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A Second Quorum Sensing Regulon in *Burkholderia pseudomallei*

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ABSTRACT

*Burkholderia pseudomallei*, a Gram-negative soil bacterium, is the causative agent of melioidosis. Quorum sensing is a mechanism responsible for the regulated expression of virulence genes in many bacterial pathogens. The first quorum sensing regulon in *B. pseudomallei*, *Ais/Air*, was recently identified in our laboratory. The report describes the identification of a second quorum sensing regulon, *RhlI/RhlR*, in *B. pseudomallei*. By data mining of the recently completed *B. pseudomallei* K96243 genome sequence using homologous sequences from related bacteria, we have found several putative quorum sensing genes in *B. pseudomallei*. Gene-specific primers were designed to amplify the putative quorum sensing genes, *rhlI* and *rhlR*. The primers included *BamHI* restriction sites to facilitate the cloning of the PCR products into a broad host range mobilizable plasmid, pRK415. The PCR products were sequenced to obtain the nucleotide sequences of the *B. pseudomallei* KHW, a clinical isolate. The translated amino acid sequences of the *B. pseudomallei* KHW RhlI and RhlR proteins show high homology to the RhlI and RhlR proteins, respectively, of the *P. aeruginosa* quorum sensing system.

INTRODUCTION

*Burkholderia pseudomallei* is the causative agent of melioidosis, a systemic and potential life-threatening disease in humans. The bacterium is intrinsically resistant to many antibiotics and its mechanisms of virulence are still obscure. In this study, we aim to identify quorum sensing regulons in *B. pseudomallei* using a virulent locally isolated clinical strain, KHW.

Quorum sensing is a two component gene regulation system, which controls cell density-dependent expression of diverse bacterial phenotypes. It consists of a N-acyl homoserine lactones (AHLs) autoinducer synthase, and an autoinducer regulator protein. Quorum sensing is known to regulate the production of virulence factors, motility, biofilm formation, plasmid transfer, and antibiotic resistance in several gram-negative bacterial pathogens (Lutter, et al., 2001). The strict control of virulence factor expression may be important in preventing the microorganism from alerting its host to its presence when infecting populations are small. As such, individual bacterial cells are essentially delayed from producing virulence factors until they are in a population (quorum) that is large enough to overwhelm the host (Whitehead, et al., 2001).

Our laboratory has recently identified a pair quorum sensing genes in *B. pseudomallei*, which we called *ais* and *air*. Our studies with the *ais* deletion mutant of *B. pseudomallei* suggest

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that there may be additional quorum sensing regulons in \textit{B. pseudomallei}. Moreover, in \textit{P. aeruginosa}, a closely related specie of \textit{B. pseudomallei}, at least two pairs of quorum sensing genes, \textit{lasI/lasR} and \textit{rhlI/rhlR}, are involved in the regulation of virulence (Whitehead, et al, 2001). This project describes the identification of a second pair of quorum sensing genes, \textit{rhlI/rhlR}, in \textit{B. pseudomallei}.

RESULTS AND DISCUSSION

A. Bioinformatics research of the putative regions of the second pair of quorum sensing genes of \textit{B. pseudomallei} K96243 strain.

Pairwise alignment was performed using protein sequences of quorum sensing systems (\textit{luxI/R} families) of related bacterial species as queries in a tblastn search against the \textit{B. pseudomallei} K96243 genomic translated protein database at www.sanger.ac.uk. The results identified a region at nucleotide positions 2130000 ~ 2132100 on \textit{B. pseudomallei} chromosome 2 which has high similarity (30-50\%) to quorum sensing gene families, which includes the synthase and regulator genes. The putative quorum sensing genes were named \textit{rhlI} and \textit{rhlR}. Open Reading Frame predictions of the putative \textit{B. pseudomallei} quorum sensing genes using Vector NTI\textsuperscript{TM} Suite 8 ORF analyses program, identified \textit{rhlI} coding sequence at 2131287 ~ 2131895 and \textit{rhlR} coding sequence at 2130282 ~ 2130974 on the \textit{B. pseudomallei} K96243 chromosome 2. (Fig. 1) A putative promoter sequence, gccgcctctcttgcaatcgttattgcatttgatattatttgcaga caatttca was identified (2130052 ~ 2130097) using the Neural Network Promoter Prediction at http://www.fruitfly.org/seq_tools/promoter.html. (Fig. 1.)

Multiple amino acid sequences alignment of translated \textit{B. pseudomallei} K96243 \textit{RhlI} with other AHL synthase proteins, and \textit{RhlR} with other AHL dependent transcriptional regulator proteins of related bacterial species showed that there were conserved motifs within quorum sensing systems. This strongly suggests that the \textit{B. pseudomallei} K96243 \textit{rhlI} and \textit{rhlR} sequences which we have identified are likely to be involved in quorum sensing.

Fig. 1. The physical map of putative quorum sensing genes \textit{rhlI} and \textit{rhlR} (orange arrow) on \textit{B. pseudomallei} K96243 chromosome 2 (nucleotide positions 2130000 to 2132100). Green arrows indicate the PCR primer pairs, \textit{rhlIF1/rhlIR1} and \textit{rhlRF1/rhlRR1}. The putative promoter is indicated by the dark red arrow.

B. Primers for PCR
Primer pairs, rhlIF1/rhlIR1 and rhlRF1/rhlRR1 were designed, using Vector NTI™ Suite 8 Primer Design program, to include BamHI restriction sites in the sense and antisense primers so as to facilitate the cloning of the *rhlI* and *rhlR* PCR products, respectively, into the broad host range, mobilizable vector, pRK415 (Fig. 1, Table 1). 2 nucleotides, GC, were added to the beginning of rhlRF1 and rhlIR1, to allow the cutting of the PCR product by BamHI. The primer stocks were dissolved with sterile distilled water and were diluted to 10 μM before use.

**Table 1.** Primers for the amplification and cloning of *B. pseudomallei* *rhlI* and *rhlR* (*BamHI* restriction site is in bold)

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Nucleotide Sequence</th>
</tr>
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<tbody>
<tr>
<td><em>rhlI</em></td>
<td>5’- GGCAGGGGCTCATCTTTCCATGA -3’</td>
</tr>
<tr>
<td></td>
<td>5’- GCGGATCCCTAGAGGAACGTCCAATTC -3’</td>
</tr>
<tr>
<td><em>rhlR</em></td>
<td>5’- GCGGATCCCGGGGATAAAAATATCGAACGC -3’</td>
</tr>
<tr>
<td></td>
<td>5’- ATGAAACGGGCGGCGCTAACCGAT -3’</td>
</tr>
</tbody>
</table>

**C. PCR amplification of the *B. pseudomallei* KHW *rhlI* and *rhlR***

Polymerase chain reactions were done to amplify the full-length of *rhlI* and *rhlR* including their promoter region. *B. pseudomallei* KHW, a local clinic virulent strain was used as template. The reagents and the optimal PCR thermal cycle for *rhlI* and *rhlR* are listed in Table 2A, 2B.

<table>
<thead>
<tr>
<th>Reagent</th>
<th><em>rhlI</em> Volume (μL)</th>
<th><em>rhlR</em> Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer (BioTaq)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>50mM MgCl₂ (BioTaq)</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>2mM dNTP</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>100ng/μL KHW DNA Template</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>BioTaq DNA polymerase (5u/mL)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>101M Primer 1 (rhlIF1/rhlRF1)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>101M Primer 1 (rhlIR1/rhlRR1)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>5M Betaine</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Sterile Distilled Water (H₂O)</td>
<td>8.75</td>
<td>8.5</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

**Table 1A.** PCR mixture components of *rhlI* and *rhlR* (Left)

**Table 1B.** Optimal PCR thermal cycle for *rhlI* and *rhlR* (Below)

<table>
<thead>
<tr>
<th></th>
<th><em>rhlI</em></th>
<th><em>rhlR</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>T (°C)</td>
<td>Duration</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>30’</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>30’</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1’30’</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10’</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>30’</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>30’</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1’30’</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10’</td>
<td></td>
</tr>
</tbody>
</table>

The sizes of the PCR products obtained were as expected from the *rhlI* and *rhlR* sequences predicted (Fig.2). PCR products were extracted using BIO 101 GENECLEAN® SPIN kit according to the manufacturers instructions.
D. Cloning of \textit{rhlI} and \textit{rhlR} into vector pRK415

The plasmid vector, pRK415, and purified \textit{rhlI} and \textit{rhlR} PCR products were each digested with \textit{Bam}HI (1u/ìL) for 2 hours at 37°C. Linearized pRK415 was treated with CIAP (calf intestinal alkaline phosphatase) to hydrolyze 5’-phosphate groups so as to prevent self-ligation of the vector DNA. Ligation of pRK415 and \textit{rhlI} or \textit{rhlR} was performed using T4 DNA ligase (0.3u/ìL) at 16°C overnight. The ligation products were transformed into competent \textit{E. coli} DH5αëpir by electroporation at 1.8kV. Transformants were selected on LA agar containing 25ìg/ml tetracycline, 0.01% (w/v) X-gal and 0.2mM IPTG.

E. DNA sequencing of \textit{B. pseudomallei} KHW \textit{rhlI} and \textit{rhlR}

DNA sequencing of \textit{B. pseudomallei} KHW \textit{rhlI} and \textit{rhlR} PCR products were performed using the ABI BigDye reagents and analyzed on an ABI377 automated DNA sequencer (PerkinElmer). \textit{rhlIF1/rhlIR1} and \textit{rhlRF1/rhlRR1} were used as sequencing primers. Sequence of \textit{B. pseudomallei} KHW \textit{rhlI} and \textit{rhlR} were analyzed using Vector NTI™ ContigExpress program. Pairwise sequence alignment of the \textit{B. pseudomallei} KHW \textit{rhlI} and \textit{rhlR} DNA sequences with that of \textit{B. pseudomallei} K96243 showed that they share 97% and 99% similarity, respectively. Homology search of the translated \textit{B. pseudomallei} KHW protein \textit{RhlI} and \textit{RhlR} against GenBank protein database using blastp identified several proteins which share high similarity (40-50%) with \textit{RhlI} are N-acyl-homoserine lactone synthases, and high similarity (41-49%) with \textit{RhlR} are acylhomoserine lactone dependent transcriptional regulators, respectively. The highest scores were those from \textit{Burkholderia spp.} and \textit{Pseudomonas spp.}, which are taxonomically closely related to \textit{B. pseudomallei}. This strongly suggests that the \textit{rhlI} and \textit{rhlR} genes which we have identified comprise a second quorum sensing system in \textit{B. pseudomallei}.

REFERENCES

