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Bioinformatic analysis and *in vitro* site-directed mutagenesis of conserved amino acids in BphK^{LB400}, a specific bacterial glutathione transferase

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ABSTRACT

Glutathione transferases [GSTs: EC 2.5.1.18)] are ubiquitous multifunctional prokaryotic and eukaryotic enzymes involved in the cellular detoxification and excretion of a large variety of compounds. However, our understanding of the role of bacterial GSTs in metabolism is still in its infancy. The association of bacterial GST DNA with other genes involved in degradation of toxic pollutants, including poly-chlorinated biphenyls (PCBs), indirectly suggests a role for bacterial GSTs in biodegradation. Previously, in this laboratory, a specific bacterial GST, BphK^{LB400} isolated from *Burkholderia xenovorans* LB400, was shown to be capable of dehalogenating chlorinated organic substrates rendering them less toxic. However, little is known about the specific amino acids in BphK^{LB400} involved in catalysis *in vitro*. In this study, bioinformatic analysis of BphK^{LB400} and other bacterial GSTs, including PCB degraders, identified a number of amino acids that were identical in all bacterial GST sequences analysed. Two amino acids, Cys10 and His106, were selected for *in vitro* site-directed mutagenesis studies. *In vitro* GST activity assay results suggest that these two amino acids play a role in determining the catalytic activity of BphK^{LB400}. Studies of bacterial cell extracts expressing BphK^{LB400} (wildtype and mutant) identified a specific mutant, Cys10Phe, with increased GST activity towards 1-chloro-2,4-dinitrobenzene (the model substrate for GSTs). BphK^{LB400} (mutant) with increased activity towards toxic chlorinated organic compounds could have potential for bioremediation of contaminated soil in the environment.

1. Introduction

Polychlorinated biphenyls (PCBs) are industrial compounds which were widely used from the 1920s as dielectrics and heat transfer fluids, plasticisers, wax extenders and flame retardants (Safe et al., 1985). They are highly stable organic-soluble persistent compounds which can bioaccumulate in animals. Concerns about PCBs and their toxicological effects led to the ban of PCB production in the 1970s. In humans, PCBs have been identified as probable carcinogens. In Ireland, it is estimated that there are 334,043 l of PCBs in inventoried and non-inventoried holdings (Meaney and Carty, 2002). The safe and rapid removal of PCBs from the environment is an issue of growing concern worldwide. Traditional methods of destruction, such as incineration, prove insufficient as there is a risk of producing extremely toxic dioxins and furans through partial oxidation. Research is now focusing on biological methods, such as bioremediation which can be defined as the use of biological agents such as bacteria or plants to remove and/or neutralise contaminants in the environment.

Burkholderia xenovorans LB400 contains an operon, *bph*, which confers on this bacterial strain the ability to degrade PCBs (Bopp, 1986; Hofer et al., 1993, 1994). A *bphK* gene, found in a central location within the *bph* operon of *B. xenovorans* LB400, encodes a protein belonging to a superfamily of enzymes, the glutathione transferases (GSTs: EC 2.5.1.18) (Hofer et al., 1994). Previously our laboratory reported that BphK^{LB400} shows dechlorination activity towards 4-chlorobenzoate (4-CBA), an end product of *bph*-promoted degradation of PCBs (Gilmartin et al., 2003). More recently, BphK^{LB400} was shown to catalyse the dehalogenation of 3-Chlorinated 2-hydroxy-6-oxo-6-phenyl-2,4-dienoates (HOPDAs), inhibitory PCB metabolites (Fortin et al., 2006). These HOPDAs are produced as metabolites of PCBs by the *bph* pathway and inhibit the pathway's upstream hydrolase (Fortin et al., 2006).

Many mutagenesis studies have been carried out on bacterial GSTs in order to identify specific amino acids involved in catalysis (Casalone et al., 1998; Gilmartin et al., 2005; Inoue et al., 2000; Nisida et al., 1994). Both a conserved tyrosine residue in class alpha, mu, pi and sigma GSTs and a serine residue in class theta GSTs have been identified in stabilising the thiolate form of glutathione (GSH) for GST catalysis (Armstrong, 1997; Favaloro et al., 1998; Wilce and Parker, 1994). In BphK^{LB400}, a highly conserved C-terminal motif, identified through sequence alignment with a number of PCB

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degrading bacterial GSTs, was found to be involved in substrate specificity and catalytic activity (Gilmartin et al., 2005). In a recent study of two crystal structures of BphK, a BphK-GSH-HOPDA complex and BphK-$(GSH)_2$, two conserved amino acids, Cys10 and His106, were shown to be involved in the reductive dehalogenation of chlorinated HOPDAs (Tocheva et al., 2006). Cys10 and His106 were also identified in the crystallographic structure of *Escherichia coli* GST complexed with the GSH inhibitor glutathione sulfonate (Nishida et al., 1998).

Bioinformatic analysis of GSTs shows that there are two conserved regions in the amino acid sequence. The N-terminal region spans approximately amino acids 1–75, is highly conserved, and is associated with GSH binding. The C-terminal region spans approximately amino acids 90–200, and is much less conserved. Indeed, the sequences of the C-terminal region are often too different (below 20% identity) to be detected as similar in automated searches of sequence databases. This lends support to the hypothesis that the C-terminal region of GSTs plays a crucial role in determining enzyme activity and substrate specificity (Vuilleumier, 1997). Previously in this laboratory, bioinformatic analysis was used to identify highly conserved amino acids in the C-terminal region from a number of GSTs from PCB or polyaromatic hydrocarbon degrading bacteria including *B. xenovorans* LB400, and the effect of mutating conserved amino acids on GST activity investigated (Gilmartin et al., 2005; McGuinness et al., 2006). In the current study, we expand on these bioinformatic analyses and carry out *in vitro* site-directed mutagenesis to investigate the effect of mutating Cys10 and His106 on the catalytic activity of $BphK^{LB400}$ using 1-chloro-2,4-dinitrobenzene (CDNB), the model substrate for GSTs, as a substrate.

2. Materials and methods

2.1. Bioinformatic techniques

The Uniprot protein databank provided by Expert Protein Analysis System (ExPASY) (Boeckmann et al., 2003) was screened for bacterial GSTs capable of degrading PCB's or other organic compounds using the $BphK^{LB400}$ protein sequence and a number of bacterial GSTs were identified (Table 1). A separate search was also conducted for GSTs showing sequence similarity with $BphK^{LB400}$, with >50% amino acid sequence homology to $BphK^{LB400}$. Sequence alignments were carried out using the ClustalW2 general purpose multiple sequence alignment program (Larkin et al., 2007) provided by EMBL-EBI.

2.2. In vitro site-directed mutagenesis

The *bphK* gene was amplified from *Pseudomonas fluorescens* F113RIFPCB, a strain genetically modified to contain the *bph* operon of *B. xenovorans* LB400 (Brazil et al., 1995) and cloned into the pGEM-T vector (under the expression of the *lac* promotor) in *E. coli* XLI-Blue. Cys10 and His106 $BphK^{LB400}$ mutants were generated using the Stratagene Quick Change II site-directed mutagenesis kit as per the manufacturer's instructions. The kit involves the use of a set of user-defined primers containing the mutation of interest (Table 2), which anneal to a complementary sequence in the plasmid. PCR was carried out to generate a mutated plasmid, and the mutated plasmids were transformed into competent *E. coli* XLI-Blue cells. The mutated $bphK^{LB400}$ genes were sequenced to confirm the presence of the expected mutation.

Mutants were chosen based on Swiss-PdbViewer *in silico* analysis of the effect of amino acid substitutions on the crystalline structure of $BphK^{LB400}$ (Brennan et al., 2007). Briefly, rotamer score values were obtained for each *in silico* amino acid mutation at Cys10 and His106. Swiss-PdbViewer calculates rotamer scores based on the conformation of the new amino acid side chain from libraries of rotamers within the program. Amino acid substitutions producing the greatest effect *in silico* on the crystalline structure of $BphK^{LB400}$ were chosen for mutagenesis.

Table 2
$bphK^{LB400}$ amplification and site-directed mutagenesis primers.

bphK primers	Sequence 5′–3′
PCR amplification	
bphK Fwd	CAA CAA AGC ATG AAC AAC AAC C
bphK Rev	ATG GCG TTT CTC ATT CCA GG
Site-directed mutagenesis	
His106Gly Fwd	GAG CGA ACT GGG CAA GAG CTT CAG CC
His106Gly Rev	GGC TGA AGC TCT TGC CCA GTT CGC TC
His106Pro Fwd	GAG CGA ACT GCC CAA GAG CTT CAG CC
His106Pro Rev	GGC TGA AGC TCT TGG GCA GTT CGC TC
His106Val Fwd	GAG CGA ACT GGT CAA GAG CTT CAG CC
His106Val Rev	GGC TGA AGC TCT TGA CCA GTT CGC TC
Cys10Phe Fwd	AGC CCT GGT GCC TTC TCC CTG TCA C
Cys10Phe Rev	GTG ACA GGG AGA AGG CAC CAG GGC T
Cys10Trp Fwd	AGC CCT GGT GCC TGG TCC CTG TCA C
Cys10Trp Rev	GTG ACA GGG ACC AGG CAC CAG GGC T
Cys10Tyr Fwd	AGC CCT GGT GCC TAC TCC CTG TCA C
Cys10Tyr Rev	GTG ACA GGG AGT AGG CAC CAG GGC T

2.3. Preparation of bacterial cell extract

The transformed *E. coli* cultures of $BphK^{LB400}$, wildtype and mutant, were grown in 300 ml Tryptic Soy Broth containing 100 μl ml^{-1} ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside to an OD_{600nm} of approximately 1.3–1.5. Cells were

Table 1
GSTs from bacteria that degrade PCBs and other organic compounds. (with UniProt Accession No.).

Bacterial strain	Substrate	UniProt Accession No.	Reference
Burkholderia xenovorans LB400	Polychlorinated biphenyls	Q59721	Hofer et al. (1994)
Ralstonia sp. B15	Biphenyl	Q9RAF0	Bartels et al. (1999)
Pseudomonas pseudoalcaligenes KF707	Polychlorinated biphenyls	Q52037	Kimura et al. (1997)
Pseudomonas sp. B4	Biphenyl	Q9RBS6	Bartels et al. (1999)
Cycloclasticus oligotrophus RB1	Biphenyl, phenanthrene, naphthalene	Q46153	Wang et al. (1996)
Sphingomonas paucimobilis EPA505	Fluoranthene	O33705	Lloyd-Jones and Lau (1997)
Sphingomonas sp. KMG425	Biphenyl	Q8RMI1	Shin et al. (2002)
Sphingomonas aromaticivorans F119	Toluene, biphenyl, naphthalene	O85984	Romine et al. (1999)
Sphingomonas sp. P2	Phenanthrene	Q83VK4	Pinyakong et al. (2003)
Pseudomonas sp. B7T	Biphenyl	Q9RBS4	Bartels et al. (1999)
Polaromonas naphthalenivorans CJ2	Naphtalene	A1VUV4	Copeland et al. (2006)
Pseudomonas sp. B3B	Biphenyl	Q9RBS8	Bartels et al. (1999)
Ralstonia eutropha H850	Biphenyl	Q9RLB9	Bartels et al. (1999)