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Investigation of Differential Gene Expression Patterns of Virulent and Avirulent *Burkholderia* Species

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ABSTRACT

A differential gene expression study revealed that *Burkholderia pseudomallei*, a virulent species, and *B. thailandensis*, an avirulent species, had similar gene expression patterns compared to another virulent species, *B. cepacia*. Only the differentially expressed gene fragments from *B. cepacia* were sequenced and the four fragments were amplified by arbitrary primers ACP5, ACP9, ACP10 and ACP12. The genes were identified as expressing lytic transglycosylase, porin, outer membrane autotransporter barrel domain, and two component transcriptional regulator of the LuxR family, with a 95, 94, 100, and 90% identity respectively, against *B. cenocepacia* HI2424 and AU 1054 nucleotides. Although the genes encoding these proteins are shared by most bacteria, certain factors such as growth conditions might affect the expression of the genes in some bacteria, as down regulation of gene expression in *B. pseudomallei* and *B. thailandensis* was noticed in this study.

Keywords: *B. pseudomallei*, *B. thailandensis*, *B. cepacia*, differentially expressed genes

INTRODUCTION

Burkholderia is a genus of proteobacteria consisting of several species commonly found in soil and groundwater worldwide. They are human and plant pathogens as well as environmentally important bacteria. *Burkholderia mallei* is responsible for glanders in horses; *B. pseudomallei* is the causative agent of melioidosis; and *B. cepacia* is an important source of pulmonary infection in cystic fibrosis patients.

B. thailandensis is closely related to *B. pseudomallei* but not known to cause disease. It is distinguished from *B. pseudomallei* by its ability to assimilate arabinose (Wuthiekanun *et al.* 1996).

Differentially expressed genes have been studied as biomarkers of cancer cells in humans (Choi *et al.* 2007; de Sá *et al.* 2007). With regard to bacteria, the identification of differentially expressed genes in quorum sensing-related regulons in *Vibrio vulnificus* (Shin *et al.* 2007) and gene expression in cells infected with *Salmonella typhimurium* (Kang *et al.* 2007) have been reported. Methods used were suppression subtractive hybridization (de Long *et al.* 2008), cDNA microarray (Madsen *et al.* 2008), and proteomics (Diniz *et al.* 2004).

The objective of this study was to identify the differentially expressed genes between *B. pseudomallei* and *B. cepacia* and the avirulent *B. thailandensis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Reference strains of *B. pseudomallei* (NCTC 13178), *B. thailandensis* (ATCC 700388), and *B. cepacia* (ATCC 25416) were cultured in 10 ml of Luria-Bertani medium (Sigma-Aldrich, St. Louis, MO, USA) at 37°C overnight with shaking until OD_{600} value of 1.0 was obtained.

All the following procedures were performed according to the manufacturers' recommendations.

RNA isolation

One milliliter of bacterial culture was added to 2 ml of RNAprotect Bacteria Reagent (Qiagen GmbH, Hilden, Germany) and incubated for 5 min at room temperature. RNA isolation was performed on the harvested cells using RNeasy® Mini Kit (Qiagen GmbH) and lysed in 200 µl TE buffer (30 mM Tris-Cl, 1 mM EDTA, pH 8.0) consisting of 10 mAU proteinase K (Qiagen GmbH) and 15 mg/ml lysozyme (Amresco, Solon, Ohio, USA) at room temperature for 10 min. Buffer RLT and absolute ethanol was added and 700 µl of the lysate was transferred to an RNeasy Mini spin column with subsequent centrifugation for 15 s at 10,000 × *g*. Washing and centrifugation steps were repeated 3 times and RNA was eluted from the spin column with 30 µl RNase-free water by centrifugation for 1 min at 10,000 × *g*. The recovered RNA was treated with 1 unit of DNase I (New England Biolabs, Beverly, MA, USA) and incubated at 37°C for 10 min and the RNA was subjected to electrophoresis with 1% agarose gel and quantitated by measuring the absorbance at 260 nm.

Poly(A)-tailing reaction

The addition of poly(A)-tails to the 3′-end of the RNA was done using Poly(A) Tailing Kit (Ambion, Inc., Austin, TX, USA), whereby 60 µg of RNA obtained from pervious procedures was added to the tailing reagents comprising 1X *E. coli* Poly(A) Polymerase (*E*-PAP) buffer, 2.5 mM $MnCl_2$, 1 mM ATP, and 1 unit of *E*-PAP to a final reaction volume of 100 µl and incubated at 37°C for 1 hour. Electrophoresis was carried out with the resulting poly(A)-tailed RNA with 1% agarose gel electrophoresis and quantitated.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

First strand cDNA was synthesized by mixing 3 µg RNA with 1 µM dT-ACP1 (Seegene, Inc., Seoul, Korea) and reagents from RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Inc., Burlington, ON, Canada), containing 1X reaction buffer,

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20 unit RiboLock™ Ribonuclease inhibitor, 1 mM dNTP mix, and 200 unit of RevertAid™ H Minus M-MuLV Reverse Transcriptase, to a final volume of 20 µl. The mixture was incubated at 42°C for 90 min and heated to 94°C for 2 min. The final mixture was chilled on ice for 2 min and diluted with 80 µl of DNase-free water.

Fifty nanograms of diluted first-strand cDNA was added to PCR reagents from GeneFishing™ DEG Premix Kit (Seegene), which included 0.5 µM of one of the 20 arbitrary ACPs, 0.5 µM dT-ACP2, and 1X SeeAmp™ ACP™ Master Mix, to make up a final volume of 20 µl. This PCR mixture was placed in a preheated thermal cycler and initial cycle of denaturation, annealing, and extension was done at 94°C for 5 min, 50°C for 3 min, and 72°C for 1 min, respectively. This was followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 65°C for 40 sec, and extension at 72°C for 40 sec, and a final extension at 72°C for 5 min. Five microliters of the PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide.

Cloning

The differential bands among *B. pseudomallei*, *B. thailandensis*, and *B. cepacia* were extracted and purified with GeneAll™ Gel SV Mini Kit (General Biosystem, Inc., Seoul, Korea). The PCR products were cloned into the pGEM®-T Vector System (Promega, Madison, WI, USA), and 3 µl of each purified PCR product was mixed with 1X rapid ligation buffer, 50 ng pGEM®-T Vector, and 3 Weiss units of T4 DNA ligase, and made up to a final volume of 10 µl. The reaction mixture was incubated overnight at 4°C and 2 µl was transferred to 50 µl of One Shot® TOP10 Chemically Competent Cells (Invitrogen, Carlsbad, California, USA). Following heat-shock at 42°C, 950 µl LB medium was added to the ligation reaction transformations and incubated for 1.5 hours at 37°C with shaking at 150 rpm. One hundred microliters of each transformation culture was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C.

Polymerase Chain Reaction

White colonies containing inserts were selected and resuspended in sterile distilled water and boiled for 5 min. Two microlitres of each suspension was used as a DNA template for PCR by mixing with 1X *Taq* buffer with KCl, 1.17 mM $MgCl_2$, 0.13 mM dNTP mix, 1 U *Taq* DNA polymerase, 1 µM T7 promoter primer, and 1 µM SP6 primer. Initial denaturation was at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were then electrophoresed on 1% agarose gel containing ethidium bromide at 100 V for 30 min and bands were viewed under UV light. The relevant bands were excised, purified using GeneAll™ Gel SV Mini Kit (General Biosystem), and sent for DNA sequencing (First Base, Shah Alam, Selangor, Malaysia).

Database search

The nucleotide sequence obtained was subjected to database search against *Burkholderia* nucleotides using NCBI Blast (www.ncbi.nlm.nih.gov/) and the hit presenting the highest score and sequence coverage, and the lowest E value was selected.

RESULTS

Of the 20 arbitrary primers used on the 3 organisms, 16 primers gave similar gene expression patterns while the following 4 primers ACP5, ACP9, ACP10 and ACP12, gave differential gene expression patterns after RT-PCR electrophoresis (**Table 1; Fig. 1**). *B. cepacia* showed the most variable gene expression pattern among the 3 species. An obvious differentially expressed gene pattern between *B. pseudomallei* and *B. thailandensis* was observed using primer ACP12 (**Fig. 1**). The 4 bands of ~290, 280, 210, and 250 bp were all extracted from *B. cepacia* after amplification using the 4 ACPs mentioned above (**Fig. 1**), and were identified as genes encoding lytic transglycosylase, porin, outer membrane autotransporter barrel domain, and 2 component transcriptional regulator of the LuxR family with a 95, 94, 100, and 90% identity respectively against *B. cenocepacia* HI2424 and AU 1054 nucleotides.

DISCUSSION

Genes expressing lytic transglycosylase (LT), porin, outer membrane autotransporter barrel domain, and the 2 component transcriptional regulator of the LuxR family, were seen in our study of *B. cepacia*. LTs are peptidoclycan-degrading enzymes that cleave the β-1,4 glycosidic bond between MurNAc and GlcNAc residues (Koraimann 2003). They contribute to the metabolism and remodeling of peptidoglycans and act as cell wall 'zippers' during cell division (van Asselt *et al.* 1999). LT homologue, *BapC* has been found to be associated with type III secretion genes in both *B. pseudomallei* (DeShazer *et al.* 1999) and *B. thailandensis*, whereas in *B. cepacia*, *virB1* is present (Parsons *et al*. 2001).

Porins are believed to play crucial roles in the interactions between Gram-negative bacteria and their environment (Siritapetawee *et al.* 2004) as well as in the pathway associated with bacterial infections (Marcatili *et al.* 2004). In addition, bacterial porins serve as receptors for bacteriophage and bacteriocidins (Siritapetawee *et al.* 2004), maintain the integrity of the cells (Albertí *et al.* 1995), and in pathogenic species appear to be targets for the immunological system (Siritapetawee *et al.* 2004). Porins may be also responsible for antibiotic resistance. Mass spectrometry analysis of Omp38 porin from *B. pseudomallei* and *B. thailandensis* showed sequence similarity of 76.5% to the OpcP1

Table 1 The sequences of arbitrary primers used on *Burkholderia* species that gave differential gene expression patterns.

Primers	Sequence (5′-3′)
ACP5	GTCTACCAGGCATTCGCTTCATXXXXXAGTGCGCTCG
ACP9	GTCTACCAGGCATTCGCTTCATXXXXXGATGCCGCTG
ACP10	GTCTACCAGGCATTCGCTTCATXXXXXTGGTCGTGCC
ACP12	GTCTACCAGGCATTCGCTTCATXXXXXACCGTGGACG

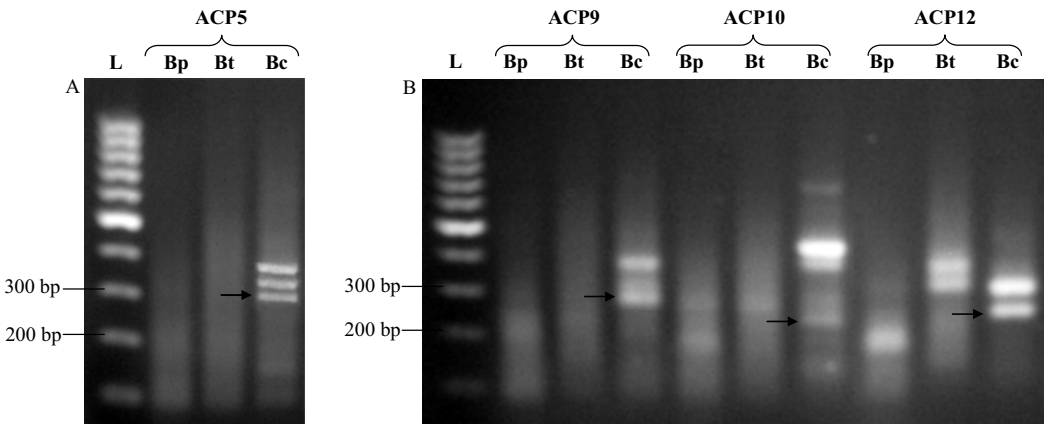

Fig. 1 Differential gene expression patterns of *B. pseudomallei*, *B. thailandensis*, and *B. cepacia* following amplification with arbitrary primers ACP5, ACP9, ACP10 and ACP12. Differential bands sequenced and identified indicated by arrows (→). L, 100 bp DNA ladder; Bp, *B. pseudomallei*; Bt, *B. thailandensis*; Bc, *B. cepacia*.

porin from *B. cepacia* (Siritapetawee *et al.* 2004).

Autotransporter β-barrel or translocator domain, a 25-30 kDa protein fragment (Konieczny *et al.* 2001), is an outer membrane-based C-terminal extension of an autotransporter which secretes or transports the N-terminal passenger domain across the outer membrane (Oomen *et al.* 2004). Proteins secreted via this pathway often confer virulence to pathogenic bacteria by mediating adhesion to host cells or actin-promoted bacterial mobility (Henderson and Nataro 2001).

Quorum sensing has been shown to regulate the production of virulence factors in several Gram negative species (Chapon-Hervé *et al.* 1997). The two components required for cell density-dependent *lux* expression are the LuxR and LuxI proteins (Engebrecht *et al.* 1983). In *B. cepacia*, *cepR*, a *luxR* homologue encodes a transcriptional regulator that has both positive and negative regulatory properties in expression of extracellular proteases, swarming motility, and biofilm production (Lewenza *et al.* 1999; Huber *et al.* 2001). *LuxR* homologues for *B. pseudomallei* and *B. thailandensis* were identified as *pmlR* and *btaR* respectively (Ulrich *et al.* 2004; Valade *et al.* 2004). Nucleotide similarity has been reported in *luxIR* alleles of *B. pseudomallei* and *B. thailandensis*, whereas *B. thailandensis btaR* and *B. cepacia luxR* homologues have identity of only 41-80% (Ulrich *et al.* 2004).

The genomes of *B. pseudomallei* and *B. thailandensis* were found to be broadly similar, encompassing 2 highly syntenic chromosomes with comparable numbers of coding regions, protein family distributions, and horizontally acquired genomic islands (Yu *et al.* 2006). Despite the differentially present genetic elements, subtractive molecular techniques have demonstrated a high degree of sequence conservation between *B. pseudomallei and B. thailandensis* (DeShazer *et al.* 2001; Reckseidler *et al.* 2001). Large regions of genomic synteny (>400 kb) were observed between the genomes of *B. pseudomallei* and *B. cepacia*. By ribosomal typing, *B. cepacia* is believed to be even more distantly related to *B. pseudomallei* than *B. thailandensis* (Ong *et al.* 2004). Hence, it was not surprising that, in our study, (i) *B. pseudomallei* and *B. thailandensis* showed similar gene expression patterns; (ii) *B. cepacia* had more distinct gene expression patterns than either *B. pseudomallei* or *B. thailandensis*; (iii) genes expressed in *B. cepacia* were mostly detected.

The genes encoding the above 4 proteins exist in all Gram negative bacteria being responsible for virulence functions. They were detected only in *B. cepacia* and not in *B. pseudomallei* and *B. thailandensis* in this study probably due to the different nucleotide alignment in the genes, or the genes were not being highly expressed in *B. pseudomallei* and *B. thailandensis*. The sequence amplified by primer ACP5 also had high compatibility with the gene encoding RpiR family transcriptional regulator of *Bacillus pumilus* when it was searched against the database of other bacterial nucleotides. RpiR protein, expressed by the *rpiB* gene, synthesizes ribose phosphate isomerase B which is involved in the metabolism of pentose phosphate in bacteria (Sørensen and Hove-Jensen 1996).

Overall, this is the first study to show that RT-PCR with arbitrary primers could be a useful and cost effective method to study the differentially expressed genes among bacteria, especially potential virulence factors besides using expression microarray. The 4 differentially expressed genes in our study were only identified in *B. cepacia.* This shows that the 4 proteins associated with the particular genes are highly expressed and could be directly involved in encoding virulence factors in *B. cepacia*. However, further investigations need to be carried out using different sets of arbitrary primers to fish out more genes and to complete differential gene expression profiles in these *Burkholderia* species.

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