



Influence of zinc and cobalt on expression and activity of parathion hydrolase from *Flavobacterium* sp. ATCC27551

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Abstract

Organophosphorus triesterases are encoded by the organophosphate degradation (*opd*) gene found in species of *Flavobacterium*, *Pseudomonas*, and *Agrobacterium*. They are involved in hydrolysis of triester bonds found in a variety of organophosphorus pesticides. In view of their potential biotechnological importance in disposal of pesticide wastes, several attempts have been made to express the *opd* gene in heterologous hosts. Increased levels of parathion hydrolase (PH) activity were previously reported in the presence of divalent metal ions such as zinc and cobalt, and it was suggested that this reflected metal ion-induced transcriptional activation of *opd*. We have now used *opd-lacZ* fusions to examine the influence of zinc and cobalt on *opd* expression, and found no effects. However, when *opd* was expressed from the *lac* promoter there was considerable increase in PH activity if the culture medium was supplemented with 1 mM zinc or cobalt although the amount of PH was unaffected. These results suggest that the increase in specific activity of PH caused by these divalent cations is due to improved folding of overexpressed protein.

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1. Introduction

Organophosphate (op) pesticides are used all over the world as effective chemical agents to

control insect pests. Persistent use of these chemicals leads to their accumulation in ecosystems and causes enormous damage to the environment, especially to the non-target organisms [1,5]. However, some soil micro-organisms display the remarkable capability of using these chemicals as their sole source of carbon and phosphate

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[8,26,30]. Certain soil bacteria that are responsible for the degradation of op-pesticides contain a highly conserved organophosphate degradation (*opd*) gene on otherwise dissimilar large indigenous plasmids [16,24,28,30]. The *opd* gene encodes parathion hydrolase (PH), a 40 kDa homodimer which contains zinc ions as a cofactor [4,9,19]. PH hydrolyses the triester bond found in all organophosphates and reduces the toxicity of these chemicals several fold. Therefore, PH has been a favourite candidate for the disposal of op-pesticide wastes and the related chemical warfare agents such as sarin and soman [18]. As wild-type bacterial strains produce low amounts of PH, attempts have been made to over-express the *opd* gene in heterologous hosts such as *Streptomyces lividans* and *Escherichia coli* [15,21,29,32]. In previous studies, increased levels of PH activity were reported when the *opd* gene was expressed in presence of the metal ions such as zinc and cobalt and based on these observations it was suggested that metal ions might enhance transcription of the *opd* gene [25]. In the present study, we have used *lacZ* transcriptional fusions to localize the likely *opd* promoter and have used these fusions together with examination of Opd (parathion hydrolase) protein levels to investigate possible metal ion-induced transcriptional activation of *opd*. Our data indicate that the effects of metal ions are all post-translational and not transcriptional.

2. Materials and methods

2.1. Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown either in LB or in M9 medium. *E. coli* HB101 cells were grown at 37 °C whereas *Pseudomonas aeruginosa* PAO1161 cells were grown at 30 °C. When required, antibiotics such as ampicillin (100 µg/ml), tetracycline (30 µg/ml), and chloramphenicol (30 µg/ml) were added to the growth medium. The M9 medium was supplemented with 1 mM filter-sterilized proline or leucine when used to grow *E. coli* HB101 and *P. aeruginosa* PAO1161, respectively.

2.2. Construction of *opd-lacZ* fusions

Transcriptional *opd-lacZ* fusions using the transcriptional *lacZ* fusion vector pMP220 are illustrated in Fig. 1. Plasmid pWWM1079 [17] was digested with *Bam*HI and the resultant 1.5 kb fragment carrying *opd* was cloned in pUC18 to generate pSM6. The *Bam*HI fragment from pSM6 was then cloned into the *Bg*II-digested pMP220 and the orientation of *opd* was determined using the single *Pst*I site in the fragment. The resultant plasmid was designated pSM7. Plasmid pSM7 contains two recognition sites for both *Pst*I and *Sph*I. In each case, one site

Table 1
Strains and plasmids

Strains	Genotypes	Source or reference
<i>E. coli</i> HB101	<i>supE44 hsdS20 (r_Bm_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	[7]
<i>P. aeruginosa</i> PAO 1161	Leu ⁻ Amp ⁺	[23]
Plasmids		
pMP220	Tet ^r , promoter probe vector	[31]
pSM5	Cm ^r , <i>opd</i> gene cloned in expression vector pMMB206 under the control of <i>lacZ</i> promoter	[27]
pSM6	Amp ^r , 1.5 kb <i>Bam</i> HI fragment containing <i>opd</i> gene cloned in pUC18	This work
pSM7	Tet ^r , 1.5 kb <i>Bam</i> HI fragment containing <i>opd</i> gene cloned in pMP220	This work
pSM8	Tet ^r , upstream region of <i>opd</i> gene cloned in pMP220 as <i>Bam</i> HI– <i>Pst</i> I fragment	This work
pSM9	Tet ^r , upstream region of <i>opd</i> gene cloned in pMP220 as <i>Bam</i> HI– <i>Sph</i> I fragment	This work
pSM10	Tet ^r , upstream region of <i>opd</i> gene cloned in pMP220 as <i>Bam</i> HI– <i>Xba</i> I fragment	This work
pSM11	Tet ^r , upstream region of <i>opd</i> gene cloned in pMP220 as <i>Bg</i> II– <i>Xba</i> I fragment	This work

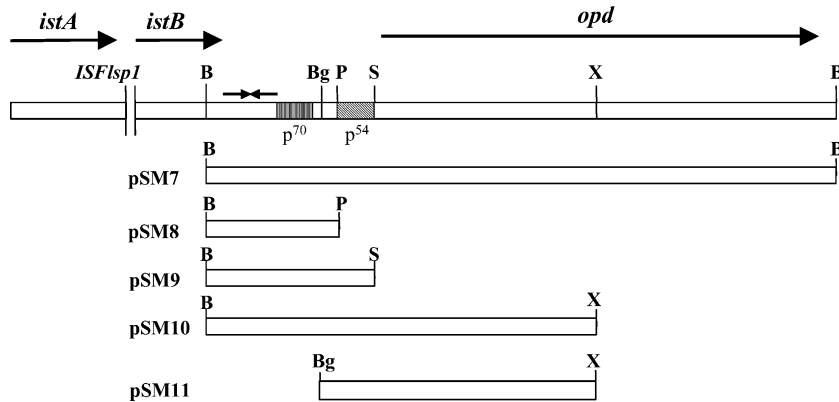


Fig. 1. A Schematic diagram showing the construction of transcriptional *opd-lacZ* fusions. Plasmids pSM7 to pSM11 carry upstream regions of the *opd* gene as various restriction fragments in promoter test vector pMP220. The transcriptional terminator of *istB* of IS element *ISFlspI* is shown with inverted arrows. The hatched boxes indicate the putative promoters, σ^{70} and σ^{54} . Abbreviations B, Bg, P, S, and X represent restriction sites for *Bam*HI, *Bgl*II, *Pst*I, *Sph*I, and *Xba*I, respectively.

is upstream of the translational start site for *opd* and the other is in the multiple cloning site of the vector. Therefore, pSM7 was digested either with *Pst*I or *Sph*I and the vector containing the upstream region of *opd* was self-ligated to give *opd-lacZ* fusions. The resulting plasmids were designated pSM8 and pSM9, respectively. Similarly, pSM7 has two *Xba*I sites, one in *opd* downstream of the translational start site and the other in the multiple cloning site of the vector downstream of the *Bgl*II site. Plasmid pSM7 was digested with *Xba*I and the vector containing the upstream region of the *opd* gene was self-ligated to give plasmid pSM10. Plasmid pSM6 was digested with *Bgl*II and *Xba*I, and the 819 bp fragment containing the upstream region of *opd* was cloned into pMP220 digested with similar enzymes to generate pSM11. The *Bgl*II site is located between putative σ^{70} and σ^{54} promoter motifs (Figs. 1 and 2), and therefore in pSM11, the upstream region of *opd* containing only the putative σ^{54} promoter was fused to *lacZ*.

2.3. Triparental mating

All *opd-lacZ* fusions and pMP220 were mobilized into *P. aeruginosa* PAO1161 by non-quantitative tri-parental plate mating. *E. coli* strains carrying the *opd-lacZ* fusions or pMP220 were used as donors, *P. aeruginosa* PAO1161 was the

recipient, and *E. coli* HB101 (pRK2013) was the helper strain [10]. Mixtures of log phase cultures of donor, recipient, and helper strains, in a ratio of 1:2:1, were plated on an LB plate and mating proceeded for 36 h. Exconjugants were then selected on tetracycline and ampicillin plates. Expression plasmid pSM5 [27] was mobilized into *P. aeruginosa* PAO1161 using *E. coli* strain HB101 (pSM5) as donor and exconjugants were selected on chloramphenicol and ampicillin plates.

2.4. β -Galactosidase assays

For β -galactosidase assays, all the *opd-lacZ* fusion plasmids as well as the vector pMP220 were transferred into *E. coli* HB101 and *P. aeruginosa* PAO1161. Strains were grown in M9 medium and β -galactosidase activity was monitored following standard procedures [13]. Experiments were replicated three times and six independent assays were carried out on each occasion. Values quoted are the averages of all experiments.

2.5. Influence of Co and Zn ions on *opd* gene expression

Overnight cultures of *E. coli* and *P. aeruginosa* PAO1161 strains containing *opd-lacZ* fusions were grown in LB with an appropriate antibiotic. Cells were harvested from 1 ml of overnight culture,

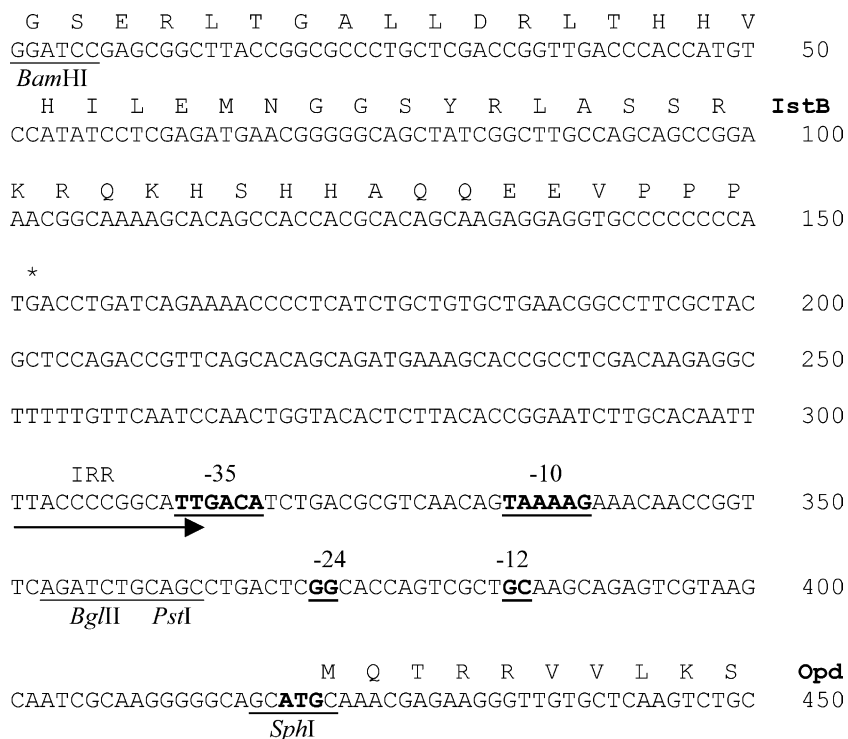


Fig. 2. The nucleotide sequence of the upstream region of the *opd* gene. The locations of the coding regions for the 3' end of *istB* and the 5' end of *opd* are indicated. The proposed right-hand inverted repeat (IRR) is shown with an arrow. Putative σ^{70} - and σ^{54} -dependent promoter motifs (–35, –10 and –24, –12, respectively) are shown in bold, underlined type. Relevant restriction sites are indicated and underlined.

washed with citrate–saline buffer, inoculated into 50 ml of M9 medium, and grown to an A_{600} of 0.5. Each culture was then divided into two parts, one of which was supplemented with 10 μM CoCl_2 , and grown for a further 3 h. Cells were then harvested from 0.1 ml of culture to assay β -galactosidase activity. *E. coli* and *P. aeruginosa* PAO1161 strains carrying pMP220 were used as a negative control. Similar experiments to assess the influence of Zn ions contained 10 μM ZnCl_2 instead of CoCl_2 .

2.6. Expression of parathion hydrolase in the presence of zinc and cobalt

Escherichia coli and *P. aeruginosa* PAO1161 strains containing pSM5 were grown overnight in M9 supplemented with chloramphenicol. This overnight culture (200 μl) was inoculated into 20 ml of fresh M9 medium and grown to an A_{600} of 0.5. Cultures were induced with 1 mM IPTG before

dividing them into two parts. One part was supplemented with 10 μM ZnCl_2 and the other part served as a control. Cultures were grown for another 2 h and parathion hydrolase activity was assayed spectrophotometrically by measuring the formation *p*-nitrophenol at 410 nm [15]. Similar experiments were conducted by supplementing 10 μM CoCl_2 . Bacterial cells from 1 ml of culture were harvested and proteins from these cultures were analysed on 12.5% SDS–PAGE. Care was taken to load equivalent amounts of protein in all lanes.

3. Results and discussion

3.1. Locating the *opd* promoter

Almost identical *opd* genes were previously cloned and sequenced from soil bacteria isolated from the diversified geographical regions

[12,14,25,28]. Based on sequence similarity, two promoter elements for *opd* were proposed independently. One of them shows similarity to the consensus σ^{70} -dependent promoter [14] whereas the other was suggested to have weak similarity to the promoters that depend on the sigma factor σ^{54} for transcription [11]. To date there have been no studies pertaining to the identification of the functional *opd* promoter. Therefore, in the present study an attempt was made to identify the functional promoter by cloning the upstream region of *opd* in the promoter test vector pMP220 using a variety of restriction fragments.

The *Bam*HI site 420 bp upstream of translation start for *opd* is located 150 bp before the stop codon for the upstream gene (*istB*) which encodes the ATP-binding protein of the IS element ISF1*spl* [27]. Therefore, the *opd* promoter sequence was anticipated to be downstream of *Bam*HI (Fig. 2). The restriction sites *Pst*I and *Sph*I are, respectively, located 60 bp upstream and directly over the translational start codon ATG of *opd*. When the upstream region of *opd* was fused to *lacZ* by cloning *Bam*HI–*Pst*I and *Bam*HI–*Sph*I the resulting *lacZ* fusions (pSM8 and pSM9, respectively) showed identical β -galactosidase activities when expressed in either *E. coli* or *P. aeruginosa*, suggesting that the *opd* promoter is located upstream of the *Pst*I site (Table 2). The *Pst*I site is located between the suggested σ^{70} - and σ^{54} -dependent promoters (Fig. 2). Therefore, the *opd*–*lacZ* fusion in pSM8 contains only the σ^{70} -dependent promoter. As the *lacZ* fusions with and without σ^{54} -dependent promoters showed identical β -galactosidase activity levels, the functional status of the σ^{54} promoter is questionable (Table 2).

To gain more insight into the functional status of the σ^{54} -dependent promoter, we constructed two further *opd*–*lacZ* fusions that carry either both the suggested σ^{70} - and σ^{54} -dependent promoters (*Bam*HI–*Xba*I in pSM10) or just the suggested σ^{54} -dependent promoter (*Bgl*II–*Xba*I in pSM11) (Fig. 1) and assayed for promoter activity both in *E. coli* and *P. aeruginosa*. There was no *lacZ* activation from pSM11 in both strains, clearly eliminating the proposed σ^{54} -dependent promoter as a functional sequence. Surprisingly, the *opd*–*lacZ* fusion that included the σ^{70} promoter element and part of the coding region of the *opd* gene (pSM10) showed less β -galactosidase activity in *E. coli* when compared to that of the *opd*–*lacZ* fusions that included only the σ^{70} promoter element but no such difference was noticed in *P. aeruginosa* (Table 2). One possible explanation for this observation could be the presence of a short inverted repeat (IR) sequence, which we have recently identified in the coding region of *opd* gene. We have constructed several expression plasmids to express parathion hydrolase in *E. coli* and in one of these plasmids we have destabilized the IR by modifying the sequence without changing its coding capacity (D. Siddavattam, unpublished results). Surprisingly, the expression levels of parathion hydrolase increased by 60–80-fold, indicating the involvement of IR sequence in regulation of *opd* gene expression. Of all the active *opd*–*lacZ* fusions, only pSM10 includes this IR sequence and it is only in pSM10 containing *E. coli* strains we have observed low levels of β -galactosidase activity. We are therefore tempted to assign a regulatory role to the IR sequence and further work to elucidate this sequence is in progress in our laboratory.

Table 2
Influence of zinc and cobalt ions on transcription of *opd* gene

Plasmid	<i>E. coli</i> HB101			<i>P. aeruginosa</i> PAO1161		
	Control	10 μ M ZnCl ₂	10 μ M CoCl ₂	Control	10 μ M ZnCl ₂	10 μ M CoCl ₂
<i>β-Galactosidase activity (Miller units)</i>						
pMP220	28.4 \pm 1	19.0 \pm 2	16.0 \pm 1	132.1 \pm 1	125.2 \pm 3	129.2 \pm 4
pSM8	130.4 \pm 2	159.4 \pm 4	65.4 \pm 3	899.2 \pm 7	952.4 \pm 6	988.4 \pm 5
pSM9	153.1 \pm 4	184.0 \pm 5	189.0 \pm 5	930.4 \pm 8	1179.8 \pm 7	993.9 \pm 3
pSM10	70.5 \pm 6	72.9 \pm 4	73.6 \pm 4	919.1 \pm 7	1104.2 \pm 9	1009.8 \pm 6
pSM11	28.7 \pm 3	20.2 \pm 2	18.2 \pm 1	139.9 \pm 3	126.1 \pm 2	119.8 \pm 3

The potential for a σ^{54} -dependent promoter (GGCACCAGTCGCTGC) upstream of the *opd* gene cloned from *Pseudomonas diminuta* was suggested by Wild et al. [11]. However, the structural and functional aspects of σ^{54} -dependent promoters have since been analysed extensively and all σ^{54} -dependent promoters known to date have a 10 bp gap between the conserved GG and GC dinucleotides. Furthermore, this spacing is absolutely necessary for promoter activity [2]. The proposed σ^{54} promoter sequence is therefore very unlikely to be functional as it contains an 11 bp spacing. Mulbry and Karns [14] cloned and sequenced the *opd* gene from *Flavobacterium* sp. and identified a possible σ^{70} -dependent promoter (TTGACA-N16-TAAAAG) 30 bp upstream of the promoter sequence suggested by Wild et al. This proposed sequence is a perfect match to the consensus -35 region for σ^{70} -dependent promoters and a four out of six match to the consensus -10 region. In the present study, all the *lacZ* fusions that showed promoter activity contained this sequence. As reported earlier [27], this consensus σ^{70} promoter is immediately downstream of the IRR (right border inverted repeat) of the IS element ISF1*sp1* which belongs to the IS21 family of transposons (Fig. 2). In a number of IS21 family transposons, an outwardly located -35 hexamer is located within the terminal inverted repeat (IR) sequences. The -35 hexamer found in the promoter sequence suggested by Mulbry and Karns is not found within the IRR sequence of ISF1*sp1* but it spans the IRR and adjacent sequences. This organization of -35 hexameric sequence, when considered together with the β -galactosidase data, suggests that the promoter motif proposed by Mulbry and Karns is likely to be the functional promoter of the *opd* gene.

A number of catabolic genes from *Pseudomonas* strains, including those involved in degradation of naphthalene and styrene, show low levels of expression when cloned in *E. coli* [3,6,20,22]. Optimum expression levels for these genes have always been achieved by cloning them under the control of inducible *E. coli* promoters [22]. Though experimental evidence to explain these results is scarce, it has been suggested that the poor expression may reflect a requirement for specific transcriptional

factors for the expression of catabolic genes. The *opd* genes have been cloned either from *Pseudomonas* strains or from soil bacteria that have a close taxonomic relationship with *Pseudomonas*. Consequently, we also examined expression from the same *opd-lacZ* fusions by mobilizing them into *P. aeruginosa* 001161. The resultant activities showed the same pattern of expression as observed in *E. coli* but the levels were 6–10-fold higher in *P. aeruginosa* PAO1161 (Table 2). We suggest that the elevated expression in *P. aeruginosa* PAO1161 reflects some aspect of the transcriptional machinery that is better suited to optimum expression of the *opd* gene.

3.2. Metal ions are not involved in transcriptional activation of the *opd* gene

Parathion hydrolase is a dimer that contains zinc ion as cofactor [19]. Metal ions such as zinc and cobalt show a positive influence on parathion hydrolase activity and earlier investigators suggested that this could reflect metal ion-dependent transcriptional activation of *opd* gene expression [25]. To examine this hypothesis, we grew both *E. coli* and *P. aeruginosa* PAO1161 cells containing *opd-lacZ* fusions in minimal medium supplemented with 10 μ M of either ZnCl₂ or CoCl₂ and monitored β -galactosidase activity (Table 2). We did not detect any changes in β -galactosidase activity levels, indicating that these metal ions do not influence the transcriptional activation of *opd* gene (Table 2). Despite earlier reports of effects due to 1 mM Zn²⁺ or Co²⁺ attempts to increase further the concentration of metal ions in the minimal medium showed adverse effects on the growth of the cells. However, when the cultures were grown in LB medium the increase in the concentration of metal ions to 1 mM showed no adverse effect on the growth of the cells. Therefore, we repeated the experiments by growing the cultures in LB medium supplemented with 1 mM of either zinc or cobalt chloride. As expected there was no significant change in β -galactosidase activity in the cultures grown in the presence and absence of metal ions (data not shown).

We also investigated whether parathion or its major degradation product *p*-nitrophenol (PNP)

influenced *opd* gene expression by supplementing a log phase culture *E. coli* or *P. aeruginosa* 001161 grown in minimal medium with 10 μ M of either substance. We then monitored β -galactosidase activity at several time intervals but there was no significant difference in activity to suggest that either parathion or PNP has any positive role on *opd* gene expression. Similarly, we have grown *Flavobacterium* sp. in the minimal medium [26] in the presence of these substances and found no difference in the specific activity of parathion hydrolase, clearly indicating that neither parathion nor PNP has influence on parathion hydrolase activity. These results are in agreement with our earlier findings [30] as well as reports of Sethunathan and Yoshida [26] who also reported constitutive expression of parathion hydrolase in soil bacteria.

To assess the reasons for the previously reported increased PH activity in the presence of Zn and Co ions, we used an expression plasmid pSM5, in which the *opd* gene is cloned under the control of an inducible *lac* promoter on a broad host range, low copy number vector. When the plasmid was introduced into *E. coli* and *opd* expression was induced by growing the cells in M9 medium in the presence of either cobalt or zinc ions, we observed significant increases in PH activity, most notably in the presence of 10 μ M CoCl₂ (Table 3). However, the observed increases in PH activity levels were much lower when compared with the results of Serdar et al. [25]. To verify if this was because of the low levels of metal ions used in the culture medium, we repeated the experiments by growing the cultures in LB medium supplemented with 1 mM CoCl₂. In these conditions, the enzyme activity levels, as evidenced by the production of *p*-nitrophenol/min/mg protein, were similar (49 μ mol/min/mg) to those reported by

Serdar et al. [25]. This may be because of the optimum expression of PH in nutrient-rich LB medium coupled with the availability of excess amounts of metal ions required for the overexpressed enzyme.

We further examined whether the elevated PH activity was due to increased stability of the overexpressed protein in the presence of metal ions by analysing the expressed protein on SDS-PAGE. We found no significant difference in parathion hydrolase concentration between the cultures grown in the presence or absence of metal ions (Fig. 3). Taken together with the data from the *opd-lacZ* fusions, we conclude that the observed

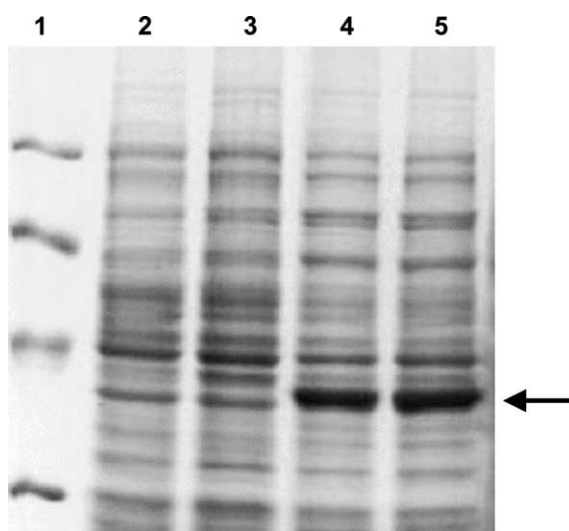


Fig. 3. Expression of parathion hydrolase in the presence of cobalt ions. Lane 1, molecular weight markers; lane 2, protein extract from *E. coli* strain HB101; lane 3, protein extract from uninduced cultures of HB101 (pSM5); lanes 4 and 5, protein extracts from induced cultures of HB101 (pSM5) grown in the presence and in the absence of cobalt ions, respectively. Approximately, 40 μ g protein was loaded in each lane.

Table 3
Influence of cobalt and zinc ions on parathion hydrolase activity

Strain	-IPTG	+ 1 mM IPTG	1 mM IPTG + 10 μ M CoCl ₂	1 mM IPTG + 10 μ M ZnCl ₂
<i>E. coli</i>	0.0	0.0	0.0	0.0
<i>E. coli</i> (pSM5)	0.1	2.6	20.8	12.0
<i>P. aeruginosa</i>	0.0	0.1	0.0	0.0
<i>P. aeruginosa</i> (pSM5)	0.1	6.8	30.3	20.4

Enzyme activity is expressed as μ moles of *para*-nitrophenol produced/mg protein/min.

enhancement in PH activity is not due to the transcriptional activation of *opd* but is rather due to the conversion of apoenzyme, produced under induced conditions, to active enzyme in the presence of metal ions.

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