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Conservation of a novel protein associated with an antibiotic efflux operon in *Burkholderia cenocepacia*

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Abstract

Burkholderia cenocepacia is a significant problem in individuals with cystic fibrosis and is a member of the *B. cepacia* complex of closely related antibiotic resistant bacteria. A salicylate-regulated antibiotic efflux operon has been identified in *B. cenocepacia* and one of its four genes, *llpE*, is without parallel in previously reported efflux operons. PCR amplification and sequencing of *llpE* from *B. cepacia* complex isolates demonstrated the highest prevalence in *B. cenocepacia* with a high degree of sequence conservation. While at least one non-synonymous mutation was identified between isolates from different genomovars, only synonymous differences were identified within the IIIA and IIIB sub-groups of *B. cenocepacia*. Structural modeling suggests that LlpE is a member of the α/β hydrolase enzyme family. Identification of strong structural homology to hydrolases and a high degree of conservation in *B. cenocepacia* suggests an enzymatic function for LlpE, benefiting survival in the cystic fibrosis lung.

Keywords: *Burkholderia cenocepacia*; Antibiotic efflux; Hydrolytic enzymes

1. Introduction

Burkholderia cepacia complex strains are important pulmonary pathogens in patients with cystic fibrosis. Although isolated from only 3.1% of patients over the age of five [1], this organism may be associated with severe morbidity and mortality in the cystic fibrosis population [2]. The complex is a group of at least ten related genomovars [3–7]. Although virtually all genomovars have been isolated from individuals with cystic fibrosis, most cystic fibrosis isolates are genomovars II and III, now called *B. multivorans* and *B. cenocepacia*, respectively [8,9]. Of these, *B. cenocepacia* has been most commonly associated with epidemic spread and increased clinical virulence [3,10]. Based on *recA* typing, *B. cenocepacia* has subsequently been divided into four sub-groups, of which genomovars IIIA and IIIB are most prevalent in cystic fibrosis [5].

Multiple antibiotic resistance is a characteristic of all *B. cepacia* complex isolates, particularly those from

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individuals with cystic fibrosis. Antibiotic efflux is a common mechanism of resistance in Gram-negative bacteria [11]. Efflux pumps have been implicated in resistance to diverse antibiotics [12–19] and other toxic compounds including dyes, detergents, disinfectants, and fatty acids [11,19].

We previously identified efflux-mediated multidrug resistance to chloramphenicol, trimethoprim, and ciprofloxacin in *B. cenocepacia* [20,21]. The *ceo* efflux operon responds to salicylate as an inducer and as a substrate of efflux [21]. The genes *ceoA*, *ceoB*, and *opcM* have significant homologies to the resistance/nodulation/cell division family of proteins, members of which are components of multidrug efflux systems. A fourth gene, *llpE*, has been identified in the cluster and is co-transcribed with *ceoA*, *ceoB*, and *opcM* [21], but its role in efflux is unclear. There is no counterpart of LlpE in any other efflux operons characterized to date. In this study, we elaborate on the potential role of LlpE in efflux, examining its prevalence in the *B. cepacia* complex, establishing a putative structural model for LlpE based on homologous proteins, and speculating on a possible enzymatic function in cystic fibrosis.

2. Materials and methods

2.1. Culture conditions

Strains used in this study are listed in Table 1. *B. cepacia* complex strains were grown in L-broth at 37 °C, 250 rpm.

2.2. PCR conditions

Genomic DNA for amplification of *llpE* was isolated from *B. cepacia* complex isolates using the DNeasy tissue Kit (Qiagen, Valencia, CA). For assessing the prevalence of *llpE*, genomic DNA was PCR amplified using primers llpE-F1 (5′GGCCTGGAAGCTTGCTTCG-G3′) and llpE-R (5′GCATTAGTCCATGGTTATTC-GGGACGGTTCGGC 3′) [21]. PCR used the GC Rich PCR system (Roche Applied Science, Indianapolis, IN), 100 ng of template DNA, 20 pmol of each primer, and 200 μM deoxynucleotides. An initial denaturation at 95 °C for 4 min was followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C for 30 s and elongation at 72 °C for 60 s, with a 5 s increment in elongation temperature per cycle for the last 20 cycles. After a final extension at 72 °C for 7 min, the samples were examined by agarose gel electrophoresis. PCR bands from selected strains were gel-purified and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems/Perkin–Elmer, Foster City, CA).

2.3. Phylogenetic analysis

Based on zonal analysis, a recent tool for visualizing gene evolution [22], an unrooted protein phylogram was constructed based on *llpE* coding sequences from 12 strains of the *B. cepacia* complex. This phylogram was created to give information on inter- and intra-nodal synonymous variations, where each node represents a structural variant. ClustalX 1.83 was used to align the various DNA and protein sequences. The aligned DNA dataset was the input in the PAUP*4.0b program to generate a maximum likelihood unrooted DNA phylogram, which was finally converted to a protein phylogram incorporating the synonymous and non-synonymous changes from the aligned protein sequence dataset.

2.4. Homology modeling

Comparative models for LlpE were built with the help of the Robetta server [23,24]. Robetta is a fully automated full-chain protein structure prediction server and the LlpE model was built from a homologous protein (E-value 2E-28, sequence identity 38%) detected with BLAST [25] and aligned by the K*Sync alignment method [23]. Loop regions were assembled ab initio by the Rosetta program fragments and optimized to fit the aligned template structure [26].

3. Results

3.1. Sequence analysis

The region upstream of *ceoABopcM* from K61-3 was sequenced and shown to code for a 286 amino acid protein. This gene, termed *llpE*, is the first gene in the *ceo* antibiotic efflux operon [21]. The N-terminal 70 amino acids of LlpE were analyzed for presence of signal peptide, using SignalP V2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/) [27]. LlpE is predicted to be a non-secretory protein and to have a signal peptide that is cleaved between amino acids 22 and 23. Analysis of LlpE by PSORT (http://psort.nibb.ac.jp/) [28], a program for prediction of protein localization, predicts a periplasmic or outer membrane localization.

3.2. Identification of homologs

Subjecting LlpE to a BLAST search [25] did not reveal homology to previously characterized *B. cepacia* lipases, none of which are associated with antibiotic resistance [29,30]. However, the search did reveal homology to various eukaryotic and prokaryotic lipases and esterases belonging to the α/β hydrolase family of proteins. These proteins have diverse functions, but