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Endorsing functionality of *Burkholderia pseudomallei* glyoxylate cycle genes as anti-persistence drug screens

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Abstract

Isocitrate lyase (ICL) and malate synthase (MS) are key glyoxylate cycle enzymes shown to be required for the persistence and virulence of *Candida albicans* and of *Mycobacterium tuberculosis* in macrophages because the up-regulation of glyoxylate genes and the corresponding enzymes could replenish C4 carbohydrates from C2 compounds in a persistent pathogen. In this study, the *ace* (acetate) genes (*aceA* and *aceB*) of a persistent pathogen, *Burkholderia pseudomallei* (ATCC 23343), encoding an ICL and a MS, respectively, were isolated and fully sequenced. The genes, *aceA* (1.3 kb) and *aceB* (1.6 kb) were cloned and expressed as tagged fusion proteins in *Escherichia coli* BL21 (DE3). The molecular weights of the predicted enzymes (ICL, 47.7 kDa and MS, 59.1 kDa) were consistent with ICLs and MSs reported so far. Phylogenetic analysis of these genes revealed significant identity (80–90%) with most bacterial ICLs and MSs. Comparative structural modeling and the localization of major ICL and MS family domains in the deduced peptide sequences showed interestingly significant similarity with isozymes from known pathogens. Specific activities of expressed ICL (589.27 nmol min^{-1} mg^{-1}) and MS (485.54 nmol min^{-1} mg^{-1}) were also demonstrated. Taken together, these results provide evidence for the functionality of glyoxylate cycle genes in *B. pseudomallei* and may thus be useful for designing antimicrobials targeted at the glyoxylate cycle.

Keywords: *Burkholderia*; Glyoxylate cycle; Persistence; Isocitrate lyase; Malate synthase

1. Introduction

In Bacteria, Archea and unicellular Eukarya, the glyoxylate cycle operates when carbon source is restricted to C2 compounds (acetate, acetyl CoA) [1]. In eukaryotes, this pathway can operate to synthesize carbohydrates from stored fats, as seen in germinating seedlings [2,3] and in nematode worms [4]. The presence and functionality of the glyoxylate cycle in humans, however, is not certain.

The necessity of the glyoxylate enzymes in pathogenesis was emphasized by studies on human and plant pathogens [5–8]. Recent work showed that the glyoxylate cycle enzymes are required for the persistence and virulence of *Mycobacterium tuberculosis* and *Candida albicans* in murine macrophages, by diverting carbon from beta-oxidation of fatty acids into the glyoxylate pathway to sustain a nutrient-starved intracellular infection [9]. Since this cycle is thought to be non-existent in mammals, the glyoxylate cycle enzymes were immediately implicated as targets of antimicrobial drug therapy. Owing to its importance in physiology and pathogenesis, the glyoxylate genes have been extensively studied in organisms from all three domains of life. However, the role of the genes in persistent human pathogens remains poorly understood.

Burkholderia pseudomallei causes melioidosis, an infective disease of rising concern in Southeast Asia and North Australia [10]. *B. pseudomallei* is thought to be a potential agent of bioterrorism due to the relative ease of its weaponization [11]. This is of special concern since no licensed vaccine against melioidosis is currently available [12]. Numerous medical and defense-driven interests have focused on elucidating the virulence factors of *B. pseudomallei* [13,14]. However, no study has yet directly implicated

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the glyoxylate cycle genes as possible virulence factors even though it is known that *B. pseudomallei*, like *C. albicans* and *M. tuberculosis*, is a persistent intracellular pathogen of macrophages [15]. Moreover, the glyoxylate cycle and the *acetate* (*ace*) genes are yet to be described in *B. pseudomallei*. Hence, in order to facilitate further experimentation on the glyoxylate pathway as a possible factor of pathogenesis, the glyoxylate genes *aceA* and *aceB* encoding isocitrate lyase (ICL) and malate synthase (MS), respectively, were cloned and functionally analyzed in this study.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

The source of *B. pseudomallei* was ATCC 23343. *Escherichia coli* was cultivated at 37 °C in Luria-Bertani (LB) medium. When necessary, filter-sterilized ampicillin (100 μg/ml) or kanamycin (50 μg/ml) (both from Sigma) was added to the medium.

2.2. DNA isolation and manipulation

Isolation and manipulation of DNA were done according to [16]. Restriction enzymes (New England Biolabs) and T4 DNA ligase (Promega) were used according to the instructions of the manufacturers. DNA fragments were isolated from 0.8% agarose gels (Gibco BRL) using a gel DNA extraction kit (Qiagen).

2.3. Primer design, cloning and DNA sequencing

Oligonucleotide primers were designed based on predicted bacterial ICL (*aceA*) and MS genes (*aceB*) deposited in the GenBank. The primers used to amplify *aceA* were OL668 (5′-TTTGGATCCTCGCGTCAACAACAGG-3′) with a *Bam*HI site (underlined) and OL669 (5′-TTTGAATTCTCAGGCGACTTTCTGG-3′) with an *Eco*RI site (underlined). The *aceB* gene was cloned using OL672 (5′-TTTGAATTCACCACGACGCTGAAGC-3′) carrying an *Eco*RI site (underlined), and OL673 (5′-TTTCTCGAGTCAGATCTCTTCGTAG-3′) with a *Xho*I site (underlined). PCR amplifications were carried out using *Pfu* DNA polymerase (Stratagene). The amplified blunt-end product was cloned directly into the pCR-BluntII-TOPO cloning vector according to the manufacturer's instructions (Invitrogen) prior to transformation into *E. coli* TOP10. The recombinant plasmid construct was extracted using the Wizard *Plus* SV Minipreps DNA Purification System (Promega) and sequenced twice on both strands to ascertain its identity. DNA sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit. Electrophoretic separation of the completed sequencing reaction was performed using an ABI 377 automated DNA sequencer.

2.4. Computer analysis and phylogenetic tree construction

The sequences used for multiple sequence alignment were downloaded from GenBank, and SWISS-PROT databases. Promoter prediction was carried out using the Prokaryotic Promoter Prediction by Neural Network software distributed by the Baylor College of Medicine, Houston, USA. M_r was deduced using computational programs at the ExPASy molecular biology server of the Geneva University Hospital and the University of Geneva. Multiple-alignment, phylogenetic analyses and neighbor-joining trees were constructed using the CLUSTAL W program package [17]. Trees were visualized using the TreeView 1.6.1 program, distributed by the University of Glasgow, UK. Enzyme signature was located using ScanPROSITE [18]. Protein domains were located and mapped using facilities of the ProDom database [19]. Comparative structural modeling was achieved using SWISS-MODEL based on information from the Protein Data Bank (http://www.rcsb.org/pdb/). In particular, the solved structures of *M. tuberculosis* (PDB ID: 1F61) and *E. coli* (PDB ID: 1IGW) were used to derive our ICL structure.

2.5. Heterologous expression and purification of fusion enzymes

The pGEX 6P-1 system (Amersham) was employed for heterologous expression of the *aceA* and of *aceB* genes, using *E. coli* BL21 (DE3) (Novagen) as the expression host. Primers were designed with suitable restriction enzyme recognition sites to allow in-frame insertion into the pGEX 6-1 vector. This placed the inserted coding sequence under the control of a *tac* promoter, in-frame with an ATG start codon located in the vector. The inserted gene was fully sequenced on both strands to confirm its identity and frame of insertion. The resulting plasmid was used to transform electrocompetent *E. coli* BL21 (DE3) cells as described [20]. Expression of glutathione-*S*-transferase (GST)-tagged fusion enzymes was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Clontech) to a final concentration of 0.1 mM. Cultures were harvested after cultivation at ambient temperatures (22–25 °C) with shaking at 200 rpm.

2.6. Purification of fusion enzymes and determination of enzyme activities

Bacterial cells were lysed by sonication using MSE Soniprep with a 1/8 in. (3 mm) probe in TDE buffer (136 mM sodium chloride, 50 mM potassium chloride, 50 mM EDTA, 25 mM Tris pH 7.5) and cell debris removed by centrifugation at 12,000 rpm. Fusion proteins were purified as per manufacturer's instructions (Amersham). The resulting purified fusion enzymes were first analyzed by SDS-PAGE and followed by enzyme activity assays and protein quantitation using fresh preparations. The spectrophotometric MS and ICL enzyme activity assays were performed as described [21,22]. Repre-