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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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ABSTRACT

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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 El Tor *Vibrio cholera*, 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 ß 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 *Heterorhabditis* (Boemare, 2002). Upon entering insect hemolymph, bacteria are targeted by an array of induced cationic AMPs (Lemaitre & 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-encapsulated mutant and its derivative B5055nmΔ*lpxM*, a double mutant non-encapsulated *lpx*M 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 1

Bacterial Strains and Plasmids

Strain or plasmid	Genotype and relevant characteristic	Reference or source
Strains		
Photorhabdus luminescens TT01	Wild type isolated from *Heterorhabditis bacteriophora* nematode TH01	(Fischer-Le Saux *et al.*, 1999)
Klebsiella pneumoniae		
B5055nm	B5055, mutation in *wza/wzb*, non-mucoid	(Clements *et al.*, 2007)
B5055nmΔ*lpxM*	Double mutant, non-mucoid, mutation in *lpxM*	(Clements *et al.*, 2007)
Escherichia coli		

Continued :

XL1-Blue	(Δ(mrcA)183Δ(mcrCB-hsdSMR-mrr *173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB laqIq ZΔM15 Tn10(Tet^r)*	Stratagene
S17.1	*pro* r- n- TpRSmR RP4-2-Tc::Mu::Tn*7 recA thi*	(Simon, 1983)
Plasmids		
pUC19	Ap^R cloning vehicle	Biolabs
pBBR1-MCS5	Gm^R *mob* broad host range vector	(Kovach, 1995)
p*msbB*	pUC19 carrying *msbB* from TT01	This study
pBR-*lpxM*	pBR322 carrying *lpxM* from B5055	(Clements *et al.*, 2007)
pBB-*msbB*	pBBR1MCS-5 carrying *msbB* from TT01	This study
pJQ200 KS	Gm^R *sacRB mob oriV* (p15A replicon) suicide vector	S. Forst
pUC-*msbB*-ΩCm	4.7 kb *Xba*I-*Sac*I fragment containing the 620bp *msbB* upstream region- ΩCm- the 618bp *msbB* downstream region, cloned into pUC/*Xba*I-*Sac*I	This study
pJQ-msbB-ΩCm	4.7 kb XbaI-SacI fragment containing the 620bp msbB upstream region- ΩCm- the 618bp msbB downstream region, cloned into pJQ200KS/XbaI-SacI	This study
pHP45- ΩCm	Ap^R Cm^R interposon ΩCm	(Fellay *et al.*, 1987)

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'-GCGCGTGCAGCCGCTGTCGTTTTGTATTGC–3' and

5'- GCGCGAGCTCATCCCGCTTAATCCAACGAC-3' including the restriction enzymes sites *Pst*I and *Sac*I, respectively. The 1.25 kb resulting fragment was hydrolyzed by *Pst*I and *Sac*I, 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 p*msbB* 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 methanesulfate) were prepared by2-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 ΩCm (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’ -GCGCTCTAGAGCCACATAAAGGGGTGGATT- 3’/

5’ -CGCGGATCCGCCAGTAACGAGGATGCAGGT- 3’ (upstream region) and

5’- CGCGGATCCGGCCGCTGTTTCCGGTTTAT- 3’/

5’ - GCGCGAGCTCGCACTTTACCGTGGCGTTAC- 3’ (downstream region).

The PCR products were restricted with *Xba*I/*Bam*HI and *Bam*HI/*Sac*I enzymes couples whose restriction sites are underlined respectively. The antibiotic resistance cassette ΩCm was isolated as a *Bam*HI 3.5 kb DNA fragment from the pHP45- ΩCm plasmid. This 3.5 kb DNA fragment was then ligated to the *Bam*HI sites between the upstream and downstream *msbB* regions and cloned into the pUC19/*Xba*I-*Sac*I vector to generate pUC-*msbB*-ΩCm. The full-length tripartite 4.7 kb DNA fragment *msbB*-ΩCm was gel purified, restricted with *Xba*I and *Sac*I, and cloned in the corresponding restriction sites of the pJQ200KS vector yielding pJQ-*msbB*-ΩCm. The pJQ200KS plasmid is a derivative of pACYC184 carrying the *sacB* gene and the *mob* site from RP4. The pJQ-*msbB*-ΩCm 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^R and Sac^R exconjugants were selected on 3% 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).

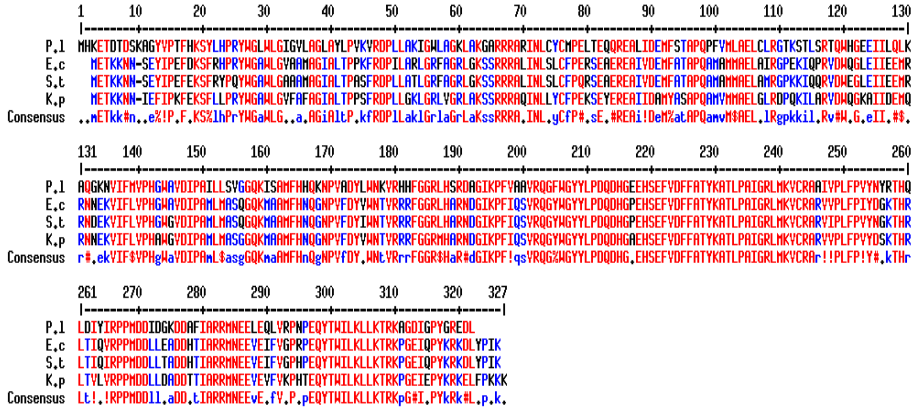


Figure 1. Multiple sequence alignment of LpxM (msbB) proteins from *E. coli* K12 (AAC74925**), *S. typhimurium* (**AAL20805**), *K. pneumoniae* MGH78578, and *P. luminescens* TT01, showing significant sequence homology. All alignments and consensus were generated with Multalin software version 5.3.3 (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_{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 B5055nm 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 $\mu g.ml^{-1}$), colistin (1 $\mu g.ml^{-1}$), and cecropin A (1 $\mu g.ml^{-1}$). 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)[a] Values of Three Cationic Antimicrobial Peptides (AMPs) for the *K. pneumoniae* B5055nm Strain, the *lpxM* Knockout Mutant and Complemented *lpxM* Mutants

	MIC values (µg.ml^{-1}) of cationic AMPs		
Strains	**Polymyxin B**	**Colistin**	**Cecropin A**
B5055nm	0.5	6.25	1.6
B5055nm*lpxM*/pUC19	0.125	0.4	0.4
B5055nm*lpxM*/p*msbB*	0.5	1.6	0.8-1.6
B5055*lpxM*/pBR-*lpxM*	0.5	6.25	0.8-1.6

[a] 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 A_{600nm} 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 Ω^R cassette. During three independent experiments, screening for *msbB* mutants led to the selection of six isolated exconjugants that were Gm^S, Cm^R and Sac^R. Such phenotypes indicate that these exconjugants have integrated the Ω^r 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 Ω^R cassette into the *msbB*-downstream locus *yebA* (encoding a putative peptidase precursor YebA). No pJQ200 plasmid-derived fragments were detected in any of the analysed sequences therefore corroborating the loss of the plasmid-encoded Gm^R and Sac^S phenotypes within the six selected exconjugants.

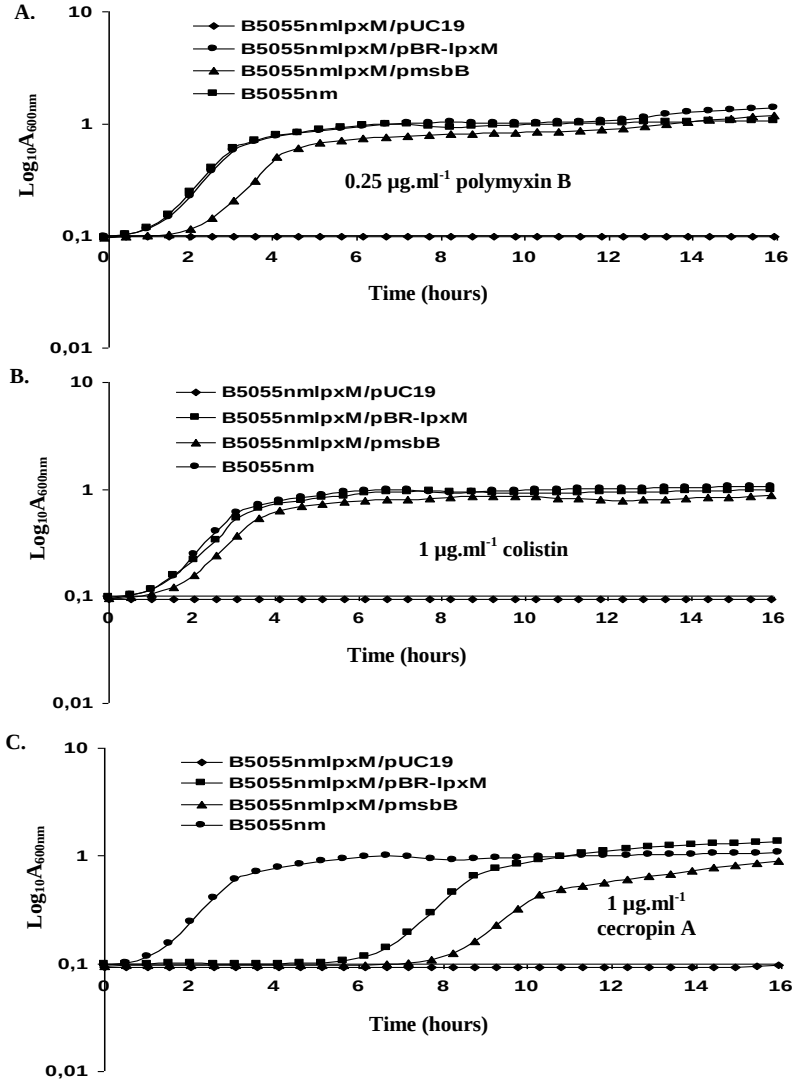


Figure 2. Growth kinetics of *K. pneumoniae* B5055nm strain and its *lpxM* isogenic mutant harboring the pUC19 vector (B5055nm*lpxM*/pUC19), the pBR322 vector plus the parental *lpxM* gene (B5055nm*lpxM*/pBR1*lpxM*) or the pUC19 vector plus *Photorhabdus msbB* gene (B5055nm*lpxM*/p*msbB*).

Indeed, the plasmid-derived cloned DNA fragment containing the Ω^R 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 Ω^R 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 consequently reduced their ability to grow and survive in sucrose at high concentrations.

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REFERENCES

Ausubel, F.M, Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G. and Struhl, K. 1993. *Current protocols in molecular biology*. John Wiley and sons, New York, p. 1–15.

Bauer, M.E. and Shafer, W.M. 2015. On the *in vivo* significance of bacterial resistance to antimicrobial peptides. *Biochim. Biophys. Acta*, 1848(11, Part B): 3101-3111.

Bennett, H.P. and Clarke, D.J. 2005. The *pbgPE* operon in *Photorhabdus luminescens* is required for pathogenicity and symbiosis. *J. Bacteriol.*, 187: 77-84.

Boemare, N.E. 2002. Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus*. In R. Gaugler (ed.), *Entomopathogenic nematology*. CABI Publishing, Wallingford, United Kingdom, p. 35-56.

Brillard, J., Duchaud, E., Boemare, N., Kunst, F. and Givaudan, A. 2002. The PhlA hemolysin from the entomopathogenic bacterium *Photorhabdus luminescens* belongs to the two-partner secretion family of hemolysins. *J. Bacteriol.*, 184: 3871-3878.

Bulet, P., Hetru, C., Dimarcq, J.L. and Hoffmann, D. 1999. Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol.*, 23: 329-344.

Clementz, T., Bednarski, J.J. and Raetz, C.R. 1996. Function of the *htrB* high temperature requirement gene of *Escherchia coli* in the acylation of lipid A: HtrB catalyzed incorporation of laurate. *J. Biol. Chem.*, 271: 12095-12102.

Clementz, T., Zhou, Z. and Raetz, C.R. 1997. Function of the *Escherichia coli* msbB gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipid A. Acylation by MsbB follows laurate incorporation by HtrB. *J. Biol. Chem.*, 272: 10353-10360.

Clements, A., Tull, D., Jenney, A.W., Farn, J.L., Kim, S.H., Bishop, R.E., McPhee, J.B., Hancock, R.E., Hartland, E.L., Pearse, M.J., Wijburg, O.L., Jackson, D.C., McConville, M.J. and Strugnell, R.A. 2007. Secondary acylation of *Klebsiella*

pneumoniae lipopolysaccharide contributes to sensitivity to antibacterial peptides. J. *Biol. Chem*., 282: 15569-15577.

Cullis, P.R. and Hope, M.J. 1985. Physical Properties and Functional Roles of Lipids in Membranes. *In*: Biochemistry of lipids and membranes (D.E. Vance and J.E. Vance, Eds), Benjamin Cummings, Menlo Park, p. 25-72.

D'Hauteville, H., Khan, S, Maskell, D.J., Kussak, A., Weintraub, A., Mathison, J., Ulevitch, R. J., Wuscher, N., Parsot, C., Sansonetti, P.J. 2002. Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. *J. Immunol.,* 168: 5240-5251.

Delihas, N. 2008. Small mobile sequences in bacteria display diverse structure/function motifs. *Mol. Microbiol.,* 67: 475-481.

Derzelle, S., Turlin, E., Duchaud, E., Pages, S., Kunst, F., Givaudan, A. and Danchin, A. 2004. The PhoP-PhoQ two-component regulatory system of *Photorhabdus luminescens* is essential for virulence in insects. *J. Bacteriol.,* 186: 1270-1279.

Dimarcq, J.L., Bulet, P., Hetru, C. & Hoffmann, J. 1998. Cysteine-rich antimicrobial peptides in invertebrates. *Biopolymers,* 47: 465-477.

Fahy, E., Subramaniam, S., Brown, H.A., Glass, C.K., Merrill, A.H., Jr., Murphy, R.C., Raetz, C.R., Russell, D.W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., Van Meer, G., Van Nieuwenhze, M.S., White, S.H., Witztum, J.L. and Dennis, E.A. 2005. A comprehensive classification system for lipids. *J. Lipid Res.,* 46: 839-861.

Fellay, R., Frey, J. and Krisch, H. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. *Gene,* 52: 147-154.

Fischer-Le Saux, M., Viallard, V., Brunel, B., Normand, P. and Boemare, N.E. 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. luminescens subsp. nov., *P. luminescens* subsp. akhurstii subsp. nov., *P. luminescens* subsp. laumondii subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. temperata subsp. nov. and *P. asymbiotica* sp. nov. *Int. J. Syst. Bacteriol.,* 49 Pt 4: 1645-1656.

Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M. and Miller, S.I. 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol. Microbiol.,* 27: 1171-1182.

Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M. and Miller, S.I. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell,* 95: 189-198.

Hancock, R.E. 1999. Host defence (cationic) peptides: what is their future clinical potential? *Drugs,* 57: 469-473.

Hancock, R.E. and Diamond, G. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.,* 8: 402-410.

Helander, I.M., Kilpelainen, I. and Vaara, M. 1994. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant pmrA mutants of *Salmonella typhimurium*: a 31P-NMR study. *Mol. Microbiol.,* 11: 481-487.

Karow, M. and Georgopoulos, C. 1992. Isolation and characterization of the *Escherichia coli msbB* gene, a multicopy suppressor of null mutations in the high-temperature requirement gene *htrB*. *J. Bacteriol.,* 174: 702-710.

Karow, M., Fayet, O. and Georgopoulos, C. 1992. The lethal phenotype caused by null mutations in the *Escherichia coli htrB* gene is suppressed by mutations in the *accBC*

operon, encoding two subunits of acetyl coenzyme A carboxylase. *J. Bacteriol.*, 174: 7407-7418.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M. 2nd and Peterson, K.M. 1995. Gene, Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes.

Lemaitre, B. and Hoffmann, J. 2007. The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.*, 25: 697-743.

Low, K.B., Ittensohn, M., Le, T., Platt, J., Sodi, S., Amoss, M., Ash, O., Carmichael, E., Chakraborty, A., Fischer, J., Lin, S.L., Luo, X., Miller, S.I., Zheng, L., King, I., Pawelek, J.M., Bermudes, D. 1999. Lipid A mutant *Salmonella* with suppressed virulence and TNFalpha induction retain tumor-targeting *in vivo*. *Nat. Biotechnol.*, 17: 37-41.

Matson, J.S., Yoo, H.J., Hakansson, K. and Dirita, V.J. 2010. Polymyxin B resistance in El Tor *Vibrio cholerae* requires lipid acylation catalyzed by MsbB. *J. Bacteriol.*, 192: 2044-2052.

Murray, S.R., Bermudes, D., de Felipe, K.S. and Low, K.B. 2001. Extragenic suppressors of growth defects in *msbB Salmonella*. *J. Bacteriol.*, 183: 5554-5561.

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.*, 67: 593-656.

Parillo, J.E. 1993. Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.*, 328: 1471-1477.

Raetz, C.R. and Whitfield, C. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem*., 71: 635-700.

Raetz, C.R., Reynolds, C.M., Trent, M.S. and Bishop, R.E. 2007. Lipid A modification systems in gram-negative bacteria. *Annu. Rev. Biochem.*, 76: 295-329.

Russell, J.A. 2006. Management of sepsis. *N. Engl. J. Med.* 355: 1699-1713.

Sato, M., Tsuchiya, H., Kato, M., Yamamoto, K., Kanematsu, N., Takagi, N. and Namikawa, I. 1988. Sucrose concentratio ns in the growth medium influence the membrane lipids of *Streptococcus mutans*. *Letters in Applied Microbiology*, 6: 69-73.

Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology*, 1: 784-791.

Somerville, J.E. Jr., Cassiano, L. and Darveau, R.P. 1999. *Escherichia coli msbB* gene as a virulence factor and a therapeutic target. *Infect. Immun*., 67: 6583-6590.

Starner, T.D., Swords, W.E., Apicella, M.A. & McCray, P.B., Jr. 2002. Susceptibility of nontypeable *Haemophilus influenzae* to human beta-defensins is influenced by lipooligosaccharide acylation. *Infect. Immun.*, 70: 5287-5289.

Steeghs, L., de Cock, H., Evers, E., Zomer, B., Tommassen, J. and van der Ley, P. 2001. Outer membrane composition of a lipopolysaccharide-deficient *Neisseria meningitidis* mutant. *Embo. J.*, 20: 6937-6945.

Vorachek-Warren, M.K., Ramirez., S., Cotter, R.J. and Raetz, C.R. 2002. A triple mutant of *Escherichia coli* lacking secondary acyl chains on lipid A. *J. Biol. Chem.*, 277: 14194-14205.

Xu, H., Ling, J., Gao, Q., He, H., Mu, X., Yan, Z., Gao, S. and Liu, X. 2013. Role of the lpxM lipid A biosynthesis pathway gene in pathogenicity of avian pathogenic *Escherichia coli* strain E058 in a chicken infection model. *Vet. Microbiol.*, 166: 516-526.