COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Presentation of Functional Organophosphorus Hydrolase Fusions on the Surface of *Escherichia coli* by the AIDA-I Autotransporter Pathway

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ABSTRACT: We report, the surface presentation of organophosphorus hydrolase (OPH) and green fluorescent protein (GFP) fusions by employing the adhesin-involved-in-diffuse-adherence (AIDA-I) translocator domain as a transporter and anchoring motif. The surface location of the OPH–GFP fusion protein was confirmed by immunofluorescence microscopy, and protease accessibility, followed by Western blotting analysis. The investigation of growth kinetics and stability of resting cultures showed that the presence of the AIDA-I translocator domain in the outer membrane neither inhibits cell growth nor affects cell viability. Furthermore, the surface-exposed OPH–GFP was shown to have enzymatic activity and a functional fluorescence moiety. These results suggest that AIDA-I autotransporter is a useful tool to present heterologous macromolecule passenger proteins on the bacterial surface. Our strategy of linking GFP to OPH and the possibility to employ various bacterial species as host has enormous potential for enhancing field use.

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KEYWORDS: surface display; organophosphorus hydrolase fusions; AIDA-I autotransporter

Introduction

Neurotoxin organophosphate compounds are widely used in many pesticides and chemical nerve agents (Donarski et al., 1989; Tuovinen et al., 1994). Organophosphorus hydrolase (OPH), isolated from soil microorganisms, shown to degrade a broad spectrum of toxic organophosphates (Grimsley et al., 1997; Mulbry and Karns, 1989) with high turnover rate is of great interest for bioremediation and nerve agent degradation. The use of natural isolates as biocatalysts is an attractive strategy for treatment of these compounds. However, the transport limitation of substrates across the cell membrane is a barrier affecting whole-cell biocatalytic efficiency (Chen and Mulchandani, 1998). A way to enhance OPH biocatalytic efficiency is the surface display of OPH. Several reports have indicated that OPH could be surface displayed by either the Lpp-OmpA fusion system or the ice nucleation protein (INP) system (Richins et al., 1997; Shimazu et al., 2001).

The exploitation of genetically engineered microorganisms to perform biodegradation of organophosphate compounds is an important task. Enumerating and assessing of genetically engineered microorganisms in polluted soil and aquatic environments can be assisted by using stable marker systems with an easily detectable phenotype. The green fluorescent protein (GFP), a unique marker that can

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be identified by non-invasive methods, requires no cofactor for its fluorescence and can also be expressed as a fusion protein at either the *N*- or *C*-terminus. As a marker for ecological studies, GFP was introduced into microbes without influencing the metabolism of the various hosts (Halfhill et al., 2005; Jansson, 2003; Tresse et al., 1998).

Fusion strategies to employ GFP for facilitating the online monitoring of OPH expression have raised considerable interest (Wu et al., 2000, 2001). However, the translocation of high-molecular-weight OPH–GFP fusions in gram-negative bacteria may be hampered by the cell envelope. Large molecules require specialized transport mechanisms to cross the cell membrane. The molecular machineries involved in these secretion processes vary widely in complexity, and various gene products and complex interplay of the exported proteins with the accessory components are often required (Pugsley et al., 1997).

The simplest and probably also most widely realized protein secretion mechanism is the autotransporter pathway (Henderson et al., 1998). The autotransporters are synthesized as precursor proteins containing all structural requirements for the transport to the cell surface without any additional needed protein. Among this family, AIDA-I, an *Escherichia coli* adhesin involved in diffuse adherence initially described by Benz and Schmidt (1989, 1992a,b); Konieczny et al. (2001); Suhr et al. (1996), is synthesized as a 132-kDa preproprotein featuring a signal peptide which is cleaved during transport through the inner membrane, a 78 kDa adhesin (passenger) domain, and a 45 kDa translocator. These features already indicate that this autotransporter has a large capability in translocating relatively large passengers. It is an easy-to-handle system and a wide variety of recombinant proteins has already been functionally expressed on the bacterial surface by using the AIDA system (Jose, 2006; Müller et al., 2005). More than 10^5 recombinant molecules have been reported per single cell without reducing cell viability or cell integrity (Jose et al., 2001). In addition, AIDA occurs naturally in *E. coli* and was therefore considered a superior tool for the surface presentation in its homologous host.

In the present study, we developed a fusion strategy to facilitate the online monitoring of OPH surface presentation and used high-molecular-weight OPH–GFP fusions as a passenger in autodisplay. The aim of this study was to evaluate whether the AIDA system would be suitable to achieve surface presentation and stable online monitoring of active OPH to perform biodegradation of organophosphate compounds.

Materials and Methods

Please see Supplemental Material.

Results

Construction of OPH–GFP–AIDA autotransporter fusion proteins

For successful surface presentation of a recombinant passenger protein by the autotransporter pathway, a gene needs to be constructed that encodes an artificial precursor protein. This precursor consists of a signal peptide at the *N*-terminus, the passenger (OPH–GFP fusion protein), a linker region and the β-barrel structure of the AIDA

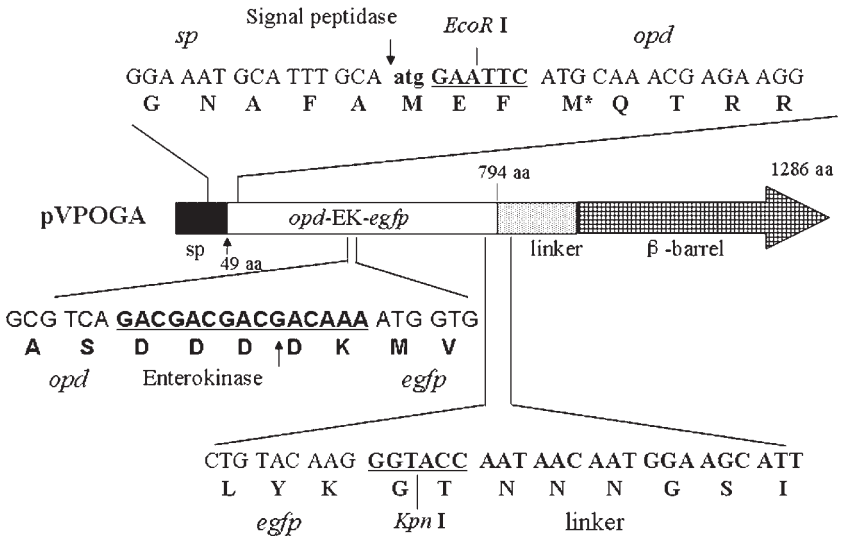

Figure 1. Structure of the opd-egfp-autotransporter fusion proteins. The environment of the fusion sites between OPH–GFP and the autotransporter domains are given as sequences. The signal peptidase cleavage site is marked by an arrow. The starting amino acid (methinine) of OPH is marked with an asterisk. Restriction sites are underlined. The opd with the egfp gene are linked by an EK cleavage site. The autotransporter domain (from 794 aa to 1286 aa in AIDA-I) is designed for the transport of the recombinant fusions.

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translocator (Fig. 1). For this purpose the plasmid pVPOGA in which the AIDA-I autotransporter fusion proteins is under the control of a tightly regulated *tac* promoter was constructed (Please see Supplemental Material for details). Since surface presentation of the fusion protein requires an *omp*T negative host strain, pVPOGA was transformed into *E. coli* UT5600.

Surface expression of OPH–GFP fusion using the AIDA translocator

To examine whether OPH–GFP fusions was successfully synthesized on the cell surface, the UT5600 producing OPH–GFP–AIDA fusion protein were fractionated into whole-cell lysate and outer membrane fraction and the fractions analyzed by Western blot analyses using the rabbit anti-OPH antibody and rabbit anti-GFP antibody, respectively. The antibodies were subsequently detected with alkaline phosphatase conjugated goat anti-rabbit IgG. The bands corresponding to the 120 kDa fusion protein were detected in whole-cell lysates and the outer membrane fraction. As expected, no signals were detected in the control (UT5600 cell harboring the shuttle vector pVLT33) and the soluble fraction (Fig. 2a and b). Additional confirmation of the presentation of fusion proteins on the cell surface was demonstrated by enterokinase (EK) digestion analysis. EK recognizes an Asp–Asp–Asp–Asp–Lys sequence and cleaves the peptide bond after the lysine residue in the peptide linker between GFP and OPH. As shown in Figure 2a (lane 5), after incubation and digestion of the membrane fraction with EK, no signal was found on the nitrocellulose membrane after incubating with anti-OPH antibody, whereas Western blot analysis using anti-GFP antibody revealed a prominent band corresponding to a molecular mass of about 80 kDa which is the calculated molecular mass of the GFP–AIDA fusion protein (Fig. 2b, lane 6), demonstrating the surface expression of the GFP-part of the fusion protein.

Surface localization of OPH–GFP fusion

The presentation of OPH–GFP fusion proteins using the AIDA translocator was also confirmed by immunofluorescence microscopy. As shown in Figure 3, UT5600 control cells harboring pVLT33 were not fluorescent at all (Fig. 3a), while UT5600 cells harboring pVPOGA exhibited green fluorescence (Fig. 3b). Furthermore, upon incubation with anti-GFP probe antiserum followed by Rhodamine-conjugated secondary antibody, the transformants were brightly fluorescent (Fig. 3c), indicating that the GFP-part of the fusion protein was successfully displayed on the cell surface.

Relationship between GFP fluorescence and OPH activity

The time-course of OPH activity and GFP fluorescence expression in UT5600 cells harboring pVPOGA is shown in Table I. OPH activity was not detected and GFP fluorescence remained at the original background level at 0 h (post-induction). After 0.5 mM IPTG induction, the activity and fluorescence were rapidly and dramatically promoted in the

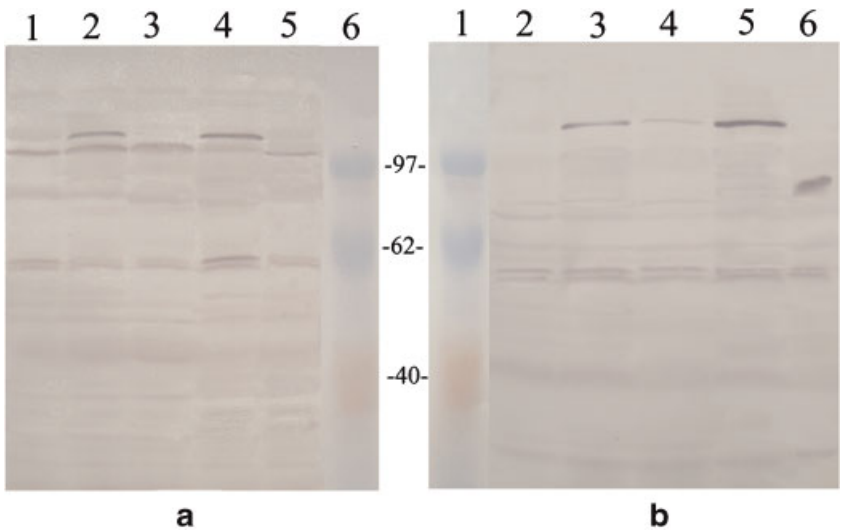

Figure 2. Expression of pVPOGA in UT5600 as assessed by Western blot analysis. **a**: Western blot analysis of differential cell fraction of UT5600 cell harboring pVPOGA incubated with anti-OPH antiserum. **Lane 1**, negative control (UT5600 cell harboring pVLT33); **lane 2**, whole-cell lysates; **lane 3**, soluble proteins; **lane 4**, outer membrane proteins; **lane 5**, EK digested outer membrane proteins; **lane 6**, marker proteins. **b**: Western blot analysis using anti-GFP antibody. **Lane 1**, marker proteins; **lane 2**, negative control; **lane 3**, whole-cell lysates; **lane 4**, soluble proteins; **lane 5**, outer membrane proteins; **lane 6**, EK digested outer membrane proteins. The molecular weight of marker proteins applied is indicated in kilodaltons. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

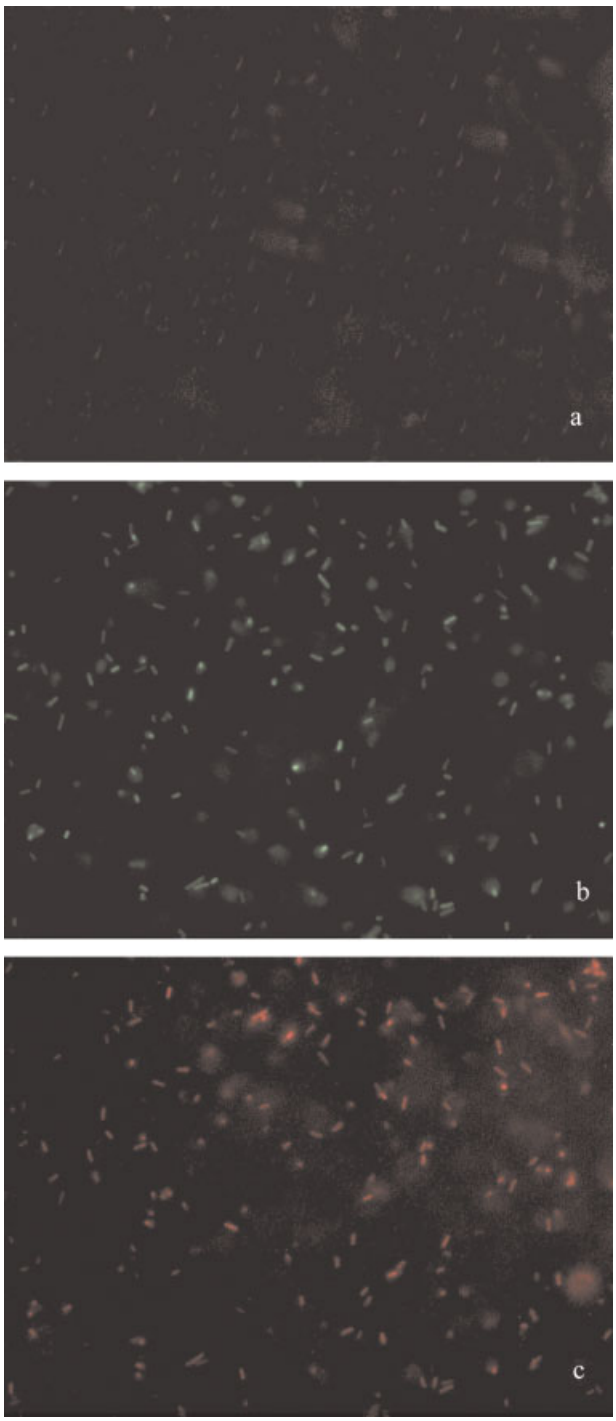

Figure 3. Immunoflurescence micrographs of UT5600 harboring pVLT33 and pVPOGA. **a**: UT5600 cells harboring pVLT33 probed with anti-GFP antiserum and fluorescently stained with Rohdamine-conjugated goat anti-rabbit IgG. **b**: Cells harboring pVPOGA under FITC filter. **c**: Cells harboring pVPOGA probed with anti-GFP antiserum and fluorescently stained with Rohdamine-conjugated IgG. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

following 24 h. The parallel increases in OPH activity and GFP fluorescence indicate that the two elements of the OPH–GFP fusion could successfully function on the cell surface.

Table I. Whole-cell OPH activity and GFP fluorescence assay.

Post-induction (h)	OPH activity (U/mL)[a]	Fluorescence intensity[a]
0	ND	46 ± 9
6	0.023 ± 0.0035	277 ± 15
12	0.0317 ± 0.0021	481 ± 26
24	0.0364 ± 0.0028	493 ± 19

[a]Values are Mean $\pm$ SD ($n = 3$).

Stability of cultures expressing OPH–GFP–AIDA fusion

To test whether expression of OPH–GFP–AIDA inhibits cell growth, growth kinetics of cells carrying pVPOGA and pVLT33 were compared. As shown in Figure 4, both cultures reached the same final cell density after 24 h of incubation. No growth inhibition was observed for cell expressing the fusion proteins. To evaluate whether expression of outer membrane protein result in membrane destabilization and cell lysis, the stability of the suspended cultures was monitored. Whole-cell activity of pVPOGA remained at almost the original level over 1-week period (Fig. 5).

Discussion

Microbial cell surface presentation has become a focus of interest due to its potential applications in biomedicine, biosensors, and vaccine development. Especially, presentation of active enzymes has been intensively pursued for its potential to be used as whole-cell biocatalysts in the fields of pharmaceutical, fine chemical, and agrochemical production. Many different proteins, including S-layer proteins, outer membrane proteins, lipoproteins, subunits of surface appendages, and autotransporters, have been successfully employed as anchoring motifs in microbial cell surface presentation (Lee et al., 2003; Samuelson et al., 2002). Among the various secretion mechanisms employed to transport ''passenger'' proteins onto the cell surface, the

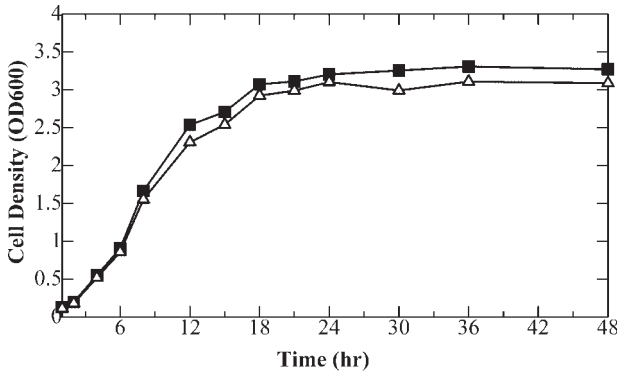

Figure 4. Cell growth kinetics of UT5600 harboring pVLT33 (■) and pVPOGA (△).

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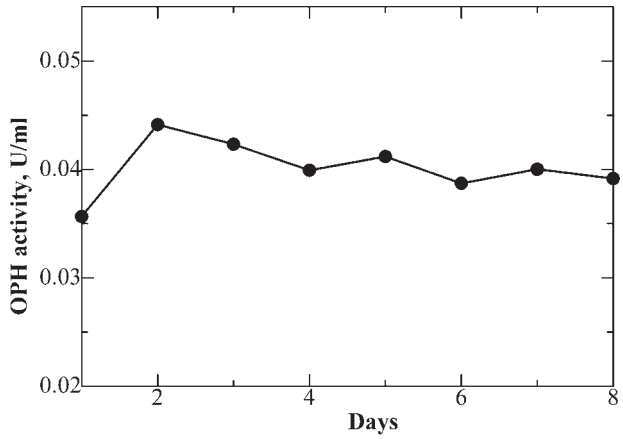

Figure 5. Whole-cell OPH activity in suspended UT5600 culture expressing OPH–GFP on surface.

autotransporter pathway is unique by its apparent simplicity. Recently, the functional β-lactamase, esterases, sorbitol dehydrogenase, bovine adrenodoxin, and protease inhibitor aprotinin had been successfully displayed by using the AIDA translocator domain (Jose and Schwichow, 2004; Jose and Zangen, 2005; Jose et al., 2001; Lattemann et al., 2000; Schultheiss et al., 2002). In this report, we describe the export of an active enzyme to the surface of *E. coli* cells by the antotransporter secretion pathway. The OPH–GFP–AIDA fusion protein was efficiently targeted to the *E. coli* UT5600 cells, and the activity of both the OPH and GFP was shown to be surface exposed. Successful presentation of the OPH–GFP fusions was confirmed by Western blotting analysis and immunofluorescence microscopy.

Surface presentation of OPH has been intensively studied in the recent decades. Multiple reports have indicated that OPH could be displayed by the Lpp-OmpA fusion system or the INP system on the surface of *E. coli*, *Pseudomonas putida*, and *Moraxella* (Richins et al., 1997; Shimazu et al., 2001, 2003). However, expression of Lpp–OmpA–OPH resulted in severe growth inhibition. Cells in liquid cultures showed a tendency for aggregation and were prone to cell lysis (Shimazu et al., 2001), In addition, this system proved to be sensitive to various secondary and tertiary structures of the passenger (Shi and Su, 2001). The INP system appears to represent a promising display system for bacteria. However, the translocation mechanism is still unknown. In the present report, our data suggest that AIDA can be used as an efficient translocator for the surface presentation of a large heterologous macromolecule (63 kDa), and no growth inhibition was observed for cell expressing large fusion proteins. This observation coincides with the surface expression of recombinant proteins beyond 10^5 molecules per single cell without reducing cell viability and cell integrity, that is, the number of colony forming units remained unaltered (Jose et al., 2001; Schultheiss et al., 2002). Above results indicate that AIDA-I is one of the more promising presentation systems for bacteria available, and can possibly be used for heterologous high-molecular-weight active molecule translocation.

As a distinct family of secreted proteins, autotransporter has been the subject of intensive studies on their structure and function. However, the last step in transport remains subject to controversial discussions. Whereas multiple studies reported that the passenger domain is transported in an unfolded state (Jose and Zangen, 2005; Lattemann et al., 2000), some results showed that the proteins in a folded conformation can be translocated through the outer membrane (Veiga et al., 1999, 2002). These data resulted in a debate about the translocation models (Oomen et al., 2004; Pohlner et al., 1987; Veiga et al., 2002). For the AIDA autotransporter a recent report showed that the AIDA translocator was mostly present as a monomer and that only a fraction of the AIDA translocator was found to form dimers on the bacterial surface (Müller et al., 2005). In the present report, the OPH, a stable unfolded enzyme which forms a homodimer in its active state (Grimsley et al., 1997), retains surface exposed activity corresponding to the report of a passenger-driven dimerization of sorbitol dehydrogenase expressed on the cell surface (Jose and Schwichow, 2004). This indicates that the AIDA translocator is able to form passenger-driven dimers that are functional on the bacterial surface. Meanwhile, According to its crystal structure, the GFP has been reported to consist of 11 antiparallel beta strands (Andresen et al., 2005). As the outer membrane translocator domain of AIDA, the beta-barrel is a beta-structure consisting of 14 beta-sheets. Our results show that OPH–GFP fusion protein is successfully transported to the cell surface of *E. coli* by AIDA translocator, indicating that the GFP domain may be translocated in unfolded state.

The OPH retained activity while fused to GFP, and the strategy of linking GFP to OPH has a wide potential for enhancing field use. To facilitate the online monitoring of OPH expressed, as noted earlier, several reports have demonstrated the potential utility of GFP for monitoring the expression and localization of OPH (Wu et al., 2000, 2001). These reports offer a possible way to monitor product level using GFP fluorescence, indicating that multiple gene strategies can be applied to a large protein when fused with GFP. However, these designs are focused on the relationship between the two parts of the OPH–GFP fusion and neglect the transport limitations for substrates as an obstacle affecting whole-cell biocatalytic efficiency. In the present study, we developed a strategy for the surface presentation of the OPH–GFP fusion by using the AIDA autotransporter translocator domain. Our design provides a proper balance between the whole-cell biocatalytic efficiency and online monitoring. Moreover, surface presentation is mediated via a middle-copy number broad-host range vector and this design has a potential application for of the functional expression of the fusion proteins on the surface of multiple hosts, such as the natural PNP degrader, *Moraxella* and *P. putida* JS444. Additionally, the fluorescence of GFP had

been shown to be pH sensitive within the physiological range (Shi and Su, 2001). The hydrolysis of organophosphate compounds would lower the medium pH due to the release of protons. Our fusion strategy could form a good basis for the future biosensor research project using these cells to evaluate fluorescence change as a function of organophosphate compounds concentration. These strategies are currently under investigation.

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