A functional msbB acyltransferase of Photorhabdus luminescens, required for secondary lipid a acylation in gram-negative bacteria, confers resistance to anti-microbial peptides

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A FUNCTIONAL MSB B ACYLTRANSFERASE OF PHOTORHABDUS LUMINESCENS, REQUIRED FOR SECONDARY LIPID A ACYLCATION IN GRAM-NEGATIVE BACTERIA, CONFERS RESISTANCE TO ANTI-MICROBIAL PEPTIDES

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ABSTRACT


Lipid A is a potent endotoxin, and its fatty acids (lauric, myristic, and sometimes palmitic acid) anchors lipopolysaccharide (LPS) into the outer leaflet of the outer membrane of most Gram-negative bacteria. The highly anionic charge of the glucosamine lipid A moiety makes the LPS a powerful attractant for cationic antimicrobial peptides (AMPs). AMPs are major component of innate immunity that kill bacteria by permeabilization of lipid bilayers. Secondary lipid A acylation of Klebsiella pneumoniae, involving the acyltransferase LpxM (formally, msbB or WaaN) that acylates (KDO)2-(lauroyl)-lipid IV-A with myristate during lipid A biosynthesis, has been associated with bacterial resistance to AMPs contributing to virulence in animal models. We investigated here the role of the msbB gene of the entomopathogenic bacterium Photorhabdus luminescens in AMP resistance, by functional complementation of the AMP susceptible K. pneumoniae lpxM mutant with the P. luminescens msbB gene. We showed that msbB (lpxM) gene of P. luminescens is able to enhance polymyxin B, colistin and cecropin A resistance of K. pneumoniae lpxM mutant, compared to the non-complemented mutant. However, we could not obtain any msbB mutant of Photorhabdus by performing allelic exchange experiments based on positive selection of sucrose highly resistant mutants. We thus suggest that msbB-mediated Photorhabdus lipid A acylation is essential for outer membrane low-permeability and that modification of lipid A composition, fluidity and osmosis-resistance have an important role in the ability of Photorhabdus to grow in sucrose at high concentrations.

Keywords: Photorhabdus luminescens, Klebsiella pneumoniae, lipid A acylation antimicrobial peptides, msbB gene
INTRODUCTION

In most of Gram-negative bacteria, the outer monolayer of the outer membranes is made up of Lipid A, a glucosamine-based saccharolipid that represents the hydrophobic moiety of lipopolysaccharide (LPS) (Raetz & Whitfield, 2002; Nikaido, 2003; Fahy et al., 2005). LPS is a complex structure which contains in addition to lipid A, two other components: core polysaccharides and O-linked polysaccharides. Lipid A is the active component of LPS endotoxin, which can promote septic shock when released from the bacterial surface during systemic infection (Parillo, 1993; Russell, 2006).

Lipid A is a highly anionic component of LPS in Gram-negative bacterial envelopes (Raetz et al., 2007). It therefore serves as powerful attractant for cationic antimicrobial peptides (AMPs) that are central to the innate humoral immune systems of mammals and insects. Many cationic AMPs are thought to act by accumulating within the cytoplasmic membrane to a critical concentration that allows the assembly of structures that permeabilize the cell (Hancock, 1999; Hancock & Diamond, 2000). For the bacterial resistance to cationic AMPs and colonization of the host-tissues, most of Gram-negative pathogens modify their lipid A backbone (for a recent review see Bauer & Shafer, 2015). In Salmonella, inducible modifications include pmr-mediated addition of aminoarabinose polar group (Helander et al., 1994; Gunn et al., 1998) as well as pagP-mediated secondary palmitate chain addition (Guo et al., 1998), which have been shown to confer bacterial resistance to AMPs from humans, vertebrates and invertebrates. Lipid A of most enterobacteria is composed of biphosphorylated glucosamine disaccharide, which is constitutively acylated with primary hydroxymyristate chains. These primary chains are further acylated with secondary laurate and myristate fatty acid chains. Addition of these acyl chains is catalyzed under non-cold-shock conditions by the acyltransferases HtrB (Clementz et al., 1996) and msbB (Clementz et al., 1997), respectively. Therefore, the presence of several (six or seven in E. coli and Salmonella) saturated fatty acid chains per molecule of LPS from enteric bacteria grown under the usual conditions, usually decreases the fluidity of the LPS layer (Cullis & Hope, 1985), by enhancing lateral interactions between fatty acid tails of neighboring LPS molecules in the outer membrane. Consequently, the properties of LPS including lipid A acylations, significantly contribute to the low-permeability of the outer membrane to a wide variety of noxious compounds such as bile salts and hydrophobic antibiotics (Nikaido, 2003). Secondary lipid A acylation catalyzed by the acyltransferase msbB was shown to mediate AMP resistance of the human pathogens K. pneumoniae and ElTor Vibrio cholerae, thereby correlating with growth and colonization attenuations in mice models of infection, respectively (Clements et al., 2007; Matson et al., 2010). Similarly, msbB mutants of Shigella flexneri, pathogenic Escherichia coli, Salmonella enterica serovar Typhimurium, are all attenuated for virulence (D’Hauteville et al., 2002; Xu et al., 2013; Somerville et al., 1999; Low et al., 1999). Secondary lipid A acylation catalyzed by HtrB was also shown to be crucial for Haemophilus influenzae resistance to human β defensin (Starner et al., 2002).

The aim of this study was to assess the role of msbB in the enterobacterium P. luminescens in order to increase our global understanding of the role of LPS in resistance to AMPs. Photorhabdus luminescens is an insect pathogen that also has a mutualistic relationship with entomopathogenic nematodes from the family Heterorhabditidae (Boemare, 2002). Upon entering insect hemolymph, bacteria are targeted by an array of induced cationic AMPs (Lemaître & Hoffmann, 2007) that are produced massively by the fat body (Dimarcq
et al., 1998; Bulet et al., 1999). *P. luminescens* TT01 strain is intrinsically resistant to many AMPs in vitro (Derzelle et al., 2004). This is the most likely reason why bacteria regurgitated by the infective nematodes or injected into the insect hemolymph are able to multiply and kill insect larvae within 24 to 48 hours after infection. The involvement of lipid A modifications in AMP resistance was previously described in *P. luminescens* and has been correlated with totally impaired virulence in insects (Derzelle et al., 2004; Bennett & Clarke, 2005). LPS is therefore thought to be an important component of *P. luminescens* that confers bacterial protection from host defense molecules.

Here, we report that the msbB gene of *P. luminescens* is able to restore polymyxin B, colistin and cecropin A resistance in the *lpxM* mutant of *K. pneumoniae*, which was previously shown to be susceptible to these compounds. We also suggest that the msbB gene is highly essential for *P. luminescens* outer membrane integrity and survival since we could not obtain the corresponding mutant, thereby precluding *in vivo* analysis in insects.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth conditions**

Permanent stocks of all bacterial strains were maintained at -80°C in Luria–Bertani (LB) broth supplemented with 17% glycerol. Bacterial strains and plasmids used in this study are listed in Table 1. *Photorhabdus luminescens* TT01 (Fischer-Le Saux et al., 1999) was routinely grown in Luria LB broth (DIFCO) or on nutrient agar (DIFCO) at 28°C. *K. pneumoniae* B5055nm, a virulent but non-mucoid mutant and its derivative B5055nmΔlpxM, a double mutant non-encapsulated *lpxM* mutant (Clements et al., 2007), *Escherichia coli* XL1-Blue (Stratagene) and S17.1 (Simon et al. 1983) were routinely grown in LB broth or on LB agar at 37°C. When required, the final concentrations of antibiotics were: 100 µg.ml⁻¹ Ampicillin (Ap), 10 µg.ml⁻¹ Gentamicin (Gm) for strains harbouring the pBBR1-MCS5 plasmid, 30 µg.ml⁻¹ Gm for strains harbouring the pJQ200KS plasmid, 20 µg.ml⁻¹ Kanamycin (Km), 20 µg.ml⁻¹ Chloramphenicol (Cm) for *E. coli* and 15 µg.ml⁻¹ Cm for *P. luminescens*.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and relevant characteristic</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Photorhabdus luminescens</em> TT01</td>
<td>Wild type isolated from <em>Heterorhabditis bacteriophora</em> nematode TH01</td>
<td>(Fischer-Le Saux et al., 1999)</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5055nm</td>
<td>B5055, mutation in <em>wza/wzb</em>, non-mucoid</td>
<td>(Clements et al., 2007)</td>
</tr>
<tr>
<td>B5055nmΔlpxM</td>
<td>Double mutant, non-mucoid, mutation in <em>lpxM</em></td>
<td>(Clements et al., 2007)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
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</tbody>
</table>
Continued:

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotypes and Vectors</th>
<th>Vendors</th>
</tr>
</thead>
</table>
| XL1-Blue         | (Δ(mrcA)183Δ(mcrCB-hsdSMR-mrr
173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacF’ proAB laqlq ZAM15 Tn10(TcR) pro r- n- TpRSmR RP4-2-Tc::Mu::Tn7 recA thi | Stratagene       |
| S17.1            | (Simon, 1983)                                                                         |                  |

<table>
<thead>
<tr>
<th>Plasmids</th>
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<tbody>
<tr>
<td>pUC19</td>
<td>Ap’ cloning vehicle</td>
<td>Biolabs</td>
</tr>
<tr>
<td>pBBR1-MCS5</td>
<td>Gm’ mob broad host range vector</td>
<td>(Kovach, 1995)</td>
</tr>
<tr>
<td>pmsbB</td>
<td>pUC19 carrying msbB from TT01</td>
<td>This study</td>
</tr>
<tr>
<td>pBR-lpxM</td>
<td>pBR322 carrying lpxM from B5055</td>
<td>(Clements et al., 2007)</td>
</tr>
<tr>
<td>pBB-msbB</td>
<td>pBBR1MCS-5 carrying msbB from TT01</td>
<td>This study</td>
</tr>
<tr>
<td>pJQ200 KS</td>
<td>Gm’ sacRB mob oriV (p15A replicon) suicide vector</td>
<td>S. Forst</td>
</tr>
<tr>
<td>pUC-msbB-ΩCm</td>
<td>4.7 kb XbaI-SacI fragment containing the 620bp msbB upstream region - ΩCm - the 618bp msbB downstream region, cloned into pUC/XbaI-SacI</td>
<td>This study</td>
</tr>
<tr>
<td>pJQ-msbB-ΩCm</td>
<td>4.7 kb XbaI-SacI fragment containing the 620bp msbB upstream region - ΩCm - the 618bp msbB downstream region, cloned into pJQ200KS/XbaI-SacI</td>
<td>This study</td>
</tr>
<tr>
<td>pH45-ΩCm</td>
<td>Ap’ Cm’ interposon ΩCm</td>
<td>(Fellay et al., 1987)</td>
</tr>
</tbody>
</table>

**Cloning and expression of the TT01 msbB gene in K. pneumoniae**

The entire msbB open reading frame of *P. luminescens* TT01 (plu2117) was PCR-amplified from TT01 genomic DNA using the oligonucleotide primers

5’-GCGCCTGCTACGGGCTGTTTGTATTGC-3’ and

5’-GCCGGAGCTCATCCCGCTTAATCCAGAC-3’ including the restriction enzymes sites PsiI and SacI, respectively. The 1.25 kb resulting fragment was hydrolyzed by PsiI and SacI, and then ligated with the pUC19 or pBBR1MCS-5 vectors hydrolyzed by the same enzymes. *E. coli* XL1-Blue was transformed with the ligation product according to standard protocols (Ausubel et al., 1993). The resulting plasmids designed pmsbB and pBB-msbB were verified by restriction and sequencing (MACROGEN, Seoul-Korea) and then were transferred into *Klebsiella* strains by standard electroporation procedure (Ausubel et al., 1993).

**Sensitivity to antimicrobial peptides**

*In vitro* susceptibility tests to determine minimum growth inhibitory concentration (MIC) values of *K. pneumoniae* strains were performed as described by Clements et al. (2007) for a range of AMPs: cecropin A (Sigma), polymyxin B sulfate (Sigma), and colistin methanesulfate (Sigma). Stock solutions of colistin, polymyxin B and cecropin A were
prepared in sterile water to obtain concentrations of 20 mg ml\(^{-1}\), 50 mg ml\(^{-1}\) and 0.1 mg ml\(^{-1}\), respectively. Briefly, 96-well microtiter plates containing decreasing concentrations of different AMPs (0.02 to 25 µg/ml of cecropin A, 0.1 to 125 µg/ml of polymyxin B sulfate, and 0.2 to 50 µg/ml of colistin methanesulfonate) were prepared by 2-fold serial dilutions of stock solutions in LB broth. Wells were inoculated with \(10^5\) K. pneumoniae CFU/ml of mid-log bacterial cultures. Growth was scored after 24–48 hours of incubation at 37°C. The microtiter plates were read by visual observation and the MIC was the lowest concentration of compound that severely inhibited cell growth. Additional in vitro kinetics of K. pneumoniae strains were determined in a Infinite 200 microplate reader (TECAN) and growth was monitored by change in absorbance at 600 nm (\(A_{600}\)) every 30 min during 16 hours.

Construction of the P. luminescens msbB mutant

In order to create a stable chromosomal mutation by allelic exchange in the TT01 msbB gene, we constructed a derivative of the pJQ200KS carrying the upstream part of the target msbB gene (620 bp extending from positions - 539 to + 33 with respect to the msbB translation initiation site), an antibiotic resistance cassette \(\Omega\)Cm (Fellay et al., 1987), and the downstream part of the target gene (618 bp extending from positions - 223 to + 395 with respect to the msbB stop codon). TT01 genomic DNA fragments were PCR-amplified with the oligonucleotide primer pairs

\[5'\,-GCCGCTCTAGAGCCACATAAAGGGTGATT-3'/\]
\[5'\,-GCCCGATCCCGCAGTAACGAGGATGCAGGT-3'\ (upstream region) and\]
\[5'\,-GCCGATCCCGCGCGCGTGTTTCCGGTTTATGCACTTTACCGTGGCGTTAC-3' \ (downstream region).\]

The PCR products were restricted with XbaI/BamHI and BamHI/SacI enzymes couples whose restriction sites are underlined respectively. The antibiotic resistance cassette \(\Omega\)Cm was isolated as a BamHI 3.5 kb DNA fragment from the pH45-\(\Omega\)Cm plasmid. This 3.5 kb DNA fragment was then ligated to the BamHI sites between the upstream and downstream msbB regions and cloned into the pUC19/XbaI-SacI vector to generate pUC-\(\Omega\)Cm-\(\Omega\)Cm. The full-length tripartite 4.7 kb DNA fragment msbB-\(\Omega\)Cm was gel purified, restricted with XbaI and SacI, and cloned in the corresponding restriction sites of the pJQ200KS vector yielding pJQ-msbB-\(\Omega\)Cm. The pJQ200KS plasmid is a derivative of pACYC184 carrying the sacB gene and the msb site from RP4. The pJQ-msbB-\(\Omega\)Cm plasmid was used to transform the E. coli strain S17.1 and was introduced into P. luminescens TT01 by mating (Brillard et al., 2002). Cm\(^\text{R}\) and Sac\(^\text{R}\) exconjugants were selected on 5% sucrose and chloramphenicol LB agar. The 632 bp msbB deletion and the omega insertion were checked by PCR analysis and sequencing (MACROGEN, Seoul, Korea).

RESULTS AND DISCUSSION

Identification of P. luminescens msbB gene and its genomic environments

The msbB gene of P. luminescens (plu2117; 969 bp, 322 amino acids) was identified by homology to E. coli K12 lpxM/msbB (AAC74925), S. enterica serovar Typhimurium msbB (AAL20805), and K. pneumoniae MGH75878 lpxM. P. luminescens MsbB shares 64.84 % and 63.55 % amino-acid identities with LpxM of each of E. coli and K. pneumoniae, respectively. Multiple alignments of LpxM (msbB) proteins are shown in Fig. 1. The msbB gene was therefore shown to be highly conserved in these enterobacteria. A second
gene, \(lpxL\) (\(htrB\)), was identified in the genome of \(P.\) \(luminescens\) TT01. \(LpxL\) (\(HtrB\)) was 27.13% identical to \(msbB\) of \(Photorhabdus\). The \(lpxL\) gene was therefore considered as a duplicate of the \(msbB\) gene present in \(P.\) \(luminescens\) TT01 genome.

Remarkable similarities in genomic organization were observed between closely phylogenetically related bacteria. Furthermore, no features indicative of mobility (tRNA, insertion sequences, and integrase genes) were identified in any of the \(msbB\) environments studied in Gram-negative bacteria, therefore indicating that the \(msbB\) gene belongs to the core genome (https://www.genoscope.cns.fr/agc/mage).

**Figure 1.** Multiple sequence alignment of \(LpxM\) (\(msbB\)) proteins from \(E.\) \(coli\) K12 (AAC74925), \(S.\) \(typhimurium\) (AAL20805), \(K.\) \(pneumoniae\) MGH78578, and \(P.\) \(luminescens\) TT01, showing significant sequence homology. All alignments and consensus were generated with Multalin software version 5.3.3 (available at http://multalin.toulouse.inra.fr/multalin/). Conserved residues are shown in red and conservative substitutions are shown in blue.

**MsbB of \(P.\) \(luminescens\) restores AMP resistance to \(K.\) \(pneumoniae\) \(lpxM\) mutant**

To determine whether \(msbB\) of \(P.\) \(luminescens\) is functional, we used \(K.\) \(pneumoniae\) \(lpxM\) mutant as a host for heterologous expression. The \(K.\) \(pneumoniae\) \(lpxM\) mutant was previously shown to have increased susceptibility to the cationic AMPs polymyxin B and colistin (Clements et al., 2007). We first assessed the role of \(P.\) \(luminescens\) \(msbB\) gene in the resistance to these cationic AMPS, by determining MIC values for \(K.\) \(pneumoniae\) \(lpxM\) mutant complemented with \(P.\) \(luminescens\) \(msbB\) gene (Table 2). The expression of the cloned \(msbB\) gene through the control of the IPTG inducible promoter \(P_{\text{lac}}\) enhanced resistance of the \(K.\) \(pneumoniae\) \(lpxM\) mutant to polymyxin B, colistin and cecropin A. Although polymyxin B and cecropin A MIC values increased to the B505nm levels, colistin MIC value was partially increased (Table 2). This MIC value increase provided direct evidence that the \(P.\) \(luminescens\) \(msbB\) gene product is functional in the heterologous model of \(K.\) \(pneumoniae\). In addition, we studied the bacterial growth in the presence of a definite concentration of each polymyxin B (0.25 µg.ml\(^{-1}\)), colistin (1 µg.ml\(^{-1}\)), and cecropin A (1 µg.ml\(^{-1}\)). Cecropin A is a homologue of insect cecropins whose expression is induced in hemolymph upon bacterial infection (Lemaitre & Hoffmann, 2007). The \(lpxM\) mutant of \(K.\)
**pneumoniae** was unable to initiate growth in LB medium added with any of the AMPs tested. However, lpxM mutant of *K. pneumoniae* complemented with plasmids harbouring either the parental lpxM gene or the msbB gene from *Photorhabdus*, grew well and thus were resistant to the AMP concentrations tested (Fig. 2). Taken together, these results indicated that the msbB gene of *P. luminescens* is functional and that such function may protect these bacteria against AMP produced in insect hemolymph upon infection. As the lpxM *K. pneumoniae* mutant did not appear to have any charge alterations, it is proposed that the method of increased susceptibility of the lpxM mutant was not due to increased binding, but rather increased insertion of either the head group or fatty acid tail into the membrane due to the reduced acyl chain numbers (Clements et al., 2007).

**TABLE 2**

Minimum Growth-Inhibitory Concentrations (MIC)* Values of Three Cationic Antimicrobial Peptides (AMPs) for the *K. pneumoniae* B5055nm Strain, the lpxM Knockout Mutant and Complemented lpxM Mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>Polymyxin B</th>
<th>Colistin</th>
<th>Cecropin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5055nm</td>
<td>0.5</td>
<td>6.25</td>
<td>1.6</td>
</tr>
<tr>
<td>B5055nm/lpxM/pUC19</td>
<td>0.125</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>B5055nm/lpxM/msbB</td>
<td>0.5</td>
<td>1.6</td>
<td>0.8-1.6</td>
</tr>
<tr>
<td>B5055/lpxM/pBR-lpxM</td>
<td>0.5</td>
<td>6.25</td>
<td>0.8-1.6</td>
</tr>
</tbody>
</table>

*As determined by the broth dilution method (three replicates). MIC values were scored after 24 hours of incubation at 37°C. Similar results were obtained with or without adding IPTG 0.2mM.

Overnight bacterial cultures were diluted (1:100) in a fresh LB medium supplemented or not with 0.2 mM IPTG and containing the indicated concentration of AMPs; 0.25 µg/mL of polymyxin B (A), 1 µg/mL of colistin (B), and 1 µg/mL of cecropin A (C). Real-time changes in A600nm were monitored over 15 hours of incubation with a microplate reader system (TECAN Infinite) at 37°C, with shaking. Growth kinetics were performed in triplicate for all strains incubated with each AMP. Data represent A600nm values of one representative experiment. Similar results were obtained with or without adding of 0.2 mM IPTG to the culture medium.

**Construction of the msbB mutant of *P. luminescens***

In order to confirm the involvement of *P. luminescens* msbB in bacterial resistance to AMPs, and to investigate its possible role in virulence within insects, we performed double allelic exchange experiments to inactivate *P. luminescens* TT01 msbB gene (See materials and methods). Unfortunately, we could not select any exconjugant in which the 632 bp fragment of the wild-type msbB gene was deleted and replaced with the ΩR cassette. During three independent experiments, screening for msbB mutants led to the selection of six isolated exconjugants that were GmR, CmR and SacR. Such phenotypes indicate that these exconjugants have integrated the ΩR cassette in their chromosomes and loosed the pJQ-msbB-ΩCm plasmid sequence harbouring at least the sacB and the Gm resistance genes. Sequencing of PCR products obtained by using primers hybridizing to the msbB flanking genomic
regions revealed sequence inversion events in the closed msbB environment. These inversions were reflected by an insertion of the ΩR cassette into the msbB-downstream locus yebA (encoding a putative peptidase precursor YebA). No pI2Q200 plasmid-derived fragments were detected in any of the analysed sequences therefore corroborating the loss of the plasmid-encoded GmR and SacS phenotypes within the six selected exconjugants.

Figure 2. Growth kinetics of *K. pneumoniae* B5055nm strain and its *lpzM* isogenic mutant harboring the pUC19 vector (B5055nm*lpzM*/pUC19), the pBR322 vector plus the parental *lpzM* gene (B5055nm*lpzM*/pBR1*lpzM*) or the pUC19 vector plus *Photorhabdus msbB* gene (B5055nm*lpzM*/pmsbB).
Indeed, the plasmid-derived cloned DNA fragment containing the Ω\(^8\) cassette flanked by the msbB-downstream and upstream regions was believed to be integrated closely to- but not into the msbB locus. In order to verify if the msbB gene were expressed within the six selected exconjugants, we performed real time RT-PCR and detected equal levels of msbB transcripts in the wild-type TT01 strain and in the six exconjugants therefore confirming that the locus was not inactivated (data not shown). We should note that parallel allelic exchange experiments allowed us, using the same cloning strategy, to successfully inactivate the mdtA gene in two different strains of *P. luminescens* (paper in preparation). Thus, these mdtA mutants should be regarded as adequate positive control of the experimental designs, indicating that msbB functionality is required for bacteria to survive during the double allelic exchange selection pressures.

Bacteria may alter their genomes in response to selective pressure from their environment. These alterations are often observed within specific genomic sequences called regions of plasticity. Although the genomic region containing msbB gene in *Photorhabdus* likely belongs to core genome, we have identified five ERIC (*Enterobacterial Repetitive Intergenic Consensus*) sequences in the genomic environment of msbB. These elements were shown to be involved in pseudogenization (formation of pseudogenes) although nothing is known to date about the nature of their mobility (Delihas, 2008). The use of sucrose as a high selection pressure on *Photorhabdus* exconjugants may have resulted in genomic rearrangements. Therefore, presence of ERIC sequences may have enhanced the frequency of deletion and inversion in exconjugants that consequently underwent genomic rearrangements in order to prevent the double allelic exchange event and the inactivation of msbB gene.

LPS layer is generally crucial for bacterial survival and there are only a small number of mutations that can be made in the lipid A molecule that do not affect the growth of the bacterium. For instance, a LPS-deficient mutant of *Neisseria meningitidis* has been constructed, and has shown a reduced growth rate (Steeghs et al., 2001). Furthermore, inactivation of *E. coli* htrB gene resulted in a conditionally lethal phenotype (when bacteria were grown in a rich medium at high temperatures) (Clementz et al., 1996) while msbB mutations in *E. coli* were not lethal (Clementz et al., 1997), and did not affect *E. coli* growth (Karow & Georgopoulos, 1992; Vorachek-Warren et al., 2002). *E. coli* HtrB and LpxM display significant sequence similarity to each others, suggesting that msbB may serve a role similar to that of HtrB (Clementz et al., 1996; Clementz et al., 1997). Indeed, msbB gene of *E. coli* was characterized as a multicopy suppressor of temperature sensitivity caused by null mutations in the high-temperature requirement gene *htrB* (Karow & Georgopoulos, 1992). This lethal phenotype was also shown to be suppressed by extragenic spontaneously arising mutations mapped to genes involved in fatty acids biosynthesis (Karow et al., 1992). Similarly, outer membrane permeability and growth defects observed in *S. typhimurium* lpxM mutants have been found to select for extragenic suppressor mutations in genes unique to *S. typhimurium* (Murray et al., 2001). One could ask if msbB mutation in *Photorhabdus* might lead to lethal changes in membrane structure and fatty acid composition since htrB and msbB have significant sequence similarity suggesting that both encoded proteins may share similar functions.

In Gram negative-bacteria, lipid A acylations including the msbB-catalyzed myristic acid incorporation were found to significantly increase saturated fatty acid tails of LPS molecules and therefore decreased the permeability of the outer membrane. This protected bacteria against a wide variety of noxious compounds that may disrupt the membrane
integrity and osmosis-resistance (Nikaido, 2003). In this study, we used sucrose in order to positively select exconjugants resistant to 4% of sucrose (w/v) that arose through a double-crossover event in which the chromosomal wild-type copy of the msbB gene were inactivated by the $\Omega^8$ cassette insertion. It has been reported that sucrose induces changes in chemical and physical properties of the bacterial membrane. Indeed, growth of the Gram-positive bacterium *Streptococcus mutans* in sucrose-containing media showed that the degree of unsaturation in the fatty acids of both total lipid and glycolipid fractions decreased when the sucrose concentration was increased. The reduction of unsaturated fatty acids by adding sucrose therefore resulted in a reduction of membrane lipid fluidity and membrane permeability (Sato *et al.*, 1988). Increasing sucrose concentration up to 6% resulted in complete inhibition of *P. luminescens* exconjugant growth (unpublished data). Taken together, these data led us to hypothesize that the expected msbB mutants of *Photorhabdus* may have been be killed by the relatively high concentration of sucrose used for screening in allelic exchange experiments. These mutants were supposed to have modified outer membrane lipid composition, high fluidity and osmosis-sensitivity that subsequently reduced their ability to grow and survive in sucrose at high concentrations.

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