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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-

sentative results of enzyme activity assays are shown from experiment sets that were repeated at least twice with independent overexpression samples for reproducibility. Protein concentration was determined using the Protein Assay Reagent (Bio-Rad) according to the manufacturer's instructions.

3. Results

3.1. PCR cloning of aceA and aceB from B. pseudomallei (ATCC23343)

The cloned and sequenced ORFs of the *aceA* and *aceB* genes correspond to nt 2626666–2627973 and 2630844–2632436 of chromosome 1 of the sequenced *B. pseudomallei* strain (K96243) at Sanger Institute, respectively, with two variant nucleotides per gene, possibly arising from strain differences. In ATCC 23343, the *aceA* variant nucleotides were nt 2626715C → T and nt 2627770C → A, resulting in a silent and T369K amino acid change, respectively. In our sequencing effort for ATCC 23343 *aceB*, we observed nt 2630969T → C and nt 2632127C → T, resulting in a L42P and a silent residue change, respectively. Using sequence alignments, both the cloned *aceA* and *aceB* sequences bear significant homologies with existing ICL and MS sequences in Swiss-Prot (accession numbers: O53752 and Q9ZH77, respectively).

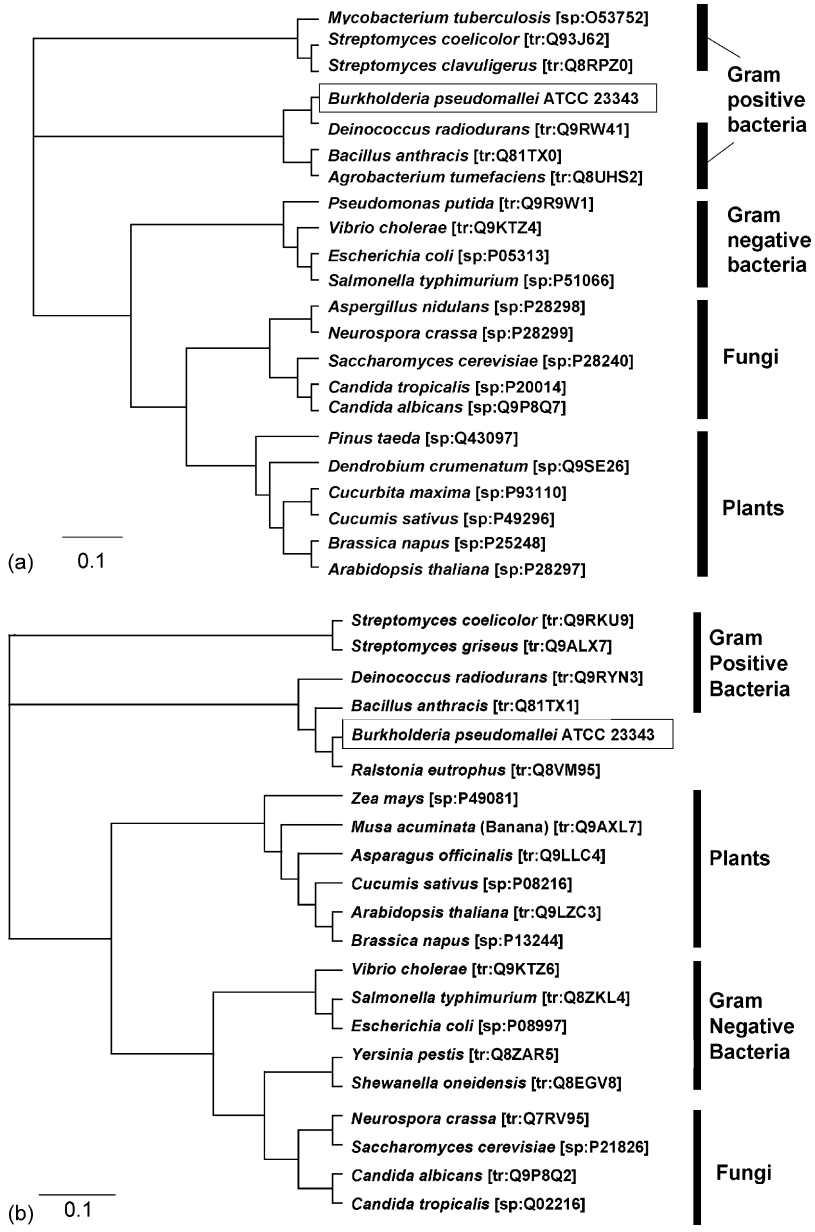

Fig. 1. Phylogenetic trees of (a) ICL and of (b) MS polypeptide sequences. The trees were constructed using neighbor-joining methods. Accession numbers of sequences are shown in brackets. Translated EMBL (TrEMBL) and SWISS-PROT sequences are denoted "tr" and "sp", respectively. Note that Gram-negative *B. pseudomallei* ICL and MS are more closely related to the Gram-positive clade.

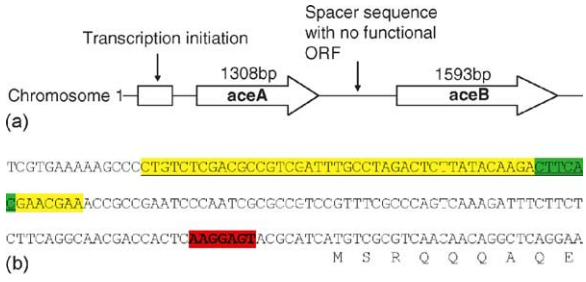

Fig. 2. (a) Gene loci of *aceA* and *aceB* on chromosome 1 of the *B. pseudomallei* genome. (b) The 5′ upstream region and a section of the *aceA* coding sequence. The predicted promoter sequence (yellow), the putative TATA box (green) and the purine rich Shine-Dalgarno-like sequence (red) are indicated. Predictions were performed using the Prokaryotic Promoter Prediction program by Neural Network (Baylor College of Medicine, Houston, USA).

3.2. Sequence and phylogenetic analyses

Successive cloning and sequencing revealed that the *aceA* and *aceB* coding sequences encode polypeptides of 435 and 530 amino acids, respectively. The deduced M_r of ICL (47,746 Da) and of MS (59,184 Da) were consistent with those of enzymes in SWISS-PROT. Both translated ICL and MS contained enzyme signature motifs corresponding to PROSITE regular expression data.

Both *aceA* and *aceB* sequences are G + C rich and have a bias of 90.1% and 92.5%, respectively, towards having a G or C in the third codon base. A codon usage bias towards G/C nucleotides is well known in the Gram positive Actinomycetes (including *Mycobacterium* spp.) but not known to be reported in Gram negative *Burkholderia* spp. This is suggestive of adaptations to specific tRNA pools for the purpose of gene regulation [23]. Interestingly, phylogenetic analyses of the amino acid sequences of ICL and MS showed a closer association with Gram positive Actinomycetes than with Gram negative proteobacteria even though *B. pseudomallei* is Gram negative (Fig. 1).

The use of promoter prediction software suggested an upstream region of *aceA*, but not of *aceB*, as the probable promoter site (Fig. 2). A −10 region that resemble the Shine-Dalgarno sequence was found (Table 1). The putative purine-rich ribosome binding site candidates were located seven bases (*aceA*) and six bases (*aceB*) upstream of their respective ATG start codons. A shared promoter for the two genes is implied from these results. This is consistent with bacterial polycistronic translational mechanisms: one promoter and multiple Shine-Dalgarno-like sequences preceding each AUG translational initiator on a single mRNA strand.

Table 1
Comparison between an *E. coli*-like promoter consensus sequence and the putative *aceA* promoter

	−10 region					
	1	2	3	4	5	6
Consensus	T	A	G	Pu	Pu	T
Percentage conservation	59	86	41	69	72	100
Observed putative promoter	A	G	G	A	G	T
Agreement with consensus	–	–	Yes	Yes	Yes	Yes

Both *aceA* and *aceB* are clustered in the genome of *B. pseudomallei*, with a spacer sequence that has insignificant homology to current GenBank sequences (data not shown). No other ORF was found immediately upstream of *aceA* or downstream of *aceB*.

High scoring pairs (HSPs) found by BLAST against the ProDom protein domain database yielded a domain map for both ICL and MS. The major identified domains matched known consensus domains of the ICL and MS families with little variation in their positions. The same sequences were used to BLAST against the NCBI human genome database and no *ace* genes were significantly identified (data not shown).

3.3. Heterologous expression and functional analysis of *B. pseudomallei* ICL and MS

To date, the only human pathogen glyoxylate genes successfully cloned and studied are those of *C. albicans* and of *M. tuberculosis*. To investigate the genes and their function, the ORFs of *aceA* and *aceB* were inserted into plasmid pGEX 6P-1 independently and transformed into separate *E. coli* BL21 (DE3) hosts in an IPTG-inducible expression system. The reading frames of both plasmids were validated by DNA sequencing on both strands. Expressed enzymes were visualized on SDS-PAGE gels and their molecular weight determined to be 48 kDa (ICL) and 59 kDa (MS), as computed initially (Fig. 3). Moreover, when the assays were repeated with the omission of substrates, or when the fusion enzymes were replaced with their sole fusion partner (GST) which was similarly expressed, enzyme activity was not detected (Fig. 4). Hence, the measured specific activities of ICL (589.27 nmol min^{-1} mg^{-1}) and MS

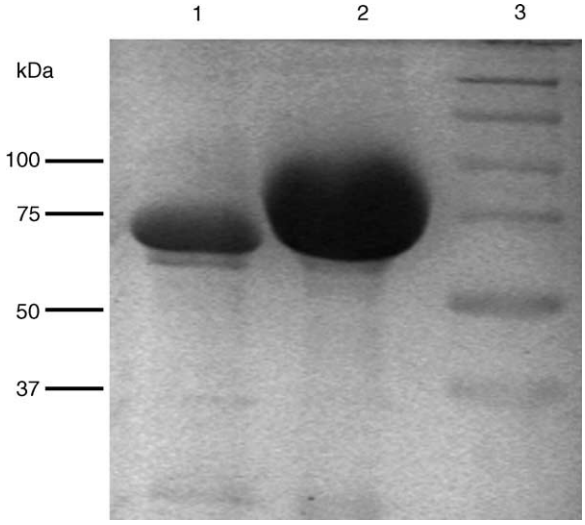

Fig. 3. SDS-PAGE analysis of overexpressed ICL and MS. Lane 1: Affinity purified ICL-GST fusion protein (74 kDa). Lane 2: Affinity purified MS-GST fusion protein (85 kDa). Lane 3: Molecular weight markers.

(485.54 nmol min^{-1} mg^{-1}) clearly showed the functionality of *aceA* and *aceB*.

4. Discussion

This study was first aimed to test the functionality of the *ace* genes in *B. pseudomallei* and to analyze the relatedness of the encoded enzymes with other microbes. Phylogenetic trees constructed using both ICL and MS amino acid sequences show close association of Gram negative *B. pseudomallei* with Gram positive bacteria but not with the Gram negative proteobacteria clade, which was thought to be close evolutionary relatives of the *Burkholderia* spp. [24]. In addition, the *ace* genes of *Deinococcus*, *Mycobacterium* and *Streptomyces* spp. have high G/C codon usage bias of nearly 90%, which the *ace* genes of *B. pseudomallei* similarly display. Plausibly, there could be a horizontal transfer of the high G + C content *ace* genes between Gram positive and Gram negative bacteria. Consequently, this might mean that the *B. pseudomallei* glyoxylate cycle genes, bearing much similarity to *M. tuberculosis* genes, could be similarly regulated at the transcriptional level and this may provide an opportunity for effective control of in-

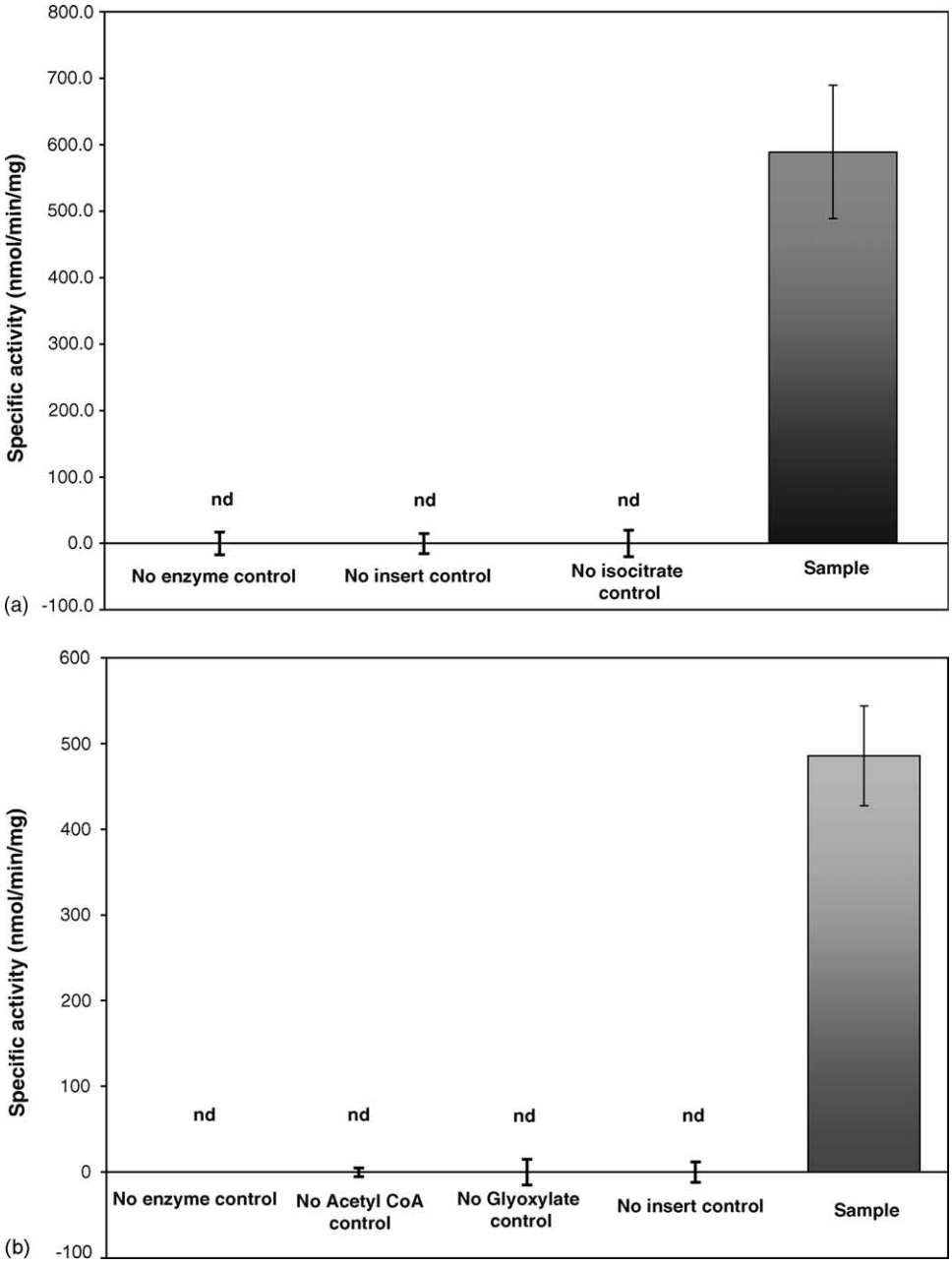

Fig. 4. Both cloned (a) ICL and (b) MS exhibited enzyme activity vis-à-vis a range of control experiments. No-enzyme, no-substrate and no-insert pGEX 6P-1 overexpressed samples were used as controls. Enzyme activities were not detected (nd) in the control experiments.

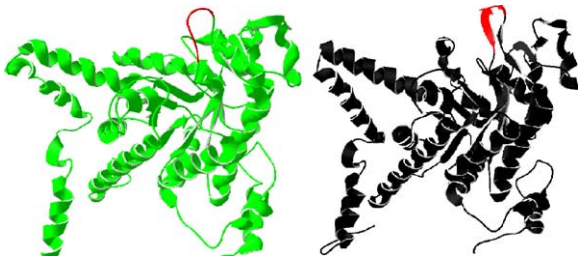

Fig. 5. Structural model of *B. pseudomallei* ICL (green) based on an *E. coli* ICL template (PDB ID: 1IGW) with the suggested hexapeptide active site highlighted in red. The solved crystal structure of the *M. tuberculosis* ICL monomer (black) (PDB ID: 1F61), is shown in the same orientation, showing their similar structure–function relationship.

tracellular persistence and virulence during disease manifestation.

The glyoxylate cycle enzyme structures of *B. pseudomallei* are not solved yet. With the aid of comparative structural modeling (Fig. 5), coordinates assigned to each amino acid residue of ICL showed that the conserved hexapeptide region containing an catalytically active cysteine was strikingly similar to related ICLs (e.g. *M. tuberculosis*) in their 3D conformation with the exception of the hexapeptide catalytic site, which was modeled as a loop instead of a beta-strand (Fig. 5). More critically, a consequence of medical importance would be the conservedness of the active domain in ICLs observed in our structural model. There is a likelihood of a shared mechanism of intracellular survival via the glyoxylate pathway during macrophage infection. Lorenz and Fink had earlier suggested that antimicrobials directed at the glyoxylate cycle be developed against persistent pathogens [9]. This study of yet another persistent pathogen, *B. pseudomallei*, with its *ace* gene sequences and the encoded glyoxylate enzyme structures deduced to be nearly identical to those of other persistent pathogens certainly lends support to the impetus to develop antimicrobials targeted at the glyoxylate cycle enzymes or *ace* genes, coding sequences of which were not found in the human genome.

In summary, although the *ace* genes and their encoded enzymes were proposed as targets of drug therapy in macrophage infections, much work is also needed to understand the broader evolutionary role of the glyoxylate pathway in persistence among microbes so as to rationally design compounds with improved anti-infective properties. Hence, such a drug would be pharmacologically attractive and clinically relevant to the treatment of not only tuberculosis, but also candidiasis and melioidosis.

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