using the oligonucleotide primers sh017 (5′-tct aga ctc gag aga tct **tgc** cct gaa tat ttc aaa gg-3′) and sh002 (5′-cac cac cag acg gtc cgt aag tg-3′). Primer sh017 adds the codon for a single cysteine residue (written in bold) and includes an *Xho*I restriction cut site that could be used for control digestions. The fragment obtained by PCR was inserted into vector pCR2.1-TOPO and recleaved with *Xba*I and *Cpo*I. Plasmid pJM1013 was also digested with *Xba*I and *Cpo*I, and the *Xba*I/*Cpo*I fragment obtained by PCR was inserted. This process resulted in plasmid pSH4, as shown in Figure 1B. The *Xho*I cut site introduced by PCR could be used to differentiate between pJM1013 and pSH4.

For inducible expression, the commercially available pET11d vector backbone from Novagen (Madison, USA) was used. It was vital for subsequent cloning purposes to delete any *Xba*I and *Bgl*II restriction sites of the vector in a first construction step. For this purpose, PCR was performed with oligonucleotide primers sh023 (5′-cct agg ggg gaa ttg tta tcc gc-3′) and sh024 (5′-tga tca cga tcc cgc gaa att aat acg-3′), and plasmid pET11d as a template. The PCR product was inserted into vector pCR2.1-TOPO, recleaved with *Avr*II and *Bcl*I, and ligated into the *Xba*I and *Bgl*II restricted vector pET11d to give pET11-SH2. *Bcl*I cutting results in sticky ends compatible with those generated by *BamH*I. The new vector, best described as completely lacking *Xba*I and *Bgl*II restriction sites, was cleaved with *Nco*I and *BamH*I. The autotransporter domains, which include the signal peptide, linear epitope, linker region, and β barrel encoding regions were amplified by PCR with pJM1013 as a template and oligonucleotides sh015 (5′-cca tgg tta aat taa aat ttg gtg ttt ttt tta cag-3′) and sh016 (5′-tga tca tta tca gaa gct gta ttt tat ccc c-3′) as primers. The PCR fragment obtained was ligated into pCR2.1 TOPO. After cleavage with *Nco*I and *Bcl*I, the fragment was inserted into vector pET11-SH22. This process resulted in plasmid pET-SH3, which allows the expression of the identical autotransporter fusion protein as that described for pJM1013; however, in this case the expression is under the control of an inducible T7/Lac promoter. Plasmid pET-SH4 was obtained by replacing the 200-bp *Nde*I/*Cpo*I fragment in pET-SH3 by the corresponding fragment containing the single cysteine residue obtained by cleavage of pSH4 with *Nde*I and *Cpo*I.

Outer membrane preparation: *E. coli* cells were grown overnight and the culture (1 mL) was used to inoculate LB medium (20 mL). Cells were cultured at 37 °C with vigorous shaking (200 rpm) for about 5 h until an OD_{578} value of 0.7 was reached. After harvesting and washing with phosphate-buffered saline (PBS), outer membranes were prepared according to the rapid isolation method reported by Hantke.[26]

For whole-cell protease treatment, *E. coli* cells were harvested, washed, and resuspended in PBS (5 mL). Trypsin was added to a final concentration of 50 mg L^{-1} and cells were incubated for 5 min at 37 °C. Digestion was stopped by washing the cells three times with PBS containing fetal calf serum (FCS, 10%) and outer membranes were prepared as described above.

For inducible expression, the strain BL21(DE3) pLysS, which harbors one of the described plasmids (pET-SH3 or pET-SH4), was grown overnight in LB medium containing ampicillin (50 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}). Chloramphenicol is needed to maintain the pLysS function to obtain more stringent control. A 1-mL aliquot of this overnight culture was used to inoculate LB medium (20 mL). Cells were cultured at 37 °C with vigorous shaking (200 rpm) for about 3 h until an OD_{578} value of 0.6 was reached. IPTG (1 mM, Roth, Karlsruhe, Germany) was added for induction. After 30 min (unless stated otherwise) cells were harvested and outer membranes were prepared as described above.

SDS-PAGE and Western blot analysis: Outer membrane preparations were diluted (1:2) with sample buffer (100 mM Tris-HCl (pH 6.8; Tris = tris(hydroxymethyl)aminomethane) containing SDS (4%), bromophenol blue (0.2%), and glycerol (20%)). The samples were boiled for 10 min at 95 °C and analyzed on an SDS-PA gel (12.5%). Proteins were visualized by Coomassie brilliant blue staining. Prestained molecular weight markers (Bio-Rad Laboratories, Hercules, USA) were used to calculate the apparent molecular weight of the outer-membrane proteins. For Western blot analysis, gels were electroblotted onto PVDF membranes with a Mini Trans-Blot apparatus from Bio-Rad laboratories (Munich, FRG), and blotted membranes were blocked in Tris-buffered saline (TBS) with FCS (3%) overnight.

Biotin maleimide labeling of whole cells prior to outer membrane preparation and Western blotting: After harvesting, whole cells were washed three times with ice-cold phosphate-buffered saline (PBS, 1 mL; sodium phosphate (10 mM), NaCl (0.9%), $MgCl_2$ (0.9%), pH 7,4), incubated with β-mercaptoethanol (2%) for 30 min, and washed five times with ice-cold PBS. Cells were collected by centrifugation and the pellet was resuspended in biotin maleimide (5 mL, 100 μM; Sigma – Aldrich, Steinheim, Germany) in PBS containing dimethylformamide (1%).[7] Cells were incubated for 15 min at room temperature then washed three times with PBS as described before. Cells labeled by this protocol were disrupted for outer membrane preparation and subsequent Western blotting. After blotting, the membranes were blocked in PBS with FCS (3%) for 1 h, then incubated with streptavidin – alkaline phosphatase conjugate (Gibco BRL, Gaithersburg, USA) diluted 1:10000 in PBS with FCS (3%) for 45 min, and washed four times with PBS.[27] A color reaction was obtained by adding incubation buffer (10 mL; NaCl (100 mM), $MgCl_2$ (5 mM), Tris-HCl (100 mM), pH 9.5) containing nitrobluetetrazolium-chloride (66 μL, 50 mg mL^{-1} in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolylphosphate-disodium salt (33 μL, 20 mg mL^{-1} in H_2O).

Immunodetection of the linear peptide epitope PEYFK used as a control: For immunodetection, membranes were incubated for 3 h with anti-PEYFK antibody Dü142[13] diluted 1:35 in TBS with FCS (3%). Prior to addition of the secondary antibody, immunoblots were rinsed three times with TBS containing Tween 20 (0.1%). Antigen – antibody conjugates were visualized by reaction with alkaline phosphatase linked goat antimouse IgG secondary antibody (KPL, Gaithersburg, USA) diluted 1:10000 in TBS containing FCS (3%). A color reaction was achieved by adding incubation buffer (10 mL; NaCl (100 mM), $MgCl_2$ (5 mM), Tris-HCl (100 mM), pH 9.5) containing nitrobluetetrazoliumchloride (66 μL, 50 mg mL^{-1} in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolylphosphate-disodium salt (33 μL, 20 mg mL^{-1} in H_2O).

Biotin maleimide labeling of whole cells and photometric analysis: A 1-mL aliquot of an overnight culture was added to LB medium (20 mL) containing ampicillin (100 μg mL^{-1}). Cells were

cultured at 37 °C with shaking at 200 rpm until an OD_{578} value of 3 was reached. Cells (1×10^9) were harvested by centrifugation at 14000g for 2 min at 4 °C. All steps were performed in 2-mL Eppendorf reaction tubes. Cells were washed three times with ice-cold PBS (1 mL, pH 7.4) then incubated in PBS (1 mL) containing 2% β-mercaptoethanol for 30 min at room temperature followed by five washing steps with ice-cold PBS (1 mL). Cells were incubated for 25 min at 30 °C in biotin maleimide (1 mL, 500 μM; Sigma Chemical Co, St. Louis, MO) dissolved in PBS containing dimethylsulfoxide (1%).[7] After three washes with ice-cold PBS containing BSA (3%), cells were incubated in PBS (1 mL) containing BSA (3%) for 1 h at room temperature, followed by centrifugation. Cells were then resuspended in streptavidin–β-galactosidase solution (6.5 μg mL^{-1} in PBS with 2% BSA) and incubated for 45 min at room temperature, followed by four washing steps with ice-cold PBS. Finally, cells were resuspended in sodium phosphate buffer (2 mL, 0.1 M) and the OD_{578} was determined. A 1-mL aliquot of this solution was added to *o*-nitrophenyl-β-D-galactoside (200 μL, 4 mg mL^{-1}) in PBS (pH 7.0) and incubated at 28 °C for 5 min. After addition of Na_2CO_3 (500 μL, 1 M), cells were pelleted by centrifugation (10 min, 5000g, 4 °C) and the absorption of the supernatant was determined at 405 nm. β-Galactosidase activity corresponding to cysteine molecules accessible at the surface was calculated in Miller units according to Giacomini's method.[15] The reaction time (5 min) and the reaction volume (1 mL) were kept constant throughout the entire measurement process.

Fluorescein maleimide labeling of whole cells and flow cytometric analysis: An overnight culture (1 mL) was added to LB medium (20 mL) containing ampicillin (100 μg mL^{-1}). Cells were cultured at 37 °C with shaking at 200 rpm until an OD_{578} value of 3 was reached. Cells (1×10^9) were harvested by centrifugation at 14000g for 2 min at 4 °C. All steps were performed in 2-mL Eppendorf reaction tubes. Cells were washed three times with ice-cold, sterile filtered PBS, suspended in fluorescein maleimide (1 mL, 10 μM; Molecular Probes Europe, Leiden, The Netherlands) dissolved in PBS containing dimethylformamide (1%), and incubated in darkness at 30 °C for 10 min. The reaction was then stopped by addition of dithiothreitol (20 mM). The surplus fluorescein maleimide was removed by washing the cells with PBS (5×1 mL). Cells were resuspended in PBS (1 mL) and diluted to a final OD_{578} value of 0.05 for subsequent FACS analysis. Fluorescence was determined in a flow cytometer (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Each figure reported represents the mean value of fluorescence of 10000 cells measured.

[1] G. Georgiou, C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson, R. Curtiss, 3rd, *Nat. Biotechnol.* **1997**, *15*, 29.
[2] J. Jose, R. Bernhardt, F. Hannemann, *J. Biotechnol.* **2002**, *95*, 257.
[3] E. Schultheiss, C. Paar, H. Schwab, J. Jose, *J. Mol. Catal. B: Enzym.* **2002**, *18*, 89.
[4] P. S. Daugherty, M. J. Olsen, B. L. Iverson, G. Georgiou, *Protein Eng.* 1999, *12*, 613.
[5] B. Stentebjerg–Olesen, L. Pallesen, L. B. Jensen, G. Christiansen, P. Klemm, *Microbiology* **1997**, *143*, 2027.
[6] C. Sousa, P. Kotrba, T. Ruml, A. Cebolla, V. De Lorenzo, *J. Bacteriol.* **1998**, *180*, 2280.
[7] C. Bos, D. Lorenzen, V. Braun, *J. Bacteriol.* **1998**, *180*, 605.
[8] D. G. Smyth, O. O. Blumenfeld, W. Konigsberg, *Biochem. J.* **1964**, *91*, 589.
[9] F. Kratz, U. Beyer, M. T. Schutte, *Crit. Rev. Ther. Drug Carrier Syst.* **1999**, *16*, 245.
[10] J. Maurer, J. Jose, T. F. Meyer, *J. Bacteriol.* **1997**, *179*, 794.
[11] J. Jose, F. Hannemann, R. Bernhardt, *ChemBioChem* **2001**, *2*, 695.
[12] J. Jose, F. Jahnig, T. F. Meyer, *Mol. Microbiol.* **1995**, *18*, 378.
[13] J. Maurer, J. Jose, T. F. Meyer, *J. Bacteriol.* **1999**, *181*, 7014.
[14] J. Jose, J. Kramer, T. Klauser, J. Pohlner, T. F. Meyer, *Gene* **1996**, *178*, 107.
[15] A. Giacomini, V. Corich, F. J. Ollero, A. Squartini, M. P. Nuti, *FEMS Microbiol. Lett.* **1992**, *79*, 87.
[16] F. Kratz, R. Muller–Driver, I. Hofmann, J. Drevs, C. Unger, *J. Med. Chem.* **2000**, *43*, 1253.
[17] A. P. Chapman, P. Antoniw, M. Spitali, S. West, S. Stephens, D. J. King, *Nat. Biotechnol.* **1999**, *17*, 780.
[18] J. J. Falke, A. F. Dernburg, D. A. Sternberg, N. Zalkin, D. L. Milligan, D. E. Koshland, Jr., *J. Biol. Chem.* **1988**, *263*, 14850.
[19] E. Schultheiss, J. Jose, unpublished results.
[20] J. C. Bardwell, K. McGovern, J. Beckwith, *Cell* **1991**, *67*, 581.
[21] X. Zhan, M. Schwaller, H. F. Gilbert, G. Georgiou, *Biotechnol. Prog.* **1999**, *15*, 1033.
[22] E. Veiga, V. de Lorenzo, L. A. Fernandez, *Mol. Microbiol.* **1999**, *33*, 1232.
[23] E. J. Kim, S. K. Yoo, *Lett. Appl. Microbiol.* **1999**, *29*, 292.
[24] M. Ueda, *Biotechnol. Adv.* **2000**, *18*, 121.
[25] H. Wernerus, J. Lehtio, P. Samuelson, S. Stahl, *J. Biotechnol.* **2002**, *96*, 67.
[26] K. Hantke, *Mol. Gen. Genet.* **1981**, *182*, 288.
[27] E. A. Bayer, M. Safars, M. Wilchek, *Anal. Biochem.* **1987**, *161*, 262.
[28] I. Benz, M. A. Schmidt, *Mol. Microbiol.* **1992**, *6*, 1539.

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