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Short communication

Biodegradation of endocrine disrupting dibutyl phthalate by a bacterial consortium expressing *Sphingobium* sp. SM42 esterase

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ABSTRACT

EsteraseG (EstG) from *Sphingobium* sp. SM42 efficiently degraded the toxic chemical, dibutyl phthalate (DBP). In this study EstG was successfully displayed on the surface of *Escherichia coli* using the *Pseudomonas aeruginosa* outer membrane protein, OprF, as a carrier protein. EstG was fused to the C-terminus of OprF at Lys^{164} resulting in export to the surface of *E. coli* that was detected by western blot analysis and flow cytometry, as well as immunofluorescence microscopy. The surface displayed EstG exhibited increased thermal stability, retaining 55, 43, and 39% of its original activity after 24 h incubation at 45, 55, and 65 °C, respectively. In addition, EstG was engineered to be efficiently secreted into the culture media of *Bacillus subtilis*, which exhibited 8-fold higher esterase activity than cell lysates. A bacterial consortium consisting of recombinant bacteria has high potential for DBP degradation by removing almost 70% of 1 mM DBP within 5 days.

1. Introduction

Cell surface display is a technique for expressing heterologous peptides or proteins on the cell surface of microorganisms, or mammalian cells by fusing a desired peptide or protein with an appropriate fusion partner [1]. The fusion partner facilitates transport of the passenger protein to be displayed across the cell envelop and anchors it onto the outer surface of the cell membrane. The most popular fusion partners are outer membrane proteins, such as OmpA, OprF, OmpS, FadL, and LamB, because they provide a number of advantages. These include: efficient secretary signals, unique membrane-spanning structures that provide fusion sites, and strong anchoring structures [2]. OprF is located in the major outer membrane of *Pseudomonas aeruginosa*. This protein plays an important role in controlling membrane integrity and cell shape, as well as acting as a nonspecific porin to allow transport of small hydrophilic molecules [3]. OprF has been reported as a successful fusion partner for expressing *Pseudomonas fluorescens* SIKW1 lipase on the surface of *Escherichia coli* [4]. Among the four fusion sites tested, a Lys^{164}-lipase fusion showed the highest whole cell lipase activity suggesting that Lys^{164} of OprF is a good fusion site for *P. fluorescens* SIKW1 lipase [4].

Display on the cell surface gives an enzyme access any external substrates and therefore membrane penetration by the substrate is not a limiting issue. An alternative strategy to avoid the substrate penetration problem is the use of *Bacillus subtilis* secretory system. *B. subtilis* has been used as a host for secretion of various commercially important products such as enzymes, biochemicals, antibiotics, and insecticides [5]. Advantages of employing this bacterium as a host are its nonpathogenicity and the capability of secreting functional extracellular proteins directly into the culture medium [6].

DBP is the most abundant phthalate ester and is commonly used as a plasticizer in the plastic industry [7]. Release of this compound into an ecosystem occurs during the production, use and disposal of plastic products [8] and significant amounts of DBP have been found to contaminate diverse environmental samples including: ground water, river, ocean, drinking water, and sediments [7]. In addition, this chemical enters the food chain by accumulating in plants, which are then consumed by humans and animals [9]. DBP is known as an endocrine-disrupting chemical that interferes with the reproductive system and behavior in humans [10]. Esterases (EC 3.1.1.1, carboxylester hydrolases) are

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Table 1
Primers and plasmids used in this study.

Primer/Plasmid	Description/Sequence	References
BT3279	5′GAGATGATGGGATCCGCTTGA 3′	[11]
BT3280	5′CGCGGGCGCAAGCTTGAGCGCCCA 3′	[11]
BT3391	5′ATCAAACTGAAGAACACCTTA 3′	This study
BT3392	5′GTCCAAGTTTCGAACCACCGA 3′	This study
pDUETestG	*estG* in high expression vector pRSFDuet1, Km^R	[11]
pOprFestG	*oprF-estG* in high expression vector pET, Ap^R	This study
pBESestG	*estG* in *B. subtilis*/*E. coli* shuttle vector pBE-S DNA, Km^R and Ap^R	This study

a group of enzymes that catalyze the hydrolysis and synthesis of short chain ester-containing molecules. These enzymes have considerable potential for production of important ester compounds required for the pharmaceutical, food, biochemical industries, as well as those of biological interest. The esteraseG gene and enzyme have been isolated from the soil bacterium *Sphingobium* sp. SM42, which can utilize toxic dibutyl phthalate (DBP) as a sole carbon source [11] and EstG has been shown to efficiently degrade DBP, both *in vitro* and *in vivo* [11]. Several studies have reported the isolation of bacterial strains that are involved in biodegradation of DBP, for example: *Arthrobacter* sp. ZH_2 [12], *Gordonia* sp. [13], and *Rhodococcus* sp. JDC-11 [14], which use phthalate 3,4-dioxygenase as a major DBP-degrading enzyme. In addition, dibutyl phthalate hydrolase has been reported as a DBP-degrading enzyme in *Acinetobacter* sp. M673 [15]. However, there are no reports focused on the biodegradation of DBP by recombinant bacterial consortia let alone those expressing surface-displayed, together with secreted, microbial esterases.

In this study, we report the efficient cell surface display of esteraseG (EstG), using OprF as a fusion carrier in *E. coli*, as well as the expression of recombinant secretory EstG in *B. subtilis*. The recombinant *E. coli* and *B. subtilis* expressing intra, extracellular, and surface-expressed EstG were employed as a bacterial consortium. By doing this, their capacity to degrade toxic DBP was higher when compared to the individual strains. This is the first report of the use of surface-displayed and secretory esterase as a DBP degrading bio-system. The results suggest that this bacterial consortium has a significant potential for application in biodegradation of toxic DBP.

2. Materials and methods

2.1. Chemicals and media

Dibutyl phthalate (DBP) and *p*-nitrophenyl butyrate (pNB) were purchased from Sigma-Aldrich (USA). All other chemicals were commercial products of biological and analytical grade. Bacterial cells were grown on solid and liquid Luria-Bertani (LB) medium supplemented with the appropriate antibiotics.

2.2. Construction of plasmids

All bacterial strains, plasmids, and primers used in this study are listed in Table 1. The N-terminus of *oprF*, encoding the first 164 amino acids, was amplified from the *P. aeruginosa* strain PAO1 chromosome using BT3391 primer, which contains an *EcoR*V site and BT3392 primer containing an *EcoR*I site. Then the 600 bp PCR product was ligated into *EcoR*V-*EcoR*I digested pETBlue2 vector (Invitrogen, USA) to produce pOprF. Plasmid pOprFestG was constructed by digestion of pDUETestG [11] with *Hinc*II and *Hind*III to obtain the *estG* gene. The *estG* fragment was inserted into *Hinc*II-*Hind*III digested pOprF. To construct pBESestG, the *BamH*I-*Hind*III estG fragment was amplified from pSM44 [11] using BT3279 and BT3280 primers. The fragment was then inserted into *BamH*I-*Hind*III digested pBE-S DNA (Takara Bio Inc., Japan) to produce pBESestG. Transformation of *B. subtilis* CU1065 with pBESestG was carried out according to the manufacturer's instructions.

2.3. Bacterial strains and growth conditions

Plasmid pOprFestG was transformed into *E. coli* DE3 and the recombinant cells were cultured in Luria-Bertani (LB) medium supplemented with ampicillin (100 mg/L). The expression of OprF-EstG fusion protein was induced with 0.2 mM IPTG when the OD_{600} was 0.4 followed by shaking for 18 h at 37 °C. *E. coli* DE3 harboring two recombinant plasmids pOprFestG and pDUETestG were cultured in LB medium supplemented with ampicillin (50 mg/L) together with kanamycin (15 mg/L). When the OD_{600} reached 0.4 the expression of surface and intracellular esteraseG was induced by adding 0.2 mM IPTG and the cells were further cultured at 37 °C. To produce secretory esteraseG, *B. subtilis* CU1065 harboring plasmid pBESestG was cultured in LB medium supplemented with kanamycin (10 mg/L) at 37 °C.

2.4. Western blotting

The presence of OprF-EstG fusion protein on the outer membrane was analyzed by 10% (w/v) SDS-PAGE. To prepare the outer membrane protein, IPTG-induced *E. coli* DE3:pOprFestG was harvested and the cell pellet was washed with 1 ml of 10 mM phosphate buffer pH 7.2. The cell sample was centrifuged at 3500*g* for 5 min at 4 °C and crude extract was prepared by resuspending the cell pellet in 0.5 ml of 10 mM phosphate buffer pH 7.2 followed by cell disruption by three cycles of sonication (20 s each). The crude lysate was obtained after centrifugation at 12,000*g* for 2 min at room temperature to remove cell debris. The supernatant was collected and centrifuged at 12,000*g* for 30 min at 4 °C to separate the membrane proteins and lipid layers. Then 0.5 ml of 10 mM phosphate buffer pH 7.2 containing 0.5% (w/v) sarcosyl was used to resuspend the pellet. After incubation at 37 °C for 30 mins, the membrane proteins were obtained by centrifugation at 12,000*g* for 30 min at 4 °C. The insoluble pellet containing membrane proteins was washed with 10 mM phosphate buffer pH 7.2 and resuspended in Tris-EDTA buffer pH 8.0. Western blot analysis was performed by transfer of the protein in SDS-PAGE gel to a nitrocellulose membrane (15 V, 45 min) followed by incubation of the membrane with 3% skimmed milk overnight at room temperature to prevent nonspecific binding. For imumunodetection of the EstG-OprF fusion protein, rabbit anti-esteraseG antibodies and peroxidase conjugated-goat anti-rabbit immunoglobulin G were used. The light-emitting nonradioactive ECL kit (Amersham Life Sciences, Buckinghamshire, UK) was used for signal detection.

2.5. Immunofluorescence microscopy and flow cytometry analysis

A culture of induced *E. coli* DE3:pOprFestG was adjusted to OD_{600} of 0.1 and the cells were harvested by centrifugation. The cell pellet was washed twice with phosphate-buffered saline (PBS) pH 7.4 and resuspended in PBS containing 3% (w/v) bovine serum