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Functional esterase surface display by the autotransporter pathway in *Escherichia coli*

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Abstract

Bacterial surface display is a promising tool for a wide variety of biotechnological applications. In this work, a carboxylesterase, EstA from *Burkholderia gladioli* was translocated to the surface of *Escherichia coli* using the autotransporter pathway. For this purpose, an artificial gene was constructed by PCR, that encodes a fusion protein of EstA and the essential autotransporter domains. Esterase activity of whole cells expressing the EstA-autotransporter fusion protein could be detected by a filter overlay assay using α-naphthylacetate as substrate as well as by an agar plate pH-assay using *p*-nitrophenylacetate as a substrate. The specific esterase activity of whole cells was determined to be 1.7 mU/mg protein with *p*-nitrophenylacetate. After differential cell fractionation, the specific esterase activity of the outer membrane fraction was determined to be 23 mU/mg protein, using the same substrate. Western blot analysis of the different cell fractions with an EstA specific antibody yielded positive signals only in the outer membrane fraction. Furthermore, the detected protein band was in the correct size, as it was predictable from the amino acid sequence of the fusion protein. In activity staining of SDS-gels using α-naphthylacetate as a substrate, it was the identical band that exhibited esterase activity. Surface exposure could be demonstrated by proteinase K digestion of the esterase domain, whereby the protease was externally added to intact cells. These results indicated that EstA is targeted to the surface of *E. coli* by the autotransporter pathway in its active form.

Keywords: Surface display; Esterase; *Burkholderia gladioli*; Autotransporter; Activity staining

1. Introduction

Among hydrolytic enzymes, esterases represent a group of broad natural variety concerning substrate and reaction type. Due to their diverse substrate specificities and their stereoselectivity, esterases have successfully been used in the synthesis of optically pure organic substances [1,2]. More recently, esterases have also been the subject in laboratory evolution approaches in order to obtain tailor-made biocatalysts [3,4]. In the evolutive approach, it is of striking advantage to express the enzyme to be evolved on the surface of a living cell. First, a substrate to be processed does not need to cross a membrane barrier but has free access. Second, by selecting a specific variant displayed on the surface, the cell, which contains the respective gene can be co-selected. This can be used for rapid sequence determination and first steps in structure prediction. Moreover, if the enzyme was improved towards a certain industrial application, enzyme-coated cells would obviate the need for enzyme preparation and purification. Therefore,

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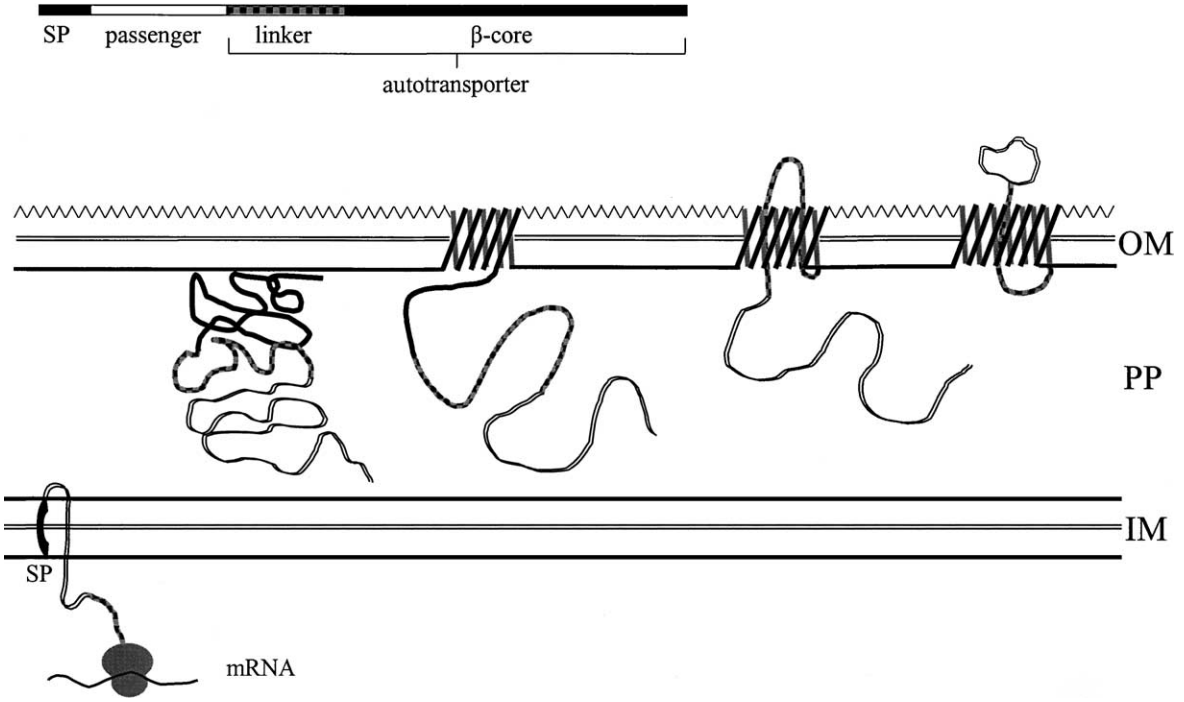

Fig. 1. Autodisplay: surface display of recombinant proteins by the autotransporter pathway in *Escherichia coli*. To be transported, the recombinant passenger must be an integral part of a precursor protein. SP: signal peptide; IM: inner membrane; PP: periplasm; OM: outer membrane.

the aim of this study was to express an esterase in its active form on the surface of *Escherichia coli* as first step towards its molecular evolution. For this purpose, the autodisplay system was used, which is based on the secretion mechanism of the autotransporter family of proteins [5] and was successfully applied before for the surface display of recombinant proteins [6–8]. To be translocated to the surface by the autotransporter pathway a recombinant passenger needs to be an integral part of a precursor protein (Fig. 1). Beside the passenger, this precursor contains a signal peptide necessary for transport across the inner membrane, a C-terminal β-barrel for transport across the outer membrane and a connecting peptide, a so-called linker to ensure full surface access. In the present study, the *EstA* gene from *Burkholderia gladioli* [9] was fused with the genes of the autotransporter domains in order to obtain such a functional precursor. The esterase activity of whole cells as well as of cellular fractions was determined by different methods and the fate of the precursor was analyzed by Western blot. The results obtained indicate, that esterase EstA can be translocated in its active form to the surface of *E. coli* by the autotransporter pathway.

2. Experimental

2.1. Bacterial strains, plasmids and culture conditions

The *E. coli* strain BL21 (DE3) [F^- *ompT hsd*S_B ($r_B^- m_B^-$) *dcm gal* (DE3)] was used for the expression of autotransporter fusion proteins. *E. coli* TOP10F′ (F′ {*lac*I^q Tn10 (Tet^R)} *mcr*A Δ(*mrr*-hsdRMS-*mcr*BC) Φ*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *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 subcloning of PCR products, were obtained from Invitrogen (Groningen, The Netherlands). For whole cell protease digestions, *E. coli* K12 DC2 [10], which

was kindly provided by H. Locher (Morphochem, Basel, Switzerland) was used. Plasmid pMSH81, which encodes EstA from *B. gladioli*, and plasmid pJM7, which encodes the AIDA-I autotransporter domains, have been described earlier [6].

Bacteria were routinely grown at 37 °C on Luria–Bertani (LB) agar plates containing 100 mg ampicillin/l. For differential cell fractionation and outer membrane preparations, cells were cultured in the presence of 10 μM ethylenediaminetetraacetate (EDTA) and 10 mM β-mercaptoethanol in liquid LB medium.

2.2. Recombinant DNA techniques

For the construction of plasmid pES01, that contains the gene encoding the esterase-autotransporter fusion protein, the EstA gene was amplified by PCR from plasmid pMSH81 [9] using oligonucleotide primers 2397 (5′-CGCTCGAGGGCGGCGGTGACGACA-AC-3′) and 2396 (5′-GGGGTACCCTTGGTGA-CG-CCGGCCG-3′). The amplified EstA gene is devoid of its own signal peptide and has four amino acid modifications, that proved to be beneficial for recombinant expression (Paar and Schwab, unpublished data; Fig. 2). Preparation of plasmid DNA, ligation, restriction digestion, transformation procedures and DNA electrophoresis were performed according to standard procedures.

2.3. Differential cell fractionation and outer membrane preparation

Bacteria were grown overnight and 1 ml of culture was used to inoculate 20 ml LB medium. Cells were cultured at 37 °C with vigorous shaking (200 rpm)

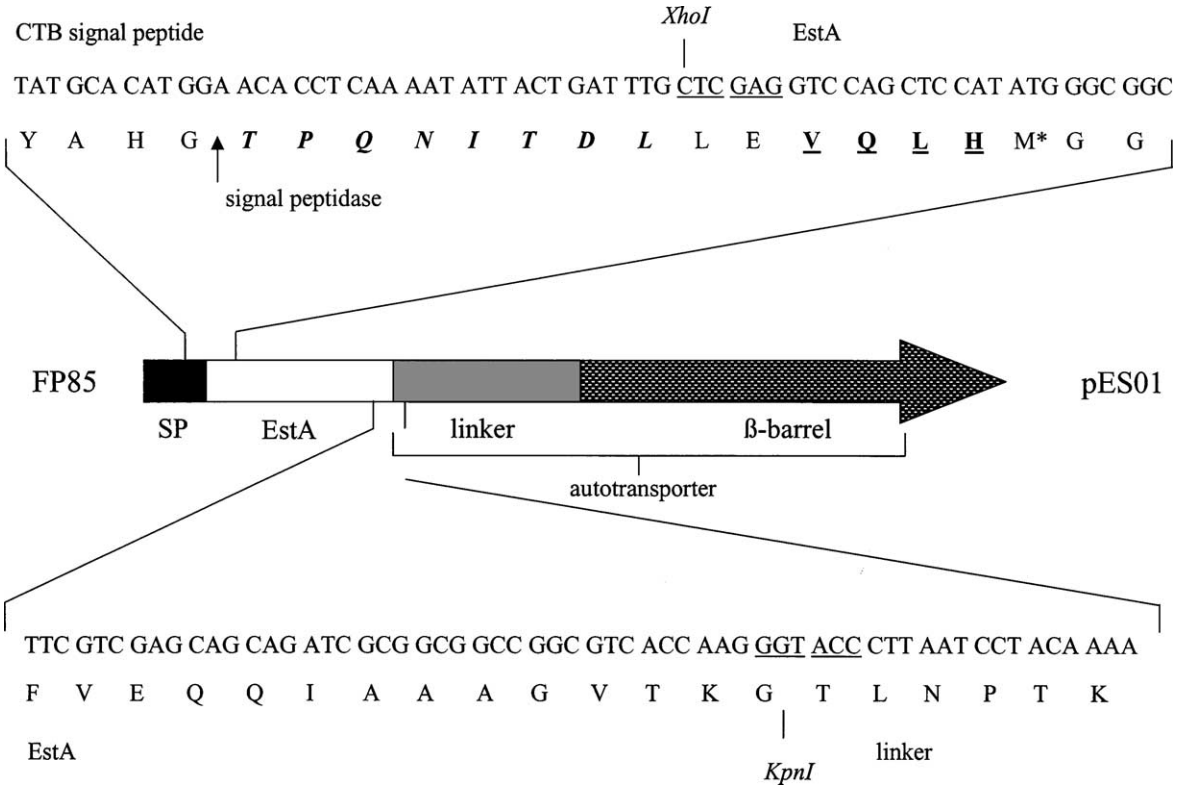

Fig. 2. Structure of the EstA-autotransporter fusion protein FP85 encoded by pES01. The environments of the fusion sites are given as sequences. The eight amino acids of CTB origin are written in italics. The signal peptidase cleavage site is marked by an arrow. Amino acid modifications of EstA proved to be beneficial for recombinant expression are underlined and the methionine replacing the starting amino acid cysteine of native EstA is marked with an asterisk.

for about 5 h until an OD_{578} of 0.7 was reached. After harvesting and washing with 0.2 M Tris–HCl, pH 8, differential cell fractionation was performed according to the method of Hantke [11]. Briefly, the cells were lysed by the addition of lysozyme (0.04 mg/ml end concentration) in the presence of 10 mM saccharose and 1 M EDTA for 10 min at room temperature. Then aprotinin was added at a final concentration of 1 μg/ml, as well as 5 ml extraction buffer (2% Triton-X-100, 50 mM Tris–HCl, 10 mM $MgCl_2$) and 0.1 mg DNAse. After 30 min on ice, the lysate was centrifuged at 1,600 × *g* for 5 min to remove intact bacteria and large debris. The clarified bacterial lysate was centrifuged at 23,500 × *g* for 60 min, and the resulting supernatant, containing soluble cytoplasmic and periplasmic proteins, was completely aspirated. The pellet was suspended in 10 ml phosphate-buffered saline (PBS) plus 1% Sarcosyl (*N*-lauryl sarcosinate, sodium salt) and centrifuged 60 min at 23,500 × *g*. The resulting supernatant representing the sarcosyl-soluble cytoplasmic membrane proteins was transferred to a fresh tube. The pelleted outer membrane proteins were washed twice with 10 ml of water, than dissolved in 30 μl water and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The soluble and cytoplasmic proteins were precipitated by adding the four-fold volume of acetone and freezing for 1 h at −20 °C. After centrifugation at 23,500 × *g* for 10 min, the pelleted proteins were dissolved in water and used for SDS-PAGE. For spectrophotometrical analysis, Triton-X-100 membrane extracts were prepared as described [12].

2.4. SDS-PAGE and Western blot analysis

For SDS-PAGE, the outer membrane isolates were diluted 1:2 with sample buffer (100 mM Tris–HCl (pH 6.8) containing 4% SDS, 0.2% bromphenol blue, and 20% glycerol), boiled for 5 min and analyzed on 12.5% acrylamide gels. For Western blot analysis, gels were electroblotted onto polyvinylidene-difluoride (PVDF) membranes and the blotted membranes were blocked in PBS with 3% dry milk powder overnight. For immunodetection, membranes were incubated with the EstA-specific antiserum, diluted 1:200 in PBS with 3% bovine serum albumin for 3 h. The immunoblots were washed three times with PBS, the secondary antibody was added and incubated for 2 h at room temperature. Antigen–antibody conjugates were visualized by reaction with horseradish peroxidase linked goat anti-rabbit IgG secondary antibody (Sigma, Deisenhofen, Germany), diluted 1:2,000 in PBS. A color reaction was achieved by adding a solution consisting of 4-chloro-1-naphthol (2 ml; 3 mg/ml in ethanol), PBS (25 ml) and H_2O_2 (10 μl; 30%).

2.5. Esterase activity assays

2.5.1. Filter assay

Esterase activity produced from colonies on agar plates was tested by a filter overlay assay according to [9]. For this purpose, cells from an overnight liquid culture were washed, diluted in PBS and an adequate dilution was plated out to result in single colonies after incubation overnight at 37 °C. A developing solution was prepared by adding 1 ml of a 0.1 M sodium nitrite solution to 1 ml Fast Blue B (Sigma, Deisenhofen, Germany; 15 mg/ml in 0.4 M HCl). After 2 min at room temperature, 40 ml of a 0.1 M sodium phosphate buffer (pH 7.0) and 1 ml of an α-naphthylacetate solution (16 mg/ml acetone) were added. Filter disks (Schleicher & Schuell 597 circles, ϕ 50 mm) were soaked with developing solution. After excess fluid was allowed to drain, filter disks were placed on the agar plates with single bacterial colonies. Esterase activity became visible by a dark purple stain of the colonies and the filter paper.

2.5.2. Agar plate pH-assay

Bacteria from an overnight liquid culture were streaked out on agar plates containing 0.1% (w/v) *p*-nitrophenyl acetate, 0.01% bromcresol purple (w/v) as an pH-indicator and 1% agar in 50 mM Tris–HCl buffer (pH 7.5) and incubated at 37 °C. According to [13], bacteria expressing active esterase alter the color of bromcresol purple to yellow, due to the acetic acid produced.

2.5.3. Specific activity determination

To quantify whole cell esterase activity, cells were grown overnight in 20 ml LB-medium at 37 °C with vigorous shaking (200 rpm). One milliliter cell culture was pelleted and resuspended in 50 mM phosphate buffer (pH 8.0) to a final OD_{578} of 10. 32 μl of this cell suspension was added to 1.6 ml of a 0.1 M

Tris–HCl buffer H 7.0) solution with *p*-nitrophenyl acetate (4 mM dissolved in DMSO) as a substrate. Esterase activity could be measured spectrophotometrically in a photometer (Unicam, Cambridge, UK) as the increase in absorption at 405 nm due to the release of *p*-nitrophenol. One unit of esterase activity was defined as the amount of enzyme releasing 1 μmol/min of alcohol under assay conditions. For these conditions, the specific absorption coefficient for *p*-nitrophenol was determined to be 6,665 ml/(μmol cm). The protein content of whole cells was measured by the method of Honn and Chavin [14] using bovine serum albumin as a standard.

2.5.4. Activity staining of SDS gels

Activity staining of renatured proteins after SDS-PAGE was performed according to [15]. Briefly, SDS was removed by washing the gel four times in 50 mM disodium hydrogen phosphate plus 12.5 mM citric acid, pH 6.3 for 30 min. The first two washes contained 25% isopropanol. Gels were then soaked with α-naphthylacetate solution (16 mg/ml acetone) and incubated for maximum 30 min at 37 °C. Esterase containing protein bands adopted a dark violet almost black color by this procedure.

3. Results and discussion

3.1. Genetic fusion of the EstA gene to the autotransporter domains of AIDA-I

The *EstA* gene was amplified by PCR from plasmid pMSH81 and fused to the autotransporter encoding domains of the adhesion involved in diffuse adherence (AIDA-I; Fig. 2). The PCR primers added an *Xho*I site at the 5′ end and a *Kpn*I site at the 3′, end of EstA. The PCR fragment was inserted into vector pCR2.1-TOPO and recleaved with *Xho*I and *Kpn*I. The restriction fragment was ligated into plasmid pJM7, that was cleaved before with the same enzymes. Plasmid pJM7 is a pBR322-derived high copy number plasmid, that directs the expression of a choleratoxin$_{\beta}$-AIDA-I autotransporter fusion protein under control of the constitutive P_{TK} promoter [6]. Cleavage with *Xho*I and *Kpn*I results in the deletion of the choleratoxin$_{\beta}$ (CTB) encoding DNA region. Insertion of the EstA PCR fragment yielded plasmid pES01, which encodes a fusion protein consisting of the signal peptide of CTB, EstA and the AIDA-I autotransporter region, including a linker region that proved to be sufficient for full surface access in earlier studies [16]. Due to the ligation procedure, the artificial gene still contains eight amino acids of mature CTB. The fusion protein encoded by pES01 was named FP85 on the basis of its predicted molecular mass of 84.9 kDa, after being processed by the signal peptidase. The correct sequence of the PCR fragment and the ligation sites were controlled by DNA sequencing. OmpT, an outer membrane protease that catalyses the sequence specific release of surface exposed proteins [17], is present in most of the available *E. coli* host strains. As the linker used in our EstA-autotransporter fusion contains an OmpT protease specific cleavage site, surface display of EstA requires an *ompT*-negative host strain. Therefore, pES01 was transformed into *E. coil* BL21 (DE3) and the expression of FP85 was analyzed by different methods.

3.2. Esterase activity of BL21 (DE3) cells harbouring plasmid pES01

In order to find out whether BL21 (DE3) pES01 cells express active EstA, they were streaked out on agar plates containing *p*-nitrophenyl acetate and bromcresol purple as a pH-indicator. As a control BL21 (DE3) pJM7, expressing the identical autotransporter domains but CTB instead of EstA were used. As can be seen in Fig. 3B only cells harbouring plasmid pES01 induced a color change of bromcresol purple to yellow, indicating an acidification and thereby active esterase formation.

E. coli BL21 (DE3) pES01 cells were mixed in equal amounts with *E. coli* BL21 (DE3) pJM7 cells as a control and plated out on LB agar to obtain single colonies. The agar plates were overlaid with substrate-soaked filter disks and spots with a light brown color as well as spots with dark purple color appeared (Fig. 3A). By plasmid isolation and restriction patterning it turned out, that the light brown spots were from colonies containing plasmid pJM7 and the dark purple spots were without exception from colonies containing plasmid pES01. This indicated that the dark purple coloring was due to esterase activity expressed by BL21 (DE3) pES01 and that the filter paper overlay assay seems to be a valid method

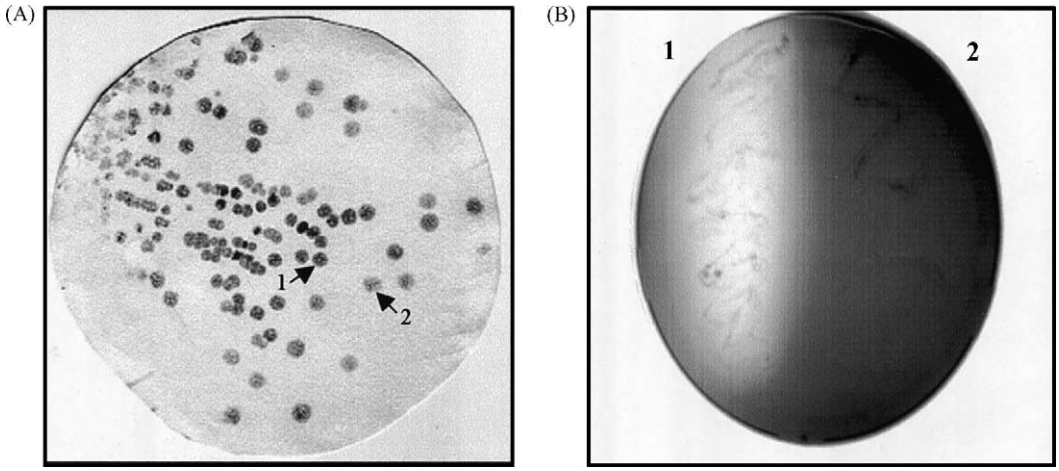

Fig. 3. Whole cell esterase screening assays. (A) Filter assay with a mixture of single colonies from *E. coli* BL21 (DE3) pES01 and *E. coli* BL21 (DE3) pJM007. (B) Agar plate pH assay. Bacteria were grown on agar plates containing *p*-nitrophenyl acetate and a pH indicator [13]. *E. coli* BL21 (DE3) pES01 expressing the EstA-autotransporter fusion protein FP85 (1) altered the color of bromcresol purple to yellow, whereas *E. coli* BL21 (DE3) harbouring pJM7 (2) applied as control did not. Agar plates were overlaid with naphthyl-soaked filter discs according to Schlacher et al. [9]. Arrow 1 marks a typical spot obtained by bacteria containing pES01 with a dark purple color. Arrow 2 marks a light brown spot caused by bacteria containing pJM7.

to differentiate between esterase positive and negative cells. It might be used to screen bacterial strains on esterase activity after surface display, an important step in the application of evolutive enzyme design. The light brown coloring appeared also with strains of *E. coli*, that were known and shown by different methods to be esterase negative (not shown). Therefore, it seems to be due to an unspecific staining by cell material and not to a residual esterase activity of BL21 (DE3).

To determine the specific esterase activity of *E. coli* BL21 (DE3) pES01 cells, expressing the autotransporter-EstA fusion protein, the release of *p*-nitrophenol from nitrophenyl acetate was followed photometrically at 405 nm (Fig. 4). For this purpose whole cells were applied to photometer cuvettes. The aim was to find out, whether the release of *p*-nitrophenol could be measured in the presence of bacteria, which have an absorption of their own at this wavelength. This is an important step towards the use of microtiter plates to screen for enzymatically active variants with chromogenic substates. By setting the obtained activity in relation to the protein content of these cells determined by the Honn and Chavin method [14] a specific esterase activity of 1.7 mU/mg protein could be calculated. The release of *p*-nitrophenol by control cells under the same conditions was negligibly low.

Taken together, these results indicate that *E. coli* BL21(DE3) harbouring the plasmid pES01, which

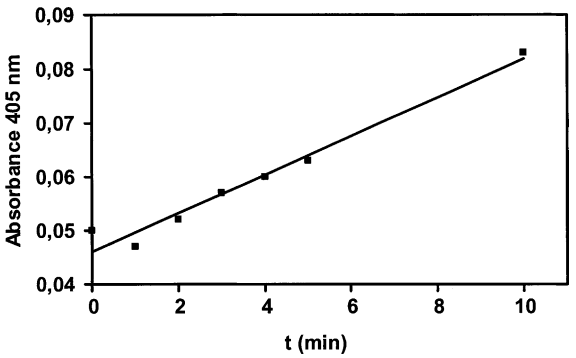

Fig. 4. Release of *p*-nitrophenol from *p*-nitrophenyl acetate by whole cells of *E. coli* BL21 (DE3) pES01. For this purpose 32 μl cell suspension with an OD_{578} of 10 was added to 1.6 ml reaction mixture in a photometer cuvette and absorption at 405 nm was recorded at different time points. For specific esterase activity, the values were set in relation to the protein content of whole cells, determined by the method of Honn and Chavin [14].

encodes the autotransporter EstA fusion protein FP85, express significant esterase activity, whereas control cells with an identical plasmid but a different passenger protein (CTB) did not. It clearly shows that EstA is active when it is expressed as a fusion to the autotransporter domains.

3.3. Surface expression of the EstA-autotransporter fusion protein

Different cell fractions were obtained by treating *E. coli* BL21(DE3) pES01 with the sarcosyl-method according to Hantke [11]. Besides the unfractionated control (whole cell fraction), only the outer membrane fraction exhibited significant release of *p*-nitrophenol from *p*-nitrophenyl acetate. The specific activity could be calculated as 23 mU/mg protein. This more than 10-fold increase in specific activity when compared with the activity of whole cells (1.7 mU/mg) was a first indication, that FP85, containing an active esterase EstA was integrated into the outer membrane. The different cell fractions were subjected to SDS-PAGE followed by Western blot analysis with an EstA-specific rabbit antiserum. Only in the outer membrane fraction, a protein of the correct size as predicted for the EstA-autotransporter fusion protein could be detected, whereas the soluble fraction, consisting of cytoplasmic and periplasmic proteins, as well as the sarcosyl-soluble membrane fraction, representing the cytoplasmic membrane proteins, were negative for FP85 (Fig. 5A).

To verify esterase activity of FP85, activity staining was performed after SDS-PAGE with different cellular fractions from *E. coli* BL21 (DE3) pES01. Therefore, proteins were renaturated inside the gels and gels were soaked with α-naphthyl acetate substrate solution. Fig. 5B shows that signals appeared in the whole cell fraction (control) and the outer membrane fraction within the same protein bands that were detected by the EstA specific antibody in Western blot experiments. In the soluble and cytoplasmic membrane fraction, no signals were obtained. This clearly shows, that the EstA-autotransporter fusion is completely transported to the cell envelope with the esterase passenger being in an active form.

In principle, due to the autotransporter secretion mechanism, the passenger, in our case EstA, might be directed to the surface or to the periplasma (Fig. 1). To clarify whole cells of *E. coli* BL21 (DE3) were subjected to proteinase K digestion. Externally added proteases are generally too large to pass the outer membrane, and therefore, degradation of the passenger domain clearly indicates its surface exposure [8]. Proteinase K was added to whole cells in a final concentration of 50 μg/ml. After washing three times with PBS containing 3% FCS to stop protease digestion, outer membranes were prepared and subjected to SDS-PAGE, Western blotting and activity staining. As can be seen in Fig. 6, the addition of proteinase K to whole cells resulted in a complete loss of esterase activity. This indicated

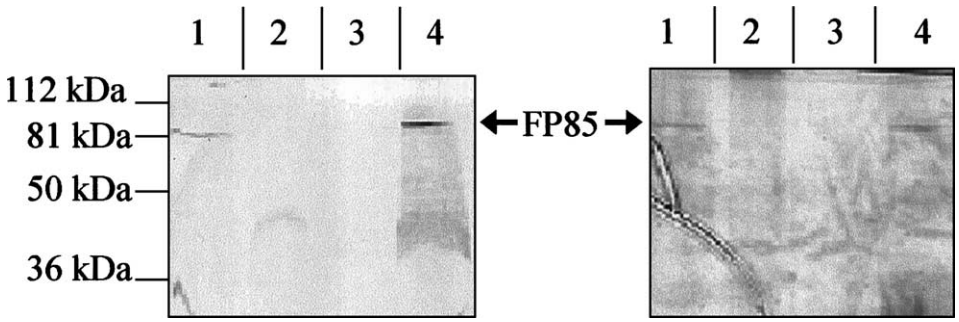

Fig. 5. Differential cell fractionation of *E. coli* BL21(DE3) pES01 cells. Lane 1, whole cell proteins; lane 2, soluble (cytoplasmic and periplasmic) proteins; lane 3, inner membrane proteins; lane 4, outer membrane proteins. (A) Western blot analysis by the use of an EstA-specific antiserum. (B) Activity staining with α-naphthylacetate after in gel renaturation of proteins [15]. Molecular weight markers are indicated at the left. The EstA-autotransporter fusion protein FP85 is marked by an arrow.

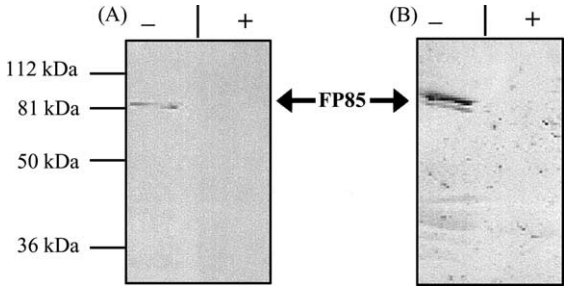

Fig. 6. Proteinase K treatment of whole cells of *E. coli* BL21 (DE3) pES01. Outer membranes were prepared after whole cells were incubated with (+) or without (−) proteinase K, separated by SDS-PAGE and subjected to (A) activity staining with α-naphthylacetate after in gel renaturation of proteins [15] or (B) Western blot analysis by the use of an EstA-specific antiserum. The molecular weight of marker proteins is indicated in kDa. The EstA-autotransporter fusion protein FP85 is marked by an arrow.

that the EstA passenger domain of the autotransporter fusion protein FP85 was accessible by externally added proteinase K, and therefore, was directed to the cell surface. In previous investigations trypsin digestion of whole cells has been used to verify surface display [6,8,18]. In our experiments, however, trypsin digestion had no influence on esterase activity of BL21(DE3) pES01 cells. To find out whether trypsin access was hindered by the lipopolysaccharide (LPS) in the cell envelope, plasmid pES01 was transformed into *E. coli* K12 DC2. Strain DC2 has an altered LPS structure that permits access of various substances to the outer membrane [10]. The addition of trypsin to whole cells of DC2 pES01 resulted in the degradation of EstA. As expected, this was also observed when proteinase K was added to DC2 pES01 cells. The natural outer membrane protein OmpA has a periplasmic moeity, that is degraded by trypsin when the periplasma is accessible for the protease [6]. In our experiments, the size and the amount of OmpA was not influenced by adding trypsin to whole cells (not shown). This indicated, that trypsin digestion of EstA with whole DC2 cells was indeed due to an improved surface accessibility and not to a leakiness of the outer membrane. In summary, our results show that the EstA-autotransporter fusion protein has integrated into the outer membrane by the β-barrel and that the EstA passenger is translocated to the surface, resulting in functional esterase surface display.

4. Conclusions

Fusion of esterase EstA from *B. gladioli* to the autotransporter domains of AIDA-I from *E. coli* [19] resulted in its translocation to the cell surface in an enzymatic active form. The activity could easily be measured with whole cells without the need of cell rupturing. Two assays were presented, a filter overlay assay and an agar plate pH assay, that will allow a rapid screening with whole cells for active esterase variants after random variation in a directed evolution approach. Therefore, the results presented here provide important basics for the evolutive design of a chiral biocatalyst using EstA as a scaffold. This is under progress currently.

Acknowledgements

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