

## Overexpression of parathion hydrolase in *Escherichia coli* stimulates the synthesis of outer membrane porin OmpF

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### Abstract

Parathion hydrolase (PH), also known as organophosphorus acid anhydrase, hydrolyses the triester linkage found in organophosphates including organophosphate pesticides and the nerve gas sarin. The enzyme is reported to be membrane-associated and the immature protein has a signal sequence of 29 amino acids. In experiments designed to examine the post-translational processing of the enzyme and to assess the distribution of the precursor and mature forms of the protein, we induced expression of the *Flavobacterium balustinum* PH structural gene, *opd*, in *Escherichia coli* strain BL21. Western blotting revealed that the induced PH was predominantly membrane-associated in *E. coli* but a protein band equivalent in size to mature PH was also found to be induced specifically in periplasmic fractions. This periplasmic protein was not PH, as it did not cross-react in Western blots, and N-terminal sequencing of the induced protein showed it to have 100% homology to the outer membrane protein OmpF.

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### 1. Introduction

Soil microorganisms have acquired genetic capabilities to degrade a variety of toxic and recalcitrant chemicals that have been deliberately released into the environment by man. Several bacterial strains that can use organophosphate (op)-pesticides as a source of carbon have been isolated from soil samples collected from diverse geographical regions [1–4]. All these soil isolates synthesize an enzyme called parathion hydrolase (PH) and in each case the genetic information responsible for degradation of op-pesticides is found on large indigenous plasmids. Genetic analysis indicates that these plasmids are not closely related but that the gene responsible for organophosphate degradation (*opd*) is highly conserved [5,6]. PH has been shown to be a membrane-associated enzyme [7] and the precursor form of

the protein contains a signal sequence of 29 amino acids. Although considerable information is available on the genetics and biochemistry of PH, there is presently very little information on the post-translational modification and membrane targeting of the enzyme.

In recent times, op-pesticide degrading bacteria have received considerable attention as potential agents of bioremediation in clean-up operations of op-pesticide polluted environments and industrial waste [8,9]. However, the levels of PH in wild-type strains are not adequate to use them effectively in such operations and several attempts have been made to overproduce PH by cloning the *opd* gene in suitable expression vectors. *Escherichia coli* was initially found to be an unsuitable host for heterologous expression of the *opd* gene [10,11]. By contrast *Streptomyces lividans* proved very effective as in this host the otherwise membrane-bound PH is processed such that the mature form of the enzyme is exported into the medium as an exoenzyme [12–14]. In the present study, we have explored further the possibility of expressing His-tagged PH from *Flavobacterium balustinum*

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in *E. coli* to study post-translational modification and to optimise the process of expression and affinity purification of the enzyme. During the course of these investigations, we have found that expression of the outer membrane porin OmpF is specifically elevated in periplasmic fractions from *E. coli* cells induced for *opd* expression. These observations suggest a possible link between phosphate metabolism and PH expression in *E. coli*.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*Escherichia coli* strain BL21 [15] was grown in LB medium (10 g tryptone, 10 g NaCl, and 5 g yeast extract in 1 L of double distilled water). Plasmid-containing strains were grown in the presence of ampicillin (250 µg/ml). Plasmid isolation and routine molecular biology techniques were performed following standard procedures.

### 2.2. Cloning of the *opd* gene in pET-23b

The pET series of vectors (Novagen Inc.) are high copy number vectors designed for the high-level expression of foreign proteins as His-tag proteins in *E. coli*. The *opd* gene to be expressed was derived from *F. balustinum* [4] and had been cloned on 1.5 kb fragment in pUC18 to give pSS15. To clone the gene in pET-23b it was necessary to create an *Nde*I site at the 5' end and *Xho*I site at the 3' end of the gene. There are no other *Nde*I or *Xho*I sites within the gene (EMBL Accession No. AJ426431) and therefore two oligonucleotides containing an *Nde*I recognition site (5'-GCA AGG GGG CCA TAT GCA AAC GAG AAG GG-3') and a recognition sequence for *Xho*I (5'-GGA TCC AGA TGC TCG AGT GAC GCC CGC-3') were designed and used to amplify the *opd* gene. The *Nde*I site contains an in-frame ATG that places the complete *opd* gene sequence directly under the transcriptional and translational control of the vector. The *Xho*I site was engineered to replace the *opd* stop codon and ensures that a 6× His-tag is added immediately after the *opd* coding sequence. A PCR was performed for 30 cycles following standard protocols, using plasmid pSS15 as template and *Pfu* polymerase. The PCR product was purified using a Qiagen PCR purification kit and the PCR product was cloned in pET-23b to give the recombinant plasmid pHYS400.

### 2.3. Expression of His-tagged parathion hydrolase

Overnight cultures of *E. coli* cells containing either the expression vector pET-23b or the recombinant expression plasmid pHYS400 were grown in LB medium with appropriate antibiotics. Twenty millilitre of LB medium was inoculated with 200 µl of overnight culture and grown for 2–3 h until the cell density reached an  $A_{600}$  of 0.5. Then each culture was divided into two equal parts and to one 10 ml culture, IPTG was added to a final concentration of 1 mM

and induction was continued for 2 h. The other half of the culture was used as control without adding IPTG. At appropriate times 1.5 ml of the culture was suspended in 100 µl of Laemmli sample buffer, boiled for 5 min and analysed on 12% SDS-PAGE.

### 2.4. Cell fractionation and localisation of parathion hydrolase in *E. coli*

Cultures (20 ml) of *E. coli* BL21 containing appropriate plasmids were induced as described above. The cell pellets collected from these cultures were then washed with 50 ml of ice cold 50 mM Tris (pH 8.0) and the cells were again harvested by centrifugation at 12,000 rpm. The cell pellet was then resuspended in 200 µl of SET buffer (0.5 M sucrose, 5 mM EDTA, 50 mM Tris (pH 8.0), and 600 µg/ml lysozyme) and incubated at 30 °C for 1 h before centrifuging at 4 °C for 30 min at 12,000 rpm. The supernatant containing the periplasmic contents were transferred to a clean tube and stored at –20 °C. The pellet containing sphaeroplasts was resuspended in 5 ml of Tris (pH 8.0). Appropriate amounts of the periplasmic, cytoplasmic, and membrane fractions were analysed in duplicate by SDS-PAGE. One of the protein gels was stained with Coomassie brilliant blue and the other was used to blot the proteins on to PVDF membrane and probed using an anti-His tag antibody (Qiagen). Western blots were performed using ECL protein detection kits (Amersham) by following the manufacturer's protocols.

### 2.5. Protein sequencing

The periplasmic fractions prepared from induced cultures were separated by SDS-PAGE. The stained gel was then blotted on to PVDF membrane and the portion of the membrane containing the appropriate band was removed and used for determination of the N-terminal sequence using an Applied Biosystems Procise Sequencer in the John Innes Centre proteomics facility. PH was purified from the cell lysate by using Hitrap-Ni affinity column. The purified protein was then analysed by SDS-PAGE and blotted onto PVDF membrane. The N-terminal sequence was performed as described above.

## 3. Results and discussion

Parathion hydrolase is a membrane-bound enzyme and it contains signal sequence of 29 amino acids [11]. The predicted sizes of the enzyme with and without the signal sequence are 40 and 37 kDa, respectively. In *Flavobacterium* sp. the precursor form of PH is processed before targeting the enzyme to the membrane. However, there are no reports about the precise sub-cellular location and post-translational processing of the enzyme. Cells containing the expression plasmid pHYS400, or the vector pET-23b were induced with IPTG together with the host strain BL21. Protein extracts from induced and uninduced cultures were

analysed by SDS-PAGE and a protein band of approximately 40 kDa was found only in the induced cultures carrying pHYS400. A similar band was absent from the uninduced strain and all controls (Fig. 1A).

When Western blots were performed using anti-His antibodies only one clear signal was obtained that identified a 40 kDa protein only present in induced cultures. This is clearly consistent with the presence of the His-tag at the C-terminus of the PH (Fig. 1B). To assess the precise cellular location of the induced protein, cells from induced and control cultures were fractionated into periplasm, cytoplasm, and membranes and again analysed both by Coomassie staining and by Western blotting (Fig. 2). In the periplasmic fractions a prominent polypeptide of the expected size for PH was present specifically in the induced pHYS400 cultures and was therefore initially construed to be the mature form of PH (Fig. 2a). However, this protein band did not give a signal when probed with the anti-His antibody (Fig. 2b). Western blotting revealed a weak signal in the cytoplasmic fraction of induced cells and a pHYS400-specific signal in the membrane fraction that showed a marked

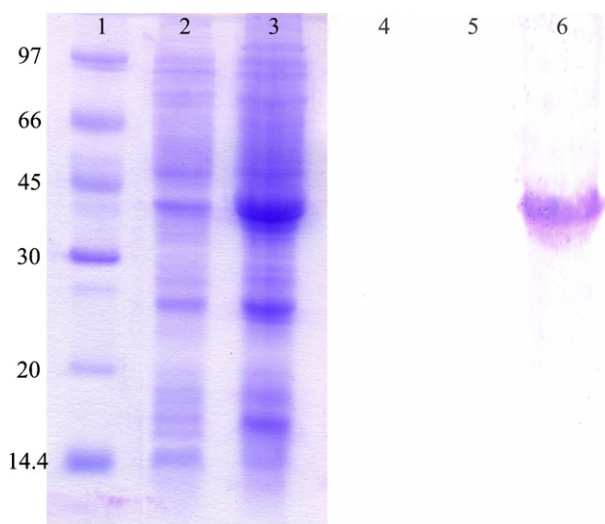


Fig. 1. Induction of parathion hydrolase (*opd*) expression in *E. coli* BL21(pHYS400). Lanes 1–3, Coomassie stained SDS-PAGE; lanes 4–6, Western blot of equivalent gel, using anti-6His to detect the His-tagged *Opd* protein; lanes 1 and 4, molecular weight markers (kDa); lanes 2 and 5, protein extracts from uninduced cultures of BL21(pHYS400); lanes 3 and 6, protein extracts from induced cultures of BL21(pHYS400).

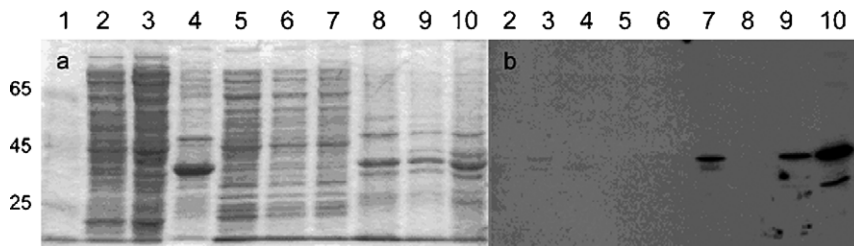


Fig. 2. Analysis of the sub-cellular location of induced parathion hydrolase. (a) Coomassie stained SDS-PAGE. Lane 1, molecular weight markers (kDa); lanes 2–4, periplasmic proteins from BL21, from uninduced and induced BL21(pHYS400); lanes 5–7, cytoplasmic proteins from BL21, uninduced and induced BL21(pHYS400); lanes 8–10, membrane proteins from BL21, uninduced and induced BL21(pHYS400). (b) Western blot of equivalent gel using anti-6His to detect the His-tagged parathion hydrolase protein.

response to induction. This signal was equivalent to the predicted size of the mature PH protein (37 kDa) (Fig. 2b). A minor signal in the membrane fraction of induced cells (Fig. 2b) is likely to be due to misprocessing or degradation of PH in *E. coli*.

To investigate this apparent paradox, the prominent protein band induced in BL21(pHYS400) was eluted from a gel and the N-terminal amino acid sequence was determined. The resultant sequence (AEIYNKDGNKVDLYG KAVGL) shows 100% identity with the outer membrane porin *OmpF* of *E. coli* K12. To assess the relationship between *OmpF* expression and induction of PH, the presence of *OmpF* was then monitored at different stages of induction by analyzing the periplasmic fraction on SDS-PAGE. By this analysis the levels of *OmpF* were undetectable before induction and its concentration increased steadily with the course of induction (Fig. 3). Hence in conclusion, whilst pHYS400-encoded PH is induced following addition of IPTG as expected and is found predominantly in the cellular membrane fraction, the major protein observed following induction is not PH but *OmpF*.

The outer membrane porin *OmpF* is normally expressed in *E. coli* in response to environmental stimuli. The expression of *OmpF* is under the control of *OmpR*-P (the phosphorylated form of *OmpR*). The phosphorylation and dephosphorylation of *OmpR* is mediated by the response regulator *EnvZ* which acts as an osmosensor to monitor

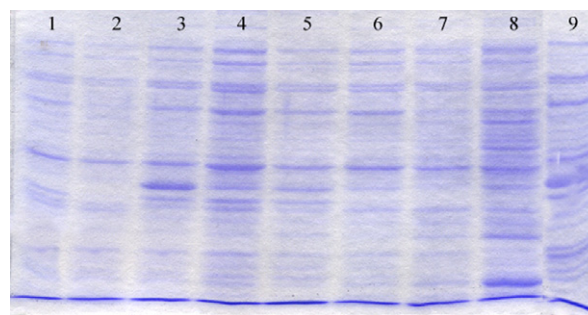


Fig. 3. Analysis of periplasmic proteins isolated at different time points after induction of parathion hydrolase. The position of *OmpF* is shown with an arrow. Lanes 1 and 2, periplasmic proteins from BL21 and BL21(pHYS400); lanes 3–7, periplasmic proteins extracted after 0, 30, 60, 90, and 120 min, respectively, of induction of parathion hydrolase in BL21(pHYS400); lanes 8 and 9, total proteins from uninduced and induced cells of BL21 (pHYS400).

changes in the external osmolarity and modifies OmpR activity accordingly by phosphorylation or dephosphorylation. OmpR is the transcriptional activator of both the *ompF* and *ompC* genes [16]. A low level of OmpR-P stimulates transcription of *ompF* and a high level of OmpR-P activates *ompC* while repressing *ompF*. Whilst the EnvZ-OmpR regulatory system is the major mechanism of controlling *ompF* expression there are a number of indications that other signal transduction pathways influence this process [17]. Recent evidence has implicated intracellular acetyl phosphate levels in the regulation of OmpR phosphorylation [18], and a second two-component system, CpxA-CpxR, has also been shown to control *ompF* expression [19]. However the signals to which both the implicated sensor kinases, EnvZ and CpxA, respond are not well characterised.

Parathion hydrolase is a homodimer containing Zn ions as a cofactor [20,21]. It does not contain any functional motifs typical of a transcriptional activator protein and there is no reason to suppose that it could directly affect transcriptional activation of *ompF*. Hence, whilst any effect of PH on *ompF* expression is likely to be an indirect consequence of some change in cellular metabolism, the precise signal cannot be predicted. PH shows significant homology to several prokaryotic and eukaryotic proteins including the product of the *E. coli* *yhfV* gene (72%). This gene is part of a potential eight gene operon, *yhfZ*, *Y*, *X*, *W*, *V*, *U*, *T*, and *S* of unknown function, although one gene, *yhfW*, is predicted to encode a protein that shows significant homology to other phosphotransferase enzymes. Given these similarities it is conceivable that *F. balustinum* *opd* expression in *E. coli* could interfere with normal metabolism, most likely through some aspect of phosphate metabolism and that this leads to the signal that stimulates OmpF expression e.g., a change in acetyl phosphate levels. It is of interest to note that in *E. coli* phosphate limitation leads to a specific elevation of the expression of another outer membrane porin, PhoE [22], and hence the metabolic signals caused by PH expression are apparently distinct from those sensed by the Pho regulon. Further studies are necessary to establish the mechanism of OmpF expression in presence of PH and of particular interest is the possibility that this effect in a heterologous background might reflect some aspect of normal Opd biology in *Flavobacterium*.

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