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Reduction of Lipopolysaccharide Content and Detoxification in *Bordetella pertussis* and *Bordetella bronchiseptica*

UNIVERSITY OF ALGARVE
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MSc in Molecular and Microbial Biology
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Declaro ser a autor a deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Sílvia Major

Project under the BacVactory program - A Technology Center for Bacterial Vaccines – on *Whole-cell and outer-membrane-vesicle vaccines* under supervision of Prof. dr. J.P.M. Tommassen at Utrecht University, Faculty of Science, Biology Department, Utrecht, The Netherlands.

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To everyone that accompanied me in this journey,
Thank you.

Resumo

O género *Bordetella* é composto por múltiplas bactérias que causam doenças a nível respiratório. Pertússis, ou tosse convulsa, é causada pelo patogénico humano *Bordetella pertussis*, enquanto *Bordetella bronchiseptica* afeta uma ampla variedade de mamíferos, mas raramente humanos. Embora raramente sejam infetados, a transmissão ocasional em humanos ocorre geralmente em indivíduos imunocomprometidos. Estirpes de *B. bronchiseptica* isoladas de diferentes espécies hospedeiras possuem características genéticas e fenotípicas únicas. Infeção por esta bactéria predispõe o animal à colonização por outros patogénicos.

Ambas as espécies demonstram uma associação clara com a idade. Na indústria suína, *B. bronchiseptica* afeta amplamente as populações de jovens leitões enquanto nos humanos a morbidade e mortalidade são mais graves em crianças infetadas com *B. pertussis.* Antes da introdução das primeiras vacinas contra a tosse convulsa, esta doença era uma das principais causas de morte. A primeira geração de vacinas desenvolvida na década de 1950 é composta de células não viáveis do patogénico. As severas reações adversas provocadas pela administração destas vacinas levaram ao desenvolvimento das vacinas de segunda geração, ou acelulares. Integram estas vacinas componentes bacterianos purificados. Indivíduos vacinados com vacinas acelulares apresentam rápido declínio da imunidade e podem ser portadores assintomáticos. A imunização contra *B. bronchiseptica*, tanto de células não viáveis quanto acelulares, foi considerada parcialmente ineficaz ou segura para os animais.

O lipopolissacarídeo (LPS) ou lipoglicano é o principal responsável pelas reações adversas das vacinas de primeira geração. O LPS é um dos principais componentes da membrana externa das bactérias Gram-negativas e constitui a camada externa da membrana externa. O LPS contribui para a assimetria lipídica na membrana externa, oferecendo uma barreira de permeabilidade eficiente a antimicrobianos e outros fatores de stress. A toxicidade do LPS deriva da sua parte lipídica, denominado lípido A. A biossíntese do lípido A, ou via de Raetz, inclui nove etapas conservadas catalisadas por enzimas. Esta via tem início com a acilação de UDP-N-acetilglucosamina (UDP-GlcNAc) pela enzima LpxA e subsequente desacetilação pela enzima LpxC. A LpxC é uma enzima dependente de zinco que se correlaciona diretamente com a quantidade de LPS presente na membrana externa. Nas espécies de *Bordetella* a estrutura do lípido A é completada após a introdução de cadeias acil secundárias catalisadas pelas aciltransferases LpxL1 e LpxL2 na cadeia acil primária. O número de cadeias acil é

determinante na atividade endotóxica da bactéria. A enzima LpxL1 não é normalmente expressa em *B. pertussis*, resultando numa estrutura com cinco cadeias acil. Em *B. bronchiseptica* a expressão de ambas as enzimas resultam em seis cadeias acil no lípido A distribuídas de forma irregular e produzindo uma estrutura assimétrica.

O LPS é transportado através da membrana interna pelo transportador ABC (cassete de ligação de ATP) MsbA e posteriormente para a camada externa da membrana externa pela maquinaria de transporte do LPS (Lpt). O transporte mediado pelo MsbA é específico para a acilação do lípido A e a sua depleção leva à acumulação de LPS e glicerofosfolípidos na membrana interna da bactéria. A superexpressão deste gene essencial mostrou compensar a inativação do gene *lpxL* no crescimento de *E. coli* e *Burkholderia cenocepacia*.

Múltiplos mecanismos foram identificados em bactérias Gram-negativas para preservar a assimetria lipídica na membrana externa. Perturbações da membrana ou rutura da biossíntese do LPS ou do seu sistema de transporte causam uma inversão compensatória de fosfolípidos para a camada externa da membrana externa de forma a acomodar a redução nos níveis de LPS. A fosfolipase PldA atua como um sensor de assimetria lipídica na membrana externa, degradando fosfolípidos que destabilizam a homeostasia. Os ácidos gordos produzidos pela hidrólise dos fosfolípidos sinalizam para a inibição da degradação da enzima LpxC e consequente aumento do LPS.

O sistema de transporte ABC, Mla, facilita o transporte retrógrado de fosfolípidos de volta para a membrana interna. Esta via é regulada positivamente após a perda de LPS. Mutações que resultam na perda de função de qualquer um dos genes que codificam o transportador Mla afetam toda a via, levando à rutura do equilíbrio lipídico na membrana externa e à acumulação de fosfolípidos na superfície celular. Após a interrupção dos níveis de LPS, a remoção destes dois sistemas permite que a membrana externa recupere a homeostasia entre as camadas da bicamada lipídica. Exemplos de bactérias Gram-negativas capazes de sobreviver na ausência de lípido A são *Acinetobacter baumanni, Neisseria meningitidis* e *Moraxella catarrhalis*.

Existe a necessidade de vacinas mais seguras e eficientes para *B. bronchiseptica* e *B. pertussis*. As vacinas acelulares são menos reativas do que as vacinas de células não viáveis devido à quantidade mínima ou inexistente de LPS. O objetivo deste estudo foi aplicar o mesmo princípio numa vacina com células não viáveis. Reduzir a quantidade de LPS e a sua endotoxicidade em ambas as espécies envolveu a interferência na biossíntese e estrutura do lípido A, com a suposição de que menos LPS e lípido A com menos cadeias

acil traduzem em bactérias menos endotóxicas. A produção das enzimas-chave da síntese, LpxA, LpxC, LpxL1 e LpxL2 foi interrompida ou regulada com promotores induzíveis. A interferência com as duas primeiras enzimas afeta a quantidade de lípido A produzido. A inativação do gene *lpxA* por meio da introdução de um marcador antibiótico foi combinada com um plasmídeo de expressão contendo o gene expresso a partir de um promotor controlável. A construção do vetor contendo o gene *lpxA* interrompido pela sequência de resistência ao antibiótico cloranfenicol falhou pois não foi possível introduzir a sequência de resistência ao antibiótico no vetor. O principal desafio foi conseguir transformantes resistentes a cloranfenicol.

A mesma estratégia foi usada para os genes *lpxL1* e *lpxL2* em simultâneo (*lpxL1-2*) e *lpxL2* em diferentes estirpes de *Bordetella*. A inativação de *lpxL1-2* foi também tentada na estirpe deficiente *B. pertussis* ΔPdlA-MlaF usando um vetor suicida para recombinação homóloga e troca alélica. A estirpe mutante permite que a célula atinja a homeostasia quando a estrutura de LPS é deficiente em cadeias acil. A inativação de *lpxL1-2* no cromossoma foi efetuada com a colaboração de uma cópia dos genes sob um promotor induzível num plasmídeo fora do cromossoma. O mutante não apresentou diferenças de crescimento em comparação com a estirpe selvagem (WT). A estrutura do LPS divergiu do controlo WT apresentando uma cadeia acil suplementar.

Numa abordagem diferente, a expressão dos genes *lpxC* e *lpxL2* foi regulada por meio de um promotor controlável inserido no cromossoma, todavia a inserção cromossómica foi inexequível.

Termos chave: *Bordetella*, lipopolissacarídeo, lípido A, acilação, knock-out, regulação.

Abstract

Bordetella species cause respiratory diseases in several animals. Pertussis, or whooping cough, is caused by the human pathogen *Bordetella pertussis*, while *Bordetella bronchiseptica* affects a broad range of mammals, but rarely humans. Severe morbidity and mortality in young children caused by *B. pertussis* led to the development of whole-cell vaccines. The reactogenicity of the vaccine produced several side effects that later led to development of second-generation acellular vaccines. Individuals vaccinated with acellular vaccines present a rapid waning of immunity and can be asymptomatic carriers. Immunization against *B. bronchiseptica*, either whole-cell or acellular, has been regarded as not fully effective or safe for the animals.

The lipopolysaccharide (LPS) is the main reason for the high reactogenicity of the first-generation vaccines. LPS is one of the main components of the outer membrane of Gram-negative bacteria. LPS contributes to the lipid asymmetry in the outer membrane offering an efficient permeability barrier to antimicrobials and other stressors. LPS endotoxicity derives from its lipid A moiety. This study aimed to reduce the LPS amount and endotoxicity in the bacterial cell through reduction of lipid A biosynthesis or by modifying its structure, with the assumption that less LPS and less-acylated lipid A translates in cells with reduced endotoxicity. Interference with lipid A biosynthesis and structure involved the inactivation of the *lpxA, lpxL2* and *lpxL1* and *lpxL2* genes simultaneously and chromosomal regulation of the expression of the *lpxC* and *lpxL2* genes. Constructs were created for the insertion of an inducible promoter that would allow for the regulation of *lpxC* and *lpxL2* expression. However, the chromosomal insertion was unfeasible. Chromosomal knock-out with a copy under an inducible promoter on a plasmid did not present fitness differences compared to the wild-type (WT) strain. LPS structure diverged from the WT control and presented hexa-acylated lipid A.

Keywords: *Bordetella*, lipopolysaccharide, lipid A, acylation, knock-out, regulation.

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1. Introduction

1.1. The *Bordetella* genus and virulence

Bordetella are Gram-negative aerobic coccobacilli bacteria of the family Alcaligenaceae [1]. The genus is composed of several species which cause respiratory diseases in several animals. *B. pertussis* is the etiologic agent of pertussis in humans, also known as whooping cough [2], while *B. bronchiseptica* is responsible for tracheobronchitis or kennel cough in dogs, atrophic rhinitis in pigs, bronchopneumonia in rabbits, and other respiratory complications in animals, but rarely in humans [3, 4]. Although humans are seldom infected when transmission occasionally happens is usually in immunocompromised individuals. Being common reservoirs, domestic animals are suspected to be the source of the transmission to humans, yet due to the rare incidence and microbiological diagnosis there is no significate evidence to support this conclusion [1, 5]. Between animals transmission is made by direct contact through respiratory secretions [6].

Since *B. bronchiseptica* can affect both companion and farm animals, studies focus more on pigs, dogs, and cats. Isolates from different host species possess unique genetic and phenotypic traits [7]. Though several viral and bacterial agents have been detected in dogs with infectious respiratory diseases, this bacterium appears to have a primary pathogenic role [6, 8]. In swine industry worldwide, respiratory diseases are the main cause of morbidity and mortality [9]. The upper respiratory illness induce by *B. bronchiseptica* demonstrates a clear association with age by largely affecting young piglets even though it can occur in older populations [6]. In a 6-year period study conducted in China, *B. bronchiseptica* was the fourth most isolated pathogenic bacterium from pig lung samples. Analogous to what happens in canines, the bacterium was found to predispose the animal to colonization from other pathogens [9].

Humans, as previously mentioned, are affected by *B. pertussis*. Transmission occurs via contaminated aerosol droplets [10]. After attachment to the ciliated respiratory epithelial cells, the bacteria is able to evade host defences and produce local and systemic damage [6]. Morbidity and mortality are more severe in young children given their immature respiratory system and being too young to receive the full course of vaccination [11]. Before the introduction of the first pertussis vaccines this disease was one of the major causes of death. From 1940 to 1948 there was more reported deaths related to pertussis than from measles, scarlet fever, diphtheria, poliomyelitis and meningitis combined in children younger than 1 year [6]. The source of infection is usually an infected family member that carries the disease asymptomatically [11].

These bacteria produce a variety of virulence factors, including filamentous hemagglutinin, pertactin and fimbriae, suitable for efficient and persistent colonization of the ciliated epithelial cells of the upper respiratory tract [3, 6, 7]. They produce toxins such as LPS and adenylate cyclase toxin which disables innate immune defenses and is essential for virulence [5]. *B. pertussis* also expresses pertussis toxin, which is important to pathogenesis and is a crucial protective antigen of the bacteria [12].

1.2. Vaccination against *B. pertussis* and *B. bronchiseptica*

Two types of vaccines are used against *B. pertussis*: a whole-cell vaccine implemented in the 1950 decade and an acellular vaccine introduced 40 years later [12]. The whole-cell vaccines are comprised of killed bacteria and were administered in combination with diphtheria and tetanus toxoids leading to a strong reduction in whooping cough incidence. Soon thereafter, a number of side effects due to the reactogenicity of the vaccine were observed, including anxiety, seizures and other neurological complications [11], which led to a decrease in vaccine acceptance and stimulated the development of the second-generation vaccines composed of purified bacterial components [13]. Even though different combinations can be found, the most used acellular vaccines incorporate five antigens: pertactin, haemagglutinin, pertussis toxin and fimbrial proteins 2 and 3 [11]. Although the acellular vaccine is safer than its whole-cell predecessor, individuals vaccinated with acellular vaccines can become asymptomatically infected and transmit the infection to susceptible individuals [14]. Whole-cell vaccines provide better priming and longer immunity [11].

In the last 20 years, pertussis epidemiology has been changing. Resurgence of *B. pertussis* has been exceedingly observed in countries that have switched to the acellular vaccine. Such occurrence is mainly due to the improved detection and diagnostic methods, vaccination coverage lower than recommended by the World Health Organization, waning of vaccine-induced immunity, and evolution of circulating bacteria to evade vaccine-induced immunity, resulting from vaccine-imposed selection [12, 14, 15]. An example of the latter is the pertactin-deficient *B. pertussis* strains being vastly predominant in countries that use the acellular vaccine [2]. Unfortunately, isolation of bacteria that do not express certain vaccine antigens is an increasing tendency. Epidemic cycles of whooping cough occur 2 to 5 years, parallel to the pre-vaccine era [10]. In 2012, there was a report of 48 thousand cases in the Unites States of America, the highest number since 1955 and resulted in the death of 16 infants [16]. The World Health Organization conclude in 2015 that true resurgence of pertussis occurred in 4 countries, all of which had transitioned exclusively to the use of acellular vaccines [10].

Similarly to pertussis, both whole-cell and acellular vaccines are available against *B. bronchiseptica* and both are either injectable or administrated via oral or intranasal route [17]. These acellular vaccines are comprised of cellular extract from the bacteria to reduce the content of endo- and exotoxins and the potential for adverse reaction [4]. Research suggests that, although the existing vaccines induce high antibody production, they are not fully effective or safe. For instance, this bacterium is repeatedly isolated from vaccinated animals suggesting that these vaccines fail to protect against infection [18, 19].

1.3. LPS structure and biosynthesis

The outer membrane of Gram-negative bacteria is mainly a bilayer composed of glycerophospholipids in the inner leaflet and LPS in the outer leaflet. The asymmetric distributed LPS creates an efficient permeability barrier to antimicrobial compounds and protection against complement-mediated lysis [20, 21]. LPS can activate pro-inflammatory cytokine production in macrophages and has shown to be crucial for colonization and survival of the bacteria *in vivo* [12, 22]. Structurally, it consists of three key regions – the hydrophobic lipid A domain, core oligosaccharide and O-antigen [23]. The lipid A, an acylated bisphosphorylated disaccharide of glucosamine (GlcN) with a net negative charge due to the phosphorylation of the sugar backbone [24], is a very important element in the LPS structure since it anchors the LPS in the outer membrane and possesses the endotoxic activity. This activity is based on the recognition of lipid A by the Toll-like receptor 4/myeloid differentiation factor 2/cluster complex (TLR4/MD-2) of macrophages and dendritic cells, which leads to either an enlarged production and secretion of proinflammatory cytokines via recruitment of the myeloid differentiation primary response protein 88 (MYD88) or low inflammation through Toll-interleukin 1 domain-containing adaptor-inducing interferon-β (TRIF) [25]. Changes in lipid A structure, such as reduction in acylation pattern, can decrease the recognition by the TLR4/MD-2 complex and change the recruited signaling pathway [24, 25].

In *B. bronchiseptica*, the fatty acyl chains in lipid A are differentially distributed on the GlcN residues producing an asymmetrical backbone. This bacterium produces hexa-acylated lipid A, with 3 primary and 3 secondary acyl chains [26]. *B. pertussis* lipid A is penta-acylated containing four primary hydroxylated acyl chains and one secondary acyl chain [25], as displayed in Figure 1.3.1. The core oligosaccharide is covalently linked to the lipid A moiety by one Kdo molecule (3-deoxy-D-manno-oct-2-ulopyranosonic acid) [23]. Attached to the outer core is the O-antigen, comprising a variable number of repeating oligosaccharide units [26]. *B. pertussis* LPS lacks the O-antigen and is also called lipooligosaccharide (LOS) [2].

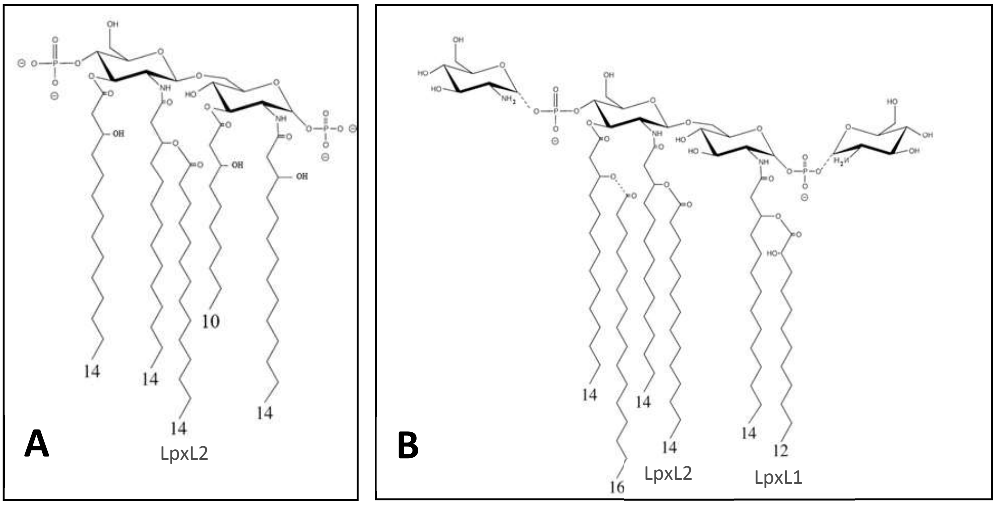


Figure 1.3.1. Lipid A structure in *Bordetella*. (A) *B. pertussis* B213 penta-acylated lipid A. (B) *B. bronchiseptica* hexa-acylated lipid A with glucosamine decorations attached to the biphosphorylated disaccharide and the secondary palmitoyl residue at the 3’ position of the primary acyl chain. LpxL2 catalyzes the transfer of the secondary C14 acyl chain to the primary acyl chain at position 2’ on both species while LpxL1 mediates the addition of a secondary 2-hydoxyl laurate at position 2 on *B. bronchiseptica* lipid A. Dashed lines represent non-stoichiometric substitutions.
Figure adapted from J. Pérez-Ortega [31].

The LPS biosynthesis pathway in *Escherichia coli*, the best studied model, takes place at the cytoplasmic interface of the inner membrane and the LPS is later transported to the outer membrane [24]. The lipid A synthesis pathway, also known as Raetz pathway (Figure 1.3.2.), includes nine conserved enzyme-catalyzed steps [20]. It begins with the acylation of UDP-N-acetylglucosamine (UDP-GlcNAc) by LpxA and subsequent deacetylation by LpxC, which is considered the first committed step of lipid A synthesis. This reaction is irreversible. LpxC is a zinc-dependent enzyme that directly correlates with the amount of LPS present in the outer membrane. LpxC is the substrate of protease FtsH. Proteolysis of the enzyme by FtsH ensures a balanced biosynthesis of LPS and glycerophospholipids [27]. Moffatt *et al.* [28] described that the full inactivation of *lpxA,* *lpxC* or *lpxD* genes resulted in a complete loss of LPS in *Acinetobacter baumannii*. Loss of LPS modifies the host immune response to the bacteria. LPS-deficient *A. baumannii* presented increased susceptibility to the antimicrobial peptide LL-37 of humans and decreased activation of host inflammatory response [28, 29].

In *E. coli* LpxL and LpxM are involved in the addition of the secondary lauroyl and myristoyl residues to the primary acyl chain [30]. However, in *Bordetella* species LpxM is not present. The acyltransferases LpxL1 and LpxL2 catalyze the transfer of secondary acyl chains to the Kdo-lipid A domain. LpxL2 resembles the *E. coli* LpxL homolog and mediates the addition of a C14 chain to the primary acyl chain at position 2' in *Bordetella* and LpxL1 mediates the transfer of a 2-hydoxyl laurate at position 2 of the primary acyl chain in *B. bronchiseptica*. The number of acyl chains is determinant in the endotoxic activity [25, 31]. Although LpxL1 is not normally expressed in *B. pertussis*, Geurtsen *et al.* [25] showed that extrachromosomal expression of *lpxL1* generated a hexa-acylated lipid A, resulting in an LPS with boosted endotoxicity activity.

After the Kdo-lipid A synthesis, the core oligosaccharide is extended producing LOS. The ABC (ATP-binding cassette) transporter MsbA flips it from the inner leaflet to the outer leaflet of the inner membrane [20]. MsbA mediated transport is specific for lipid A acylation [32] and its depletion leads to the accumulation of LOS and glycerophospholipids in the inner membrane of *E. coli* [33]. In *E. coli* and *Salmonella*, tetra-acylated can only be transported when *msbA* is overexpressed [34]. Overexpression of this essential gene was reported to compensate the *lpxL* inactivation in *E. coli* growth [32, 35] and *Burkholderia cenocepacia* [36]. The O-antigen is synthesized separately and attached after the lipid A extension of the core oligosaccharide. The LPS transport machinery (Lpt) then transports LPS or LOS to the outer leaflet of the outer membrane [24].

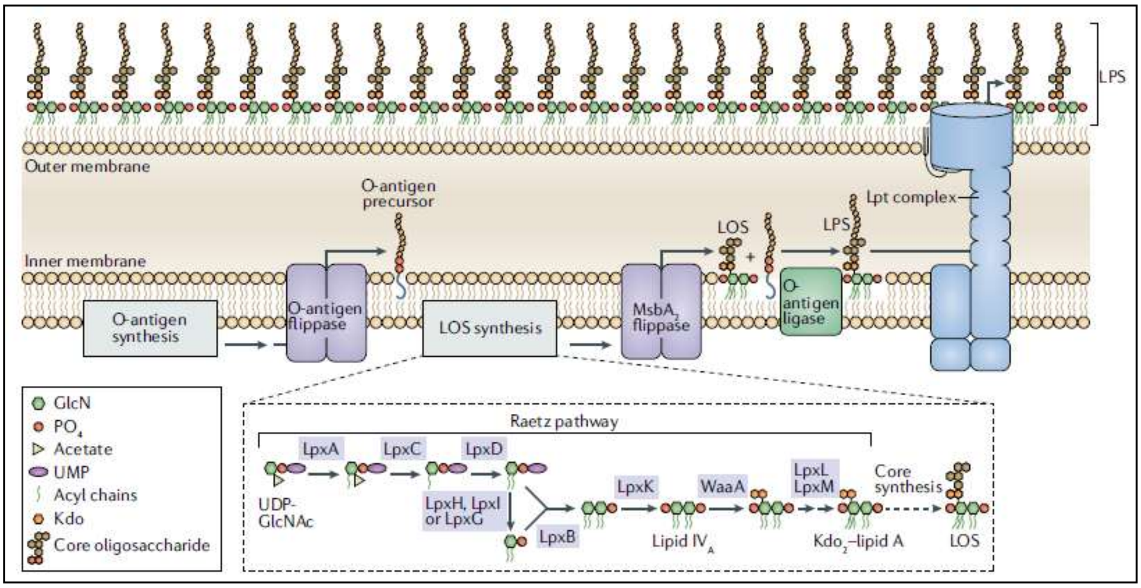


Figure 1.3.2. LPS biosynthesis in *E. coli.* Lipid A and LPS core domains biosynthesis occurs in the cytoplasm and cytoplasmatic interface of the inner membrane. Lipid A synthesis, or Raetz pathway, involves several enzymes working in succession. Afterwards, MsbA flips the LOS precursor across the inner membrane, where it is attached to the O-antigen. Lastly, the Lpt complex transports the LPS from the inner membrane to the surface of the outer membrane. Figure adapted from B. W. Simpson and M. S. Trent [24].

1.4. Membrane maintenance systems

Multiple mechanisms have been identified in Gram-negative bacteria to preserve lipid asymmetry in the outer membrane [21]. Perturbations of the membrane or disruption of either the LPS biosynthesis or its transport system cause a compensatory flipping of phospholipids to the outer leaflet of the outer membrane to accommodate the reduction in LPS levels [36, 37].

The phospholipase PldA acts as a sensor for lipid asymmetry in the outer membrane by degrading mis-localized phospholipids and generating lipid messengers that enable the cell to restore homeostasis [21]. The fatty acid produced by hydrolysis of the phospholipids signals for the inhibition of LpxC degradation and consequent increase of LPS [38].

Mla ABC transport system facilitate retrograde phospholipid transport back to the inner membrane. It is comprised of 6 proteins present in all compartments of the cell envelope. MlaA transports the phospholipids from the outer leaflet of the outer membrane to MlaC in the periplasm, from where phospholipids are delivered to the MlaFEDB complex and reintegrated into the inner membrane [39]. This pathway is upregulated upon loss of LPS [40].

Loss-of function mutations in any of the genes that encode the Mla ABC transport affects the entire pathway leading to disruption in the lipid balance on the outer membrane and to the accumulation of phospholipids at the cell surface. The inactivation of *mlaA* can be compensated by *pldA* overexpression [41]. Upon disruption of LPS levels, removal of these two systems allows the outer membrane to regain homeostasis between the leaflets of the lipid bilayer [21]. There are examples of Gram-negative bacteria that can survive in the absence of lipid A, like *A. baumanni*, *Neisseria meningitidis* and *Moraxella catarrhalis* [40]. Studies on virulence phenotypes of *A. baumanni* reveal that loss of LOS resulted in attenuated virulence [42]. Power and Trent [40] demonstrated the negative impact of Mla and PldA maintenance systems on the fitness of LOS-deficient *A. baumanni*. Mla and PldA joint elimination was enough to cause fitness improvement in the LOS-deficient mutant. In the absence of Mla and PldA the removal of phospholipids from the outer leaflet of the outer membrane is terminated, and the cells can restore homeostasis in a LOS/lipid A deficient outer membrane.

There is a need for safer and more efficient vaccines for both *B. bronchiseptica* and *B. pertussis.* Acellular vaccines are less reactogenic than the conventional whole-cell vaccines due to the non-existent or minimal amount of LPS [43]. The goal of this study was to apply the same principle to whole-cell vaccines. Reduction of LPS amounts and endotoxicity in both species was tried by interfering with lipid A synthesis and structure with the assumption that less LPS and less-acylated lipid A translates in less-endotoxic bacteria. The production of key enzymes of the synthesis, LpxA, LpxC, LpxL1 and LpxL2 was interrupted or regulated with inducible promoters. Interference with the first two enzymes affects the amount of produced lipid A. *lpxA* inactivation via introduction of an antibiotic marker was combined with an expression plasmid harboring *lpxA* expressed from a controllable promoter. The same strategy was used for *lpxL1-2* and *lpxL2* in WT *Bordetella* strains. Inactivation of *lpxL1-2* was also attempted in *B. pertussis* PdlA-MlaF deficient strain using a suicide vector for homologous recombination and allelic exchange. The mutant strain allows the cell to achieve homeostasis when LPS is under-acylated. In a different approach, *lpxC* and *lpxL2* expression was regulated through an inducible promoter inserted in the chromosome.

2. Methods and Materials

2.1. Bacterial Growth Conditions

All bacterial strains used are described in Table 2.1.1.

E. coli strains were grown in lysogeny broth (LB) [44] at 37°C and 200 rpm agitation or on LB agar plates [45]. *E. coli* WD2 was incubated at 30°C [46]. *B. pertussis* was cultivated on Bordet-Gengou (BG) agar plates (Difco) supplemented with 15% defibrinated sheep blood (Biotrading) at 35°C [47]. *B. bronchiseptica* strains were grown on LB agar plates, also at 35°C [45]. Liquid cultures were cultivated at 175 rpm and 35°C. *B. pertussis* was grown in Verwey medium [48] (pH 7.4) containing 0.001% (w/v) reduced L-glutathione instead of L-cystine and supplemented with 0.001% (w/v) $FeSO_4 \cdot 7H_2O$. To reach an initial optical density (OD) of 0.05 measured at 600 nm, the strains were formerly grown in plate and used to establish the intended OD. Strain selection involved the supplementation of the medium with 50 µg/ml nalidixic acid (nal) for *B. pertussis* and 5 µg/ml cefotaxime (ctx) for *B. bronchiseptica*, 10 µg/ml gentamicin (gen), 300 µg/ml streptomycin (str), 50 and 150 µg/ml erythromycin (ery), 100 and 200 µg/ml ampicillin (amp) and 25 µg/ml chloramphenicol (cam) were also used for transformation, cloning, electroporation, or conjugation. For induction of expression, the medium was additionally supplemented with 0.1, 0.5 or 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) [47].

Table 2.1.1. Bacterial strains and their features.

Bacterial Strain	Relevant Characteristics	Reference
Escherichia coli		
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* Φ80 Δ(*lacZ*)*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	[49]
SM10λpir	*thi thr leu tonA lacY supE recA::*RP4-2-Tc::Mu *λpir* R6K Kan^{R}	[50]
WD2	*W3110, aroA::Tn10 msbA (A270T)* harboring pMMB67EH *msbA* gene; Amp^{R}	(a)
Bordetella pertussis		
B213	Derivative of *B. pertussis* strain Tohama I; Nal^{R}, Str^{R}	[51]
B213 ΔPldA-MlaF	*pldA* and *mlaF* mutant of B213 strain; Nal^{R}, Str^{R}, Gen^{R}	(b)
B213 ΔPldA-MlaF, ΔLpxL1-2	*lpxL1-2* mutant of B213 ΔPldA-MlaF; Nal^{R}, Str^{R}, Gen^{R}	This study
B213 pMMB67EH LpxL1-2	B213 harboring pMMB67EH LpxL1-2. Nal^{R}, Amp^{R}, Str^{R}	This study
B213 pMMB67EH LpxL2	B213 harboring pMMB67EH LpxL2. Nal^{R}, Amp^{R}, Str^{R}	This study

B213 pMMB67EH LpxA	B213 harboring pMMB67EH LpxA. Nal^R, Amp^R, Str^R	This study
B213 ΔLpxL1-2 pMMB67EH LpxL1-2	*lpxL1-2* mutant of B213 harboring pMMB67EH LpxL1-2. Nal^R, Amp^R, Gen^R, Str^R	This study
Bordetella bronchiseptica		
BB-D09-SR	Derivative of BB-D09 isolated from dog; Ctx^R, Amp^R, Str^R	(a)
BB-D09-SR pMMB67EH LpxL1-2	BB-D09-SR harboring pMMB67EH LpxL1-2. Ctx^R, Amp^R, Str^R	This study
BB-D09-SR pMMB67EH LpxL2	BB-D09-SR harboring pMMB67EH LpxL2. Ctx^R, Amp^R, Str^R	This study
BB-D09-SR pMMB67EH LpxA	BB-D09-SR harboring pMMB67EH LpxA. Ctx^R, Amp^R, Str^R	This study
BB-P19	Isolated from swine; Ctx^R, Amp^R, Str^R	(c)
BB-P19 pMMB67EH MsbA	BB-P19 harboring pMMB67EH MsbA. Ctx^R, Amp^R, Str^R	This study
BB-P19 pMMB67EH LpxL1-2	BB-P19 harboring pMMB67EH LpxL1-2. Ctx^R, Amp^R, Str^R	This study
BB-P19 pMMB67EH LpxL2	BB-P19 harboring pMMB67EH LpxL2. Ctx^R, Amp^R, Str^R	This study
BB-P19 pMMB67EH LpxA	BB-P19 harboring pMMB67EH LpxA. Ctx^R, Amp^R, Str^R	This study

Kan - kanamycin
(a) Molecular Microbiology Laboratory, Department of Biology, Faculty of Science, Utrecht University, The Netherlands
(b) Eline de Jonge from Department of Molecular Microbiology and Institute of Biomembranes, Utrecht University, The Netherlands
(c) supplied by the Veterinary Microbiological Diagnostic Center (VMDC), Division of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

2.2. Preparation of Electrocompetent Cells

B. pertussis cells were grown in Verwey medium for 68 h to 72 h and *B. bronchiseptica* in LB for 16 h to 20 h in previous described conditions. Cells were centrifuged for 15 min at 4 °C, 16.639 x g rcf. The pellet from *B. pertussis* was harvested and washed twice with ultrapure water (MQ) and sucrose solution (300 mM). *B. bronchiseptica* cells were washed twice with physiological salt solution, once with MQ, and twice with sucrose solution. Pellets were resuspended in sucrose solution until an OD_{600} of 50 ± 0.5 and 40 ± 0.5 for *B. pertussis* and *B. bronchiseptica*, respectively. All steps were performed at 4°C.

The limit pulse voltage for electroporation was tested up to 25.0 kV/cm in Gene Pulser II Electroporator (Bio-Rad) with electrical resistance and capacitance of 200 ohm and 25 µF, respectively. The cells were stored at -80°C.

2.3. Recombinant DNA Approaches

Plasmids and PCR primers are described in Table 2.3.1. and Table 2.3.2., respectively. To inactivate *lpxA*, the gene contained in plasmid pCRII LpxA was interrupted by a cam-resistance cassette (Cam^R). The resistance gene was amplified by PCR from both either pEN11-Imp or pEN11-GFP plasmids with the Expand High Fidelity PCR system (Roche) using Cam^R specific primers, which introduced an AscI restriction site via reverse primer, Rv-Cam-AscI. After confirmation of the DNA sequence, both plasmid pCRII LpxA and the Cam^R PCR products were double digested with restriction enzymes AscI and SacII (Themo Scientific), ligated and the resulting DNA was used to transform *E. coli* DH5α by chemical transformation using amp and cam for transformant selection. Digestions were performed following the instructions of the manufacturer and using the advised buffer. For sticky-end ligations, T4 DNA ligase (Thermo Scientific) was used with provided buffer solution. All PCR and digestion products were isolated using the Wizard SV Gel and PCR Clean-up System (Promega) and for plasmids using E.Z.N.A. Plasmid Mini Kit II (Omega Bio-tek) and then analyzed by electrophoresis on agarose gels (120 V), using the Gene Ruler 1-kb DNA ladder (Thermo Scientific). DNA bands on gel were visualized and treated using Image Lab v6.0.1 software was used (Bio-Rad).

For *lpxA* overexpression, *Bordetella* strains were conjugated with an expression plasmid (pMMB67EH) harboring the gene either from *B. pertussis* (pMMB67EH LpxA BP) or from *B. bronchiseptica* (pMMB67EH LpxA BB). Both constructs, initially present in routine cloning strain *E. coli* DH5α, were used to transform *E. coli* SM10λpir, selected with amp (100 µg/ml) and thereafter transferred to *B. pertussis* B213, *B. bronchiseptica* BB-D09-SR and *B. bronchiseptica* BB-P19 by plate-mating conjugation as described [52]. Selection of the transconjugants involved strain specific antibiotic combined with 100 or 200 µg/ml amp for plasmid introduction. Amp-resistance of *B. bronchiseptica* strains led to the use of the higher amp concentration. The presumed transformants and transconjugants were screened for the introduction of the vector using plasmid backbone primers (pMMB-F/pMMB-R2). Each colony PCR was performed using DreamTaq DNA polymerase (Thermo Scientific) and a positive transformant and a positive transconjugant, grown in liquid culture, were stored at -80 °C in 15% glycerol.

Overexpression of *msbA* in *Bordetella* cells involved the introduction of an expression plasmid harboring the *msbA* gene (pMMB67EH MsbA) through electroporation. The plasmid was purified from *E. coli* WD2 pMMB67EH MsbA and used to electrotransform *B. pertussis* B213, *B. pertussis* B213 ΔPldA-MlaF and *B. bronchiseptica* strains.

Electrocompetent cells were plated as negative control and electroporation with pMMB67EH LpxL1-2 was done as positive control for the electroporation. Electrotransformations were conducted at room temperature and at a pulse voltage of 22.5 kV/cm. Electrocompetent cells (40 µl) were mixed with the plasmids (>150 ng/µl), electroporated, and incubated in 900 µl of the appropriate medium for 75 min at 35°C [45]. The culture was then plated using the appropriate solid medium supplemented with 100 and 200 µg/ml amp and 1mM IPTG and left to grow at 35 °C. Mutant analysis was performed as mentioned.

Table 2.3.1. Plasmids features.

Plasmid	Relevant Characteristics Description	Reference
pCRII	Cloning vector; Amp^R, Kan^R	Invitrogen
pCRII LpxA	pCRII derivative harboring *lpxA* gene	(a)
pCRII ΔLpxL2 BB	pCRII derivative harboring gentamycin interrupted *lpxL2* gene from *B. bronchiseptica*; Amp^R, Kan^R, Gen^R	(a)
pCRII ΔLpxL2 BP	pCRII derivative harboring gen interrupted *lpxL2* gene from *B. pertussis*; Amp^R, Kan^R, Gen^R	(a)
pEN11	*Neisseria* replicative plasmid; Cam^R, Kan^R	[53]
pEN11-Imp	pEN11 derivative harboring H44/76-derived *imp*	[54]
pEN11-GFP	pEN11 derivative harboring green fluorescent protein gene sequence	(a)
pKAS32	Allelic exchange suicide vector; Amp^R, Str^S	[55]
pKAS32 ΔLpxL2 BB	pKAS32 derivative harboring gen interrupted *lpxL2* gene from *B. bronchiseptica*; Amp^R, Gen^R, Str^S	This study
pKAS32 ΔLpxL1-2	pKAS32 derivative harboring gen interrupted *lpxL1-2* gene; Amp^R, Gen^R, Str^S	(a)
pMMB67EH	Broad host range, low copy-number expression vector; Amp^R	[56]
pMMB67EH MsbA	pMMB67EH derivative harboring *msbA* gene from *B. pertussis* B213; Amp^R	(a)
pMMB67EH LpxL1-2	pMMB67EH derivative harboring *lpxL1-2* gene; Amp^R	(a)
pMMB67EH LpxL2	pMMB67EH derivative harboring *lpxL2* gene; Amp^R	(a)
pMMB67EH LpxA BP	pMMB67EH derivative harboring lpx*A* gene from *B. pertussis*; Amp^R	(a)
pMMB67EH LpxA BB	pMMB67EH derivative harboring lpx*A* gene from *B. bronchiseptica*; Amp^R	(a)
pUC18	High copy cloning vector. Plac, lacZ'; Amp^R	Thermo scientific
pUC18-ybcG-tolQ	pUC18 derivative harboring *ybcG* and *tolQ* genes, Plac/Ptac, lacI^q Amp^R, Ery^R	(a)
pUC RegLpxL2 BB	pUC18 derivative harboring *lpxL2* incomplete gene, Plac/Ptac, lacI^q Amp^R, Ery^R	This study
pUC RegLpxC BB	pUC18 derivative harboring *lpxC* incomplete gene, Plac/Ptac, lacI^q Amp^R, Ery^R	This study

(a) Molecular Microbiology Laboratory, Department of Biology, Faculty of Science, Utrecht University, The Netherlands

To inactivate the *lpxL1* and *lpxL2* genes in *B. pertussis* B213 ΔPldA-MlaF, the pKAS32 plasmid harboring *lpxL1-2* (pKAS32 ΔLpxL1-2) interrupted by a gen-resistance cassette (Gen^{R}) was transferred from *E. coli* SM10λpir via plate-mating conjugation. Transconjugants were selected both at 35°C and room temperature (RT). After antibiotic selection, the mutants were analyzed by PCR. For the first crossover, i.e. the integration of the entire pKAS32 ΔLpxL1-2 plasmid via homologous recombination into the host genome, amp and gen were used for mutant selection and nal for counterselection against the donor. A second crossover event results in the excision of the plasmid from the chromosome and allows for the substitution of the WT gene by the donor sequence. Since the efficacy of plasmid excision in the transconjugants is low, *rpsL* gene present in the suicide vector aids in positive selection for plasmid loss. This gene encodes ribosomal protein S12 that, when expressed, gives a str-sensitive (Str^{S}) phenotype to the str-resistance (Str^{R}) recipient strain. Loss of the plasmid renders the cell Str^{R} again [53, 54]. Gen and str were used for selection of recombinants.

A DNA fragment coding only for LpxL2 acyltransferase, already inserted in pCRII plasmid (pCRII LpxL2), was subcloned into pKAS32. Two separate constructs with *lpxL2* gene interrupted by Gen^{R} were used i.e., pCRII ΔLpxL2 BP and pCRII ΔLpxL2 BB, with the *lpxL2* sequences deriving from *B. pertussis* and from *B. bronchiseptica*, respectively. The constructs were subcloned into pKAS32 suicide vector through double digestion, using XbaI and SacI restriction enzymes (Thermo Scientific), which are common to both donor and recipient plasmids. The resulting plasmids were then used to transform *E. coli* SM10λpir. For the double digestion it was utilized either 500 ng or 1000 ng of DNA of the donor and recipient plasmids. Ligation was performed directly with the complete double digestion product resulting from the lower DNA amount used. The double digestion resulting from the higher DNA quantity was ran on an agarose gel until band separation. The fragment containing the *lpxL2* and the resistance cassette was cleaved and used to perform ligation. Selection was performed with amp (100 µg/ml) and gen. Gen-resistant transformants were screened using primers targeting *lpxL2* (Fw-Bspp-LpxL1-mid / Rv-dwLpxL2), donor (M13-pUC-F / M13-pUC-R), and recipient (Fw-Eco91I-GemR / pkas32-Rev or Rv-GemR-Eco91I / pkas32-Rev) plasmids.

lpxL1-2 and *lpxL2* overexpression in *B. pertussis* B213 and *B. bronchiseptica* (BB-D09-SR and BB-P19) was executed as mentioned for *lpxA* overexpression. The expression plasmid harbored *lpxL1-2* (pMMB67EH LpxL1-2) or just *lpxL2* (pMMB67EH LpxL2). After introduction of the expression plasmid in the bacteria via plate-mating conjugation, the transconjugants were subjected to inactivation of *lpxL1-2* in the chromosome. *Bordetella lpxL1-2* was knocked-out by plate-mating conjugation of the bacteria with pKAS32 ΔLpxL1-2 following the same procedure as before. Selection of the transconjugants involved nal or ctx and gen after the first crossover. Str was added after the second recombinant event. Amp (100 or 200 µg/ml) and IPTG (1mM) where always included for expression plasmid maintenance. Following PCR screening with Fw-upLpxL1/ Rv-dwLpxL2 and Fw-Eco91I-GemR/ LpxL1-dw-Rev-XbaI, BglII restriction enzyme digestion was applied on the positives for the gene knockout. The restriction pattern of the knock-out diverges from the pattern deriving from the complete gene sequence.

To regulate the expression of *lpxL2* in the chromosome, an incomplete gene sequence (Reg) was PCR amplified with Expand High Fidelity PCR system from the WT *B. pertussis* B213, *B. bronchiseptica* BB-P19 and BB-D09-SR using designed primers. The same process was applied for *lpxC* regulation. The primers used on PCR amplification of *lpxC* and of *lpxL2* were constructed with a length variation of 20 to 22 overlapping bases with the same melting temperature between primer pairs. PCR products (RegLpxC and RegLpxL2) and the high copy-number vector pUC18-ybcG-tolQ were double digested with NdeI and AatII (ThermoFisher), sticky-end ligated and used to transform *E. coli* DH5α, selecting with amp and ery. Sequencing results obtained from pUC RegLpxC and pUC RegLpxL2 were aligned using BLAST - Basic Local Alignment Search Tool - against *B. pertussis* Tohama I (accession number CP039022.1) and *B. bronchiseptica* KM22 (accession number CP022962.2). An insert presenting no mutations was introduced in *Bordetella* by electroporation. Electrocompetent cells were plated as negative control and electroporation with plasmids pMMB67EH LpxA and pMMB67EH LpxL1-2 was done as positive controls. After antibiotic selection with gen and 200 µg/ml amp for BB-P19 and BB-D09-SR and 100 µg/ml amp for B213, screening involved PCRs with three pairs of primers for each gene, aligning at the *lac/tac* promoter region (PeN-sh-Fw-XbaI), the amp- (Amp for RT-PCR) and ery-resistance cassettes (ERY-FOR / ERY-REV) and downstream from *lpxC* (Rv-dw-LpxC) or *lpxL2* (3'LpxL2-R). DNA concentrations were measured using NanoDrop spectrophotometer ND-1000 (Thermo Scientific). All DNA sequencing was done by Macrogen (Amsterdam, The Netherlands) and bioinformatic analysis and plasmid constructions were assisted by NCBI, Clone Manager Suite 7 (Sci-Ed) and SnapGene Viewer (GSL Biotech LLC) software.

Table 2.3.2. Primers required for identification of bacterial isolates.

Name	Sequence (5’ → 3’)	Gene	Amplimer size (bp)
Cam-Fw-DraIII	GCGCGCGCCACAACGTGACTCTAGAGGATCGATCCG	Cam^R cassette	1203
Rv-Cam-AscI	GCGCGCGGCGCGCCTGCTGCTGGCTACCCTGTGG		
ERY-FOR	TTAAACAAGGATATATTGCAG	Ery^R cassette	430
ERY-REV	AATCGTCAATTCCTGCATG		
Fw-Bspp-LpxL1-mid	CAAATACCGCGACCGCCTG	Δ*lpxL1-2*	996
R-5'LpxL2	ATCAGGGCATTGATGCGTTC	*lpxL1-2*	1084
Fw-Eco91I-GemR	GCGCGCGGTCACCGACGCACACCGTGGAAA	Δ*lpxL1-2*	2613
LpxL1-dw-Rev-XbaI	GCGCGCTCTAGACCGTTCCAGTCCACGGTAAG		
Fw-upLpxL1	AATTCGCTCTGGCGCTGCAC	Δ*lpxL1-2*	2791
Rv-dwLpxL2	ATGACGCCGGTCACGATCTC	*lpxL1-2*	2869
pMMB-F	TGAGCTGTTGACAATTAATCAT	*lpxL1-2*	2046
pMMB-R2	TACGGCGTTTCACTTCTGAG	*lpxL2*	891
		lpxA	995
		msbA	2276
Fw-Bspp-LpxL1-mid	CAAATACCGCGACCGCCTG	*ΔlpxL2*	2895
Rv-dwLpxL2	ATGACGCCGGTCACGATCTC		
M13-pUC-F	CCCAGTCACGACGTTGTAAAACG	*lpxL2*	3173
M13-pUC-R	AGCGGATAACAATTTCACACAGG		
Fw-Eco91I-GemR	GCGCGCGGTCACCGACGCACACCGTGGAAA	Gen^R cassette + pKAS32	2028
Rv-GemR-Eco91I	GCGCGCGGTGACCGCGGCGTTGTGACAATTT		
pKAS32-Rev	CGTCGTCGACCTGAGGTAAT		
RegLpxL2-FW-NdeI	GCGCGCCATATGAGCCAATTCAAGACCCG	*lpxL2*	891
RegLpxL2-RV-AatII	GCGCGCGACGTCCTAGTGCACCCAGTAATACTGGC		861
PeNP-sh-Fw-XbaI	GCGCGCTCTAGATGTGGAATTGTGAGCGGATA	*lpxL2*	1013 (*lac*)
3'LpxL2-R	CGCCGTTTCAGTACAGCTTG		1097 (*tac*)
Amp for RT-PCR	CTATGTGGCGCGGTATTATC	*lpxL2*	6899
3'LpxL2-R	CGCCGTTTCAGTACAGCTTG		

RegLpxC-FW-NdeI	GCGCGCCATATGTTCCGACAGCGCAGTATTC	*lpxC*	924
RegLpxC-RV-AatII	GCGCGCGACGTCCTAATGGCCCGATTTGTAGGCAAC		819
PeNP-sh-Fw-XbaI	GCGCGCTCTAGATGTGGAATTGTGAGCGGATA	*lpxC*	1174 (*lac*)
Rv-dw-LpxC	CAAAAAGCCTATGGCCGCTC		1090 (*tac*)
Amp for RT-PCR	CTATGTGGCGCGGTATTATC	*lpxC*	6857
Rv-dw-LpxC	CAAAAAGCCTATGGCCGCTC		

2.4. SDS-PAGE and LPS Analysis

B. pertussis cultures were grown for 20h, as described, harvested, and resuspended to an OD_{600} of 5. Then, bacterial cells were killed at 56 °C for 60 min, centrifuged (4000 rpm, 10 min, RT) and the pellet was resuspended in lysis buffer [57] and sonicated. The lysates were mixed with loading buffer [57] to an OD_{600} of 2.5, boiled for 5 min and incubated at 65 °C for 60 min with 50 µg/ml of Proteinase K (Thermo Scientific). Samples were loaded on 16% polyacrylamide gels and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed [58]. Following electrophoresis, the gel was washed with MQ and fixated overnight in isopropanol: acetic acid: MQ (40/10/50). Oxidation was performed with fresh periodic acid (0.7 %) and then, the gel was washed 4 times in MQ for 15-minute periods. The gel was stained with silver solution (20 % $AgNO_3$), washed in MQ thrice for 15-minute periods and subsequently developed in citric acid (0.005 %) and formaldehyde (37 %) [59]. LPS was visualized with UV transilluminator. All dilutions were prepared using MQ.

2.5. Statistical Analysis

Statistical analyses were performed using GraphPad Prism v8.4.2 software using non-parametric one-way ANOVA, Kruskal-Wallis test, and Dunn’s multiple comparison test.

5. Results

5.1. Construction of pCRII Harboring *lpxA* interrrupted by Cloramphenicol resistance cassette

Aiming the reduction of LPS content in *Bordetella* cells, one of the goals was to inactivate the first enzymatic step in the lipid A synthesis, which is catalyzed by LpxA. To inactivate *lpxA*, a construct containing the gene interrupted by insertion of a cam-resistance cassette (Cam^R) was designed. First, the Cam^R cassette was amplified by PCR from plasmid pEN11-lmp (Figure 5.1.1.A) with the goal to introduce it in pCRII LpxA. Although the band size matched the expected size of the DNA fragment, it was not possible to insert it in pCRII LpxA since no transformants were obtained. Sequence analysis of the PCR product with the Cam^R cassette showed the existence of a single silent mutation and absence of the AscI restriction site. This restriction enzyme site was designed in the reverse primer to be introduced during PCR amplification from the donor plasmid. According to the service provider for the sequencing analysis, the precisions limit of the technique is approximately 1050 bp. Hence, given the extended length of the antibiotic-resistance marker (1203 bp), the sequencing of the restriction site could possibly be unreliable. To ensure that the reverse primer was indeed faltered, the source DNA for the Cam^R cassette was changed. The previous subcloning methods were repeated, with Cam^R cassette derived from pEN11-GFP (Figure 5.1.1.B) instead. The PCR product was of similar size as the one obtained from the first donor plasmid. The efficiency of pCRII LpxA and Cam^R cassette double digestion was also evaluated by agarose gel electrophoresis (Figure 5.1.1.C). Cam^R digestion could not be confirmed due to the small size of the released fragments (11 bp). The undigested plasmid (5793 bp) presented a clear 3-band pattern due to the multiple conformations the DNA can take. The fastest-migrating band marked with a bead represents the supercoiled conformation, which migrates further in the gel. The middle band, marked with plus, represents the linear form, at the expected size, and the upper most band, marked with asterisk, is the nicked DNA which is the slowest migrating band. After pCRII LpxA digestion, two fragments of 577 bp and 5216 bp were expected, with the *lpxA* located in the larger fragment. The lower band is not visible in Figure 5.1.1.C and the upper band (open arrowhead) matches the linear conformation from the undigested plasmid. This suggested that only one of the restriction enzymes digested the plasmid. Given that no growth was observed in cam and amp after transformation, single digestion was performed on pCRII LpxA to assess the digestion efficacy of each enzyme. AscI and SacII single digestion produced a single band at 6000 bp indicating a linear plasmid (data not shown). The region for primer alignment in pEN11-Imp and the location of restriction sites on both gene sequences are shown in Figure 8.1.

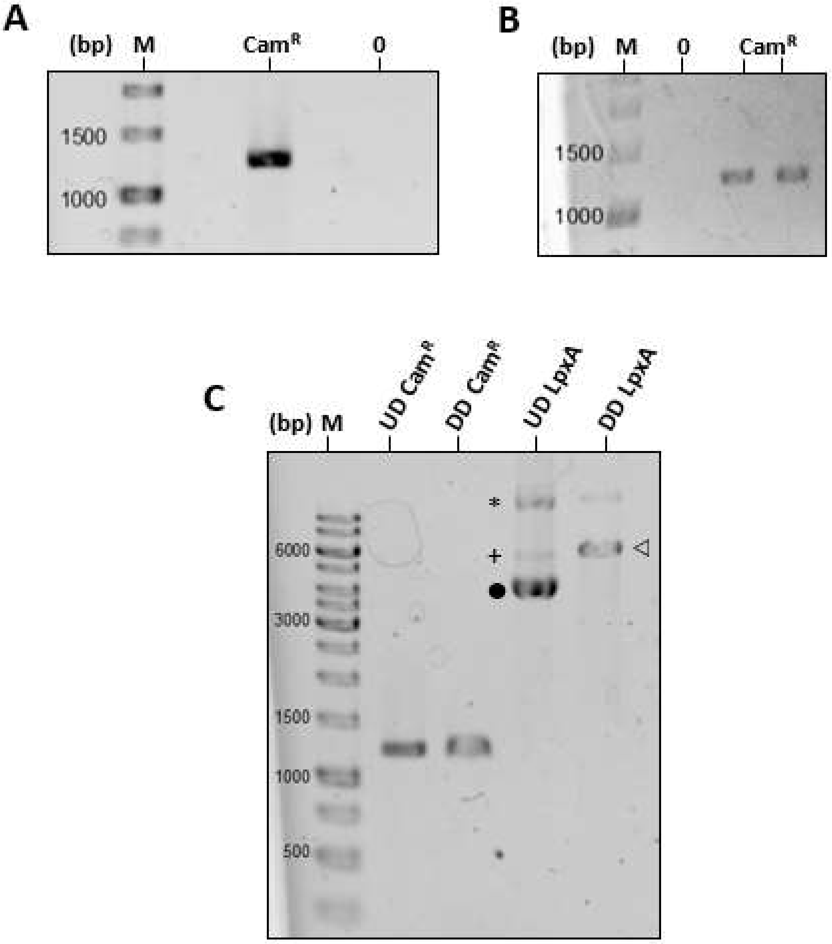


Figure 5.1.1. PCR amplification of Cam^R cassette. Digested and undigested plasmid pCRII ΔLpxA and Cam^R cassette. PCR amplification product of Cam^R cassette (1203 bp), from (A) pEN11-lmp and (B) pEN11-GFP, using primers Cam-Fw-DraIII and Rv-Cam-AscI. Lane 0 – No template control. Lane M – DNA Ladder. (C) Cam^R PCR amplification product obtained from pEN11-lmp (Cam^R) and plasmid pCRII LpxA (LpxA) before (UD) and after (DD) double digestion with restriction enzymes AscI and SacII. Digested pCRII LpxA produces a band (◁) of 5783 bp in size. (*) Nicked, (+) linear and (●) coiled DNA. Digestion and PCR products were separated by electrophoresis on 0.8% agarose gels.

5.2. Construction of pKAS32 ΔlpxL2 for *lpxL2* inactivation

The number of acyl chains in the lipid A directly correlates with its endotoxicity, typically implying that under-acylated LPS will stimulate less efficiently the host innate immune responses. Lipid A is usually hexa-acylated in *B. bronchiseptica* and penta-acylated in *B. pertussis.* By inactivating the gene encoding LpxL2 acyltransferase, this study aimed to reduce lipid A acylation. To this end, two constructs harboring *lpxL2* interrupted by Gen^R

(pCRII ΔLpxL2 BB and pCRII ΔLpxL2 BP) were double digested and subcloned into pKAS32. Figure 5.2.1.A shows the undigested pCRII ΔLpxL2 plasmids which are supercoiled and migrated further than the expected 6868 bp. The upper band represents the nicked conformation of the plasmid. Once digested, the plasmids were expected to present 2 fragments of 3877 bp and 2991 bp, with the ΔLpxL2 located in the smaller fragment, consistent with results presented in the figure. Undigested pKAS32 presented 2 bands in the supercoil and nicked configurations. After digestion, a single band of the expected size (4.4 kb) was observed.

To achieve the most effective SM10λpir transformation, yielding gen resistant transformants, 500 ng or 1000 ng of DNA from the donor and recipient plasmids was used in the double digestion. The digestion mix from both digestions was directly used for ligation or, after electrophoresis, the appropriate band was selected, purified and ligated with the plasmid. Antibiotic resistant mutants were obtained after transformation with ΔLpxL2 from *B. bronchiseptica* that used 500 ng of DNA for the double digestion, using the digestion product directly for ligation. Both pCRII and pKAS32 plasmids possess amp-resistance cassettes (Amp^R), which interfered with the selection of the transformants. The possibility for false positives is very high due to the lack of distinctive antibiotic resistance of the recipient plasmid. Nevertheless, amp and gen were used for transformant selection. Antibiotic-resistant mutants were tested using a combination of pKAS32 and gen primers. Since the orientation of Gen^R in pCRII ΔLpxL2 was unknown, PCR was performed with either the gen forward (Fw-Eco91I-GemR) or the reverse primer (Rv-GemR-Eco91I) paired with pKAS32-Rev, which aligns in the recipient plasmid. In both cases, no PCR amplification was detected. Previous works performed by this research group asserted that pKAS32 forward primer exhibited problems in PCR amplification. The lack of amplification when using the pKAS32 reverse primer was thus regarded as having a similar problem. To assess the presence of the original plasmid, primers targeting the pCRII plasmid were used (M13-pUC-F / M13-pUC-R). In addition, the presence of the insert was evaluated with primers targeting the gene sequence (Fw-Bspp-LpxL1-mid / Rv-dwLpxL2). Panel B of Figure 5.2.1. displays the colony PCR of *E. coli* SM10λ pKAS32 ΔLpxL2 BB using pCRII primers. Nine isolates, out of a total of eighteen, did not indicate the presence of the donor plasmid. The negative clones were subsequently tested for the *lpxL2,* and five of those (5, 9, 12, 15 and 18) were positive for the presence of the gene (Figure 5.2.1.C). This line of testing does not prove successful subcloning into the recipient plasmid; it merely suggests that should the gene not be in pCRII, then it ought to be in pKAS32. Clones 9 and 15 were stored for further testing.

E. coli SM10λpir transformation with pKAS32 ΔLpxL2 BP resulted in amp and gen resistant transformants. The same PCR tests for SM10λ pKAS32 ΔLpxL2 BB were applied. It was also not possible to obtain PCR amplification when the pKAS32 plasmid and the Gen^R cassette were targeted. The PCR screening for *lpxL2* presence was negative for all isolates that did not display the presence of the donor plasmid (data not shown). Therefore, it was no possible to obtain *E. coli* SM10λpir pKAS32 ΔLpxL2 BP.

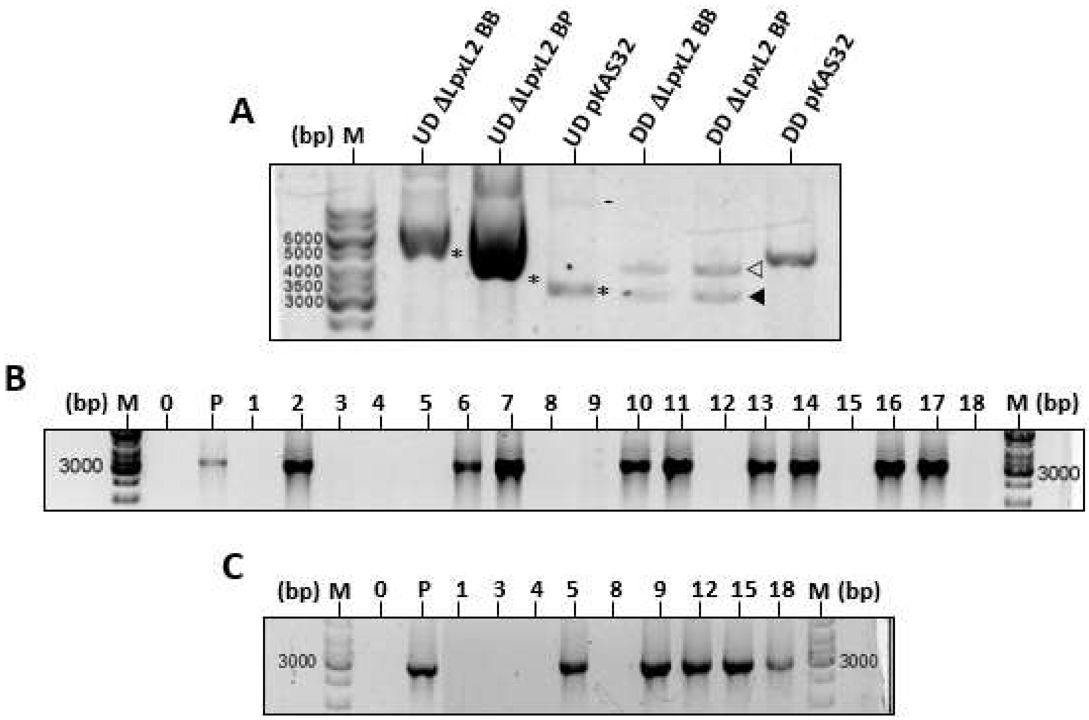


Figure 5.2.1. Digested and undigested plasmids pCRII ΔLpxL2 and pKAS32. Colony PCR of *E. coli* SM10λ after transformation with pKAS32 ΔLpxL2 BB using with specific primers for *lpxL2,* pCRII and pKAS32. (A) Undigested (UD) and double digested (DD) pCRII ΔLpxL2 BB (ΔLpxL2 BB), pCRII ΔLpxL2 BP (ΔLpxL2 BP) and pKAS32 with restriction enzymes XbaI and SacI. (-) Nicked, and (*) coiled DNA. (B) Screening for the donor plasmid with pCRII backbone primers in transformed SM10λpir isolates. (C) PCR amplification of Δ*lpxL2* with primers Fw-Bspp-LpxL1-mid / Rv-dwLpxL2 in negative isolates for the presence of pCRII. Nine out of eighteen isolates tested negative for the presence of pCRII and of those, five contained the knock-out construct. Lane 0 – No template control. Lane P – Positive control pCRII ΔLpxL2 BB. Lane M – DNA Ladder. Digestion and PCR products were separated by electrophoresis on 0.8% agarose gels.

5.3. Incomplete Inactivation of *lpxL1-2* in B213 ΔPldA-MlaF

To inactivate *lpxL1* and *lpxL2* simultaneously (*lpxL1-2*), a PldA-MlaF deficient mutant of *B. pertussis* B213 was used instead of the WT. In *A. baumannii*, the disruption of PldA and Mla has been shown to facilitate survival of the cells in the absence of LPS synthesis [40]. The *ΔlpxL1-2* construct was introduced in the *B. pertussis* B213 ΔPldA-MlaF genome by conjugation with *E. coli* SM10λpir pKAS32 ΔLpxL1-2 as donor. After the second recombination step the resulting colonies were very small and grew slowly at 35°C. Growth was nearly inexistent at RT and showed no incorporation of the knock-out after a period of 21 days (data not shown). Figure 5.3.1. displays *lpxL1-2* PCR amplification with Fw-Bspp-LpxL1-mid / R-5'LpxL2 primers after the first and second crossover events. Panel A shows the incorporation of the donor sequence into the bacterial genome of the transconjugants after the first recombinant step. After the incorporation of the construct into the chromosome, displayed in panel B, both the WT gene (1084 bp) and the *ΔLpxL1-2* (994 bp) were expected to be present, which was not the case. A second set of primers aligning in the Gen^R sequence (Fw-Eco91I-GemR) and downstream of *lpxL2* (LpxL1-dw-Rev-XbaI) produced negative results exclusively (data not shown), confirming that the cell reverted to its WT phenotype, which is contradictory to the poor growth observed. The alignment region for the mentioned primers in *B. pertussis* previous and following ΔLpxL1-2 introduction are displayed in Figure 8.3.

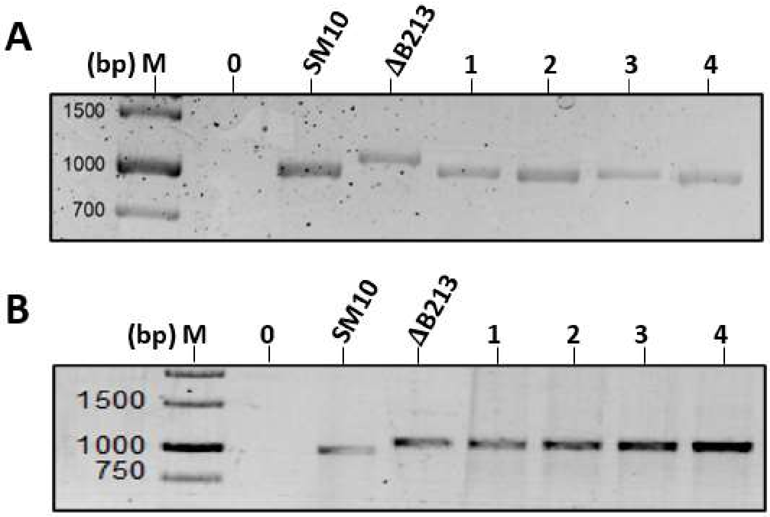


Figure 5.3.1. Colony PCR of *B. pertussis* B213 ΔPldA-MlaF after conjugation with *E. coli* SM10λpir pKAS32 ΔLpxL1-2 using gene-specific primers. PCR amplification of Δ*lpxL1-2* using Fw-Bspp-LpxL1-mid / R-5'LpxL2 primers after (A) first and (B) second recombinant step. *ΔlpxL1-2* from the donor *E. coli* SM10λpir ΔLpxL1-2 (SM10) and *lpxL1-2* from the recipient B213 ΔPldA-MlaF (ΔB213) strains were used as positive and negative controls, respectively. Lane 0 – Non-template control. Lane M – DNA Ladder. PCR products were separated by electrophoresis on 2.0% agarose gels.

Failure to knock-out *lpxL1-2* led to the attempt of overexpressing *msbA* in the same B213 mutant, the WT and the two *B. bronchiseptica* strains. MsbA is an ABC transporter responsible for the transport of LOS across the inner membrane. Studies have reported the compensation of *lpxL* inactivation in *E.coli* through *msbA* overexpression [30]. In this study, a plasmid harboring *msbA* was successfully introduced into strain BB-P19. Amp^R colonies were obtained of all four strains (B213, B213 ΔPldA-MlaF, BB-P19 and BB-D09-SR) after electroporation with pMMB67EH MsbA, although only clones obtained from BB-P19 presented positive PCR results for the presence of the plasmid (Figure 5.3.2.).

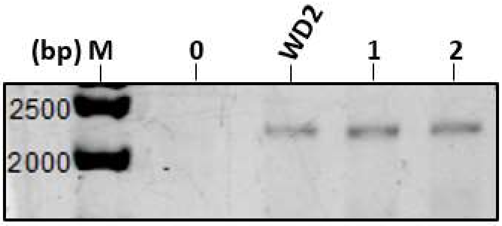


Figure 5.3.2. Colony PCR of *B. bronchiseptica* BB-19 after conjugation with *E. coli* WD2 pMMB67EH MsbA using backbone-specific primers. Partial PCR amplification of pMMB67EH MsbA using primers pMMB-F / pMMB-R2. Plasmid pMMB67EH MsbA from donor strain, *E. coli* WD2 pMMB67EH MsbA (WD2), was used as positive control. Lane 0 – No template control. Lane M – DNA Ladder. PCR products were separated by electrophoresis on 0.8% agarose gel.

5.4. *lpxA, lpxL1-2* and *lpxL2* Overexpression in *Bordetella* spp.

To facilitate inactivation of *lpxA, lpxL1-2* or *lpxL2* genes in the chromosome of B213, BB-P19 and BB-D09-SR a duplication of the target gene was introduced in the bacteria. Expression plasmids pMMB67EH LpxA BP (*lpxA* derives from *B. pertussis*), pMMB67EH LpxA BB (*lpxA* derives from *B. bronchiseptica*), pMMB67EH LpxL1-2 and pMMB67EH LpxL2 allowed for the gene to be expressed from a controllable promoter. *E. coli* SM10λpir carrying each plasmid was conjugated with the three WT strains. Selection for the transconjugants was done with nal or ctx, amp and IPTG. Screening for the presence of the plasmid used backbone primers (pMMB-F / pMMB-R2). Figure 5.4.1.A shows the incorporation of pMMB67EH LpxA BP in B213 and pMMB67EH LpxA BB in strains BB-D09-SR and BB-P19. The presence of plasmids pMMB67EH LpxL1-2 and pMMB67EH LpxL2 was confirmed in transconjugants of all 3 strains (Figure 5.4.1., panels B and C, respectively). When using pMMB67EH backbone primers, an unspecific band at around 1300 bp – 1500 bp was observed that can be seen for every construct.

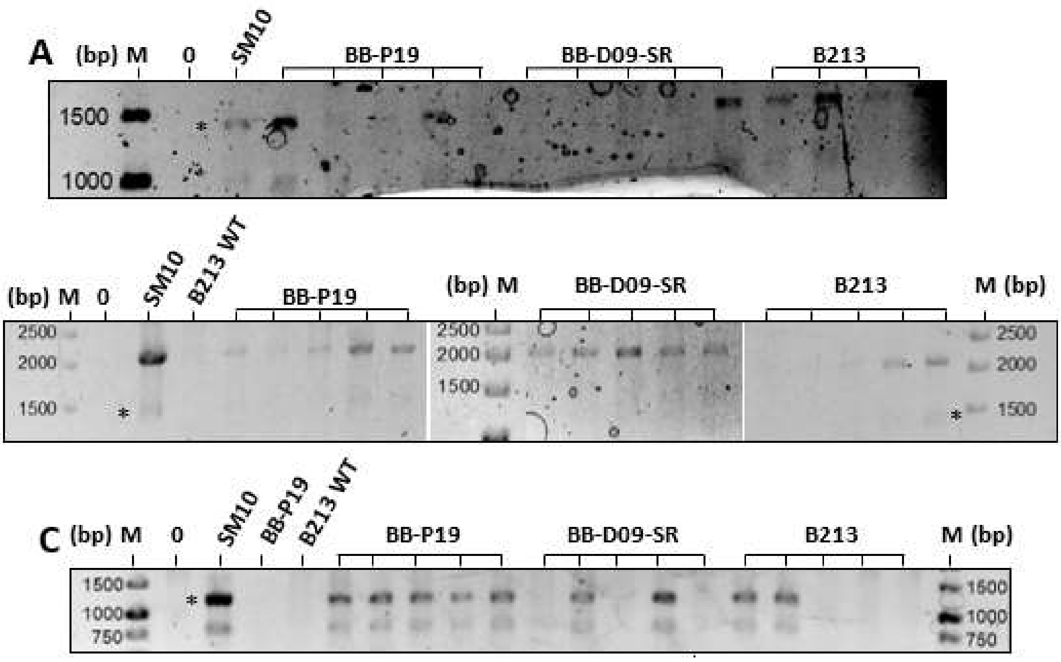


Figure 5.4.1. Colony PCR of *B. pertussis* B213 and *B. bronchiseptica* BB-D09-SR and BB-P19 after conjugation with *E. coli* SM10λpir pMMB67EH harboring *lpxA, lpxL1-2* or *lpxL2,* using backbone-specific primers. Partial PCR amplification of (A) pMMB67EH *lpxA* (995 bp), (B) pMMB67EH *lpxL1-2* (2046 bp) and (C) pMMB67EH *lpxL2* (891 bp) using primers pMMB-F / pMMB-R2. Plasmids pMMB67EH LpxL1-2, LpxL2 or LpxA of donor *E. coli* SM10λpir were used as positive controls (SM10). Negative controls used *B. pertussis* B213 or *B. bronchiseptica* BB-P19. An unspecific band (*) can be observed at 1300 bp – 1500 bp. Lane 0 – No template control. Lane M – DNA Ladder. PCR products were separated by electrophoresis on 0.8% agarose gels.

5.5. *lpxL1-2* Inactivation with extrachromosomal gene expression

lpxL1-2 knock-out was performed for *Bordetella* cells containing pMMB67EH LpxL1-2 and pMMB67EH LpxL2. To inactivate these genes, *E. coli* SM10λpir pKAS32 ΔLpxL1-2 was conjugated with the mentioned strains. Selection involved antibiotics specific to the recipient strain and to the vector. Because of the small difference in size between the WT genes (2869 bp) and the knock-out construct (2791 bp), primers annealing upstream and downstream of the knock-out genes could not be used. Instead, PCR screening was performed with primers aligning in the Gen^{R} cassette (Fw-Eco91I-GemR) and in the region of the genome downstream from the *lpxL2* (LpxL1-dw-Rev-XbaI). For this primer combination a positive control could not be used. The presence of the knock-out construct was inferred from a band of 2326 bp for the strains carrying pMMB67EH LpxL1-2 (Figure 5.5.1.A) or pMMB67EH LpxL2 (Figure 5.5.1.B). ΔLpxL1-2 was present in all three strains carrying pMMB67EH LpxL1-2 and in the *B. bronchiseptica* strains carrying pMMB67EH LpxL2. It was not possible to insert ΔLpxL1-2 in *B. pertussis* harboring pMMB67EH LpxL2. The putative mutants were tested after the second recombinant event with the same primer combination although, no PCR amplification occurred (data not shown). The growth on gen suggested the presence of the plasmid in the cell, although the negative PCR results indicate that the construct sequence was not incorporated in the chromosome.

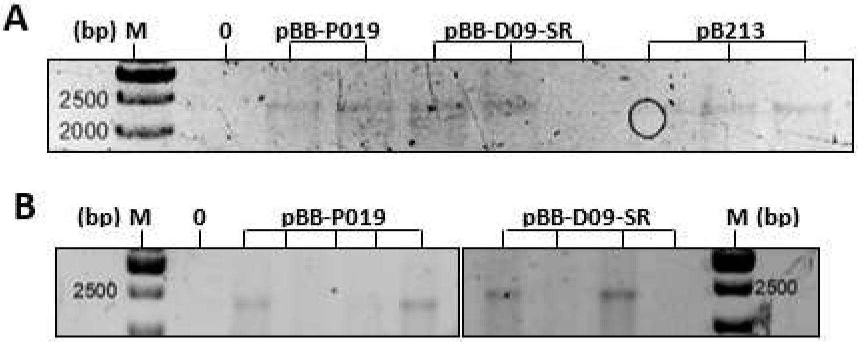


Figure 5.5.1. Colony PCR of *Bordetella* strains harboring pMMB67EH LpxL1-2 or pMMB67EH LpxL2 after conjugation with *E. coli* SM10λpir pKAS32 Δ*lpxL1-2* using primers specific to the construct and *Bordetella* genome. To assess construct introduction in the chromosome the region comprising Gen^R, *lpxL2* and downstream gene was PCR amplified using primer pair Fw-Eco91I-GemR / LpxL1-dw-Rev-XbaI. Strains B213 (pB213), BB-D09-SR (pBB-D09-SR) and BB-P19 (pBB-P19) harboring (A) pMMB67EH LpxL1-2 and (B) pMMB67EH LpxL2 were tested for the incorporation of the construct. Lane 0 – No template control. Lane M – DNA Ladder. PCR products were separated by electrophoresis on 0.8% agarose gels.

The presence of the knockout construct did not demonstrate the excision of the WT gene from the chromosome. The region containing *lpxL1-2* was PCR amplified with upstream and downstream primers (Fw-upLpxL1 / Rv-dwLpxL2) in all three WT strains and in the positive mutants. Then, the amplicon was digested with BglII. This restriction enzyme produced 2 fragments in the WT genes (547 bp, 2322 bp) and 3 fragments in the knocked-out genes (547 bp, 971 bp, 1273 bp). As can be seen in Figure 5.5.2.A two mutants deriving from B213 pMM67EH LpxL1-2 have lost the WT *lpxL1-2* genes (ΔpB213 number 1 and number 2). All mutants deriving from *B. bronchiseptica* pMMB67EH LpxL1-2 strains present a 4-band pattern suggesting the presence of the WT genes and the ΔLpxL1-2. The same can be seen in Figure 5.5.2.B, where the *B. bronchiseptica* strains carrying pMMB67EH LpxL2 display a 4-band pattern and therefore the presence of the WT *lpxL1-2*.

To force the WT genes to be excised from the chromosome the clones were re-plated in 900 μg/ml str. Results from digestion with BglII showed the same 4-band pattern seen in Figures 5.5.1. and 5.5.2. (data not shown). To inactivate *lpxL1-2* genes in the chromosome in BB-P19, BB-D09-SR and B213 all carrying pMMB67EH LpxL2 the procedure described previously was repeated. After the second crossover no growth was observed for the B213 strain. One clone from BB-D09-SR pMMB67EH LpxL1-2 and five clones from BB-P19 pMMB67EH LpxL1-2 that showed the presence of the knock-out construct were PCR amplified and digested as described. All reveal the presence of both the knock-out construct and the WT genes (data not shown).

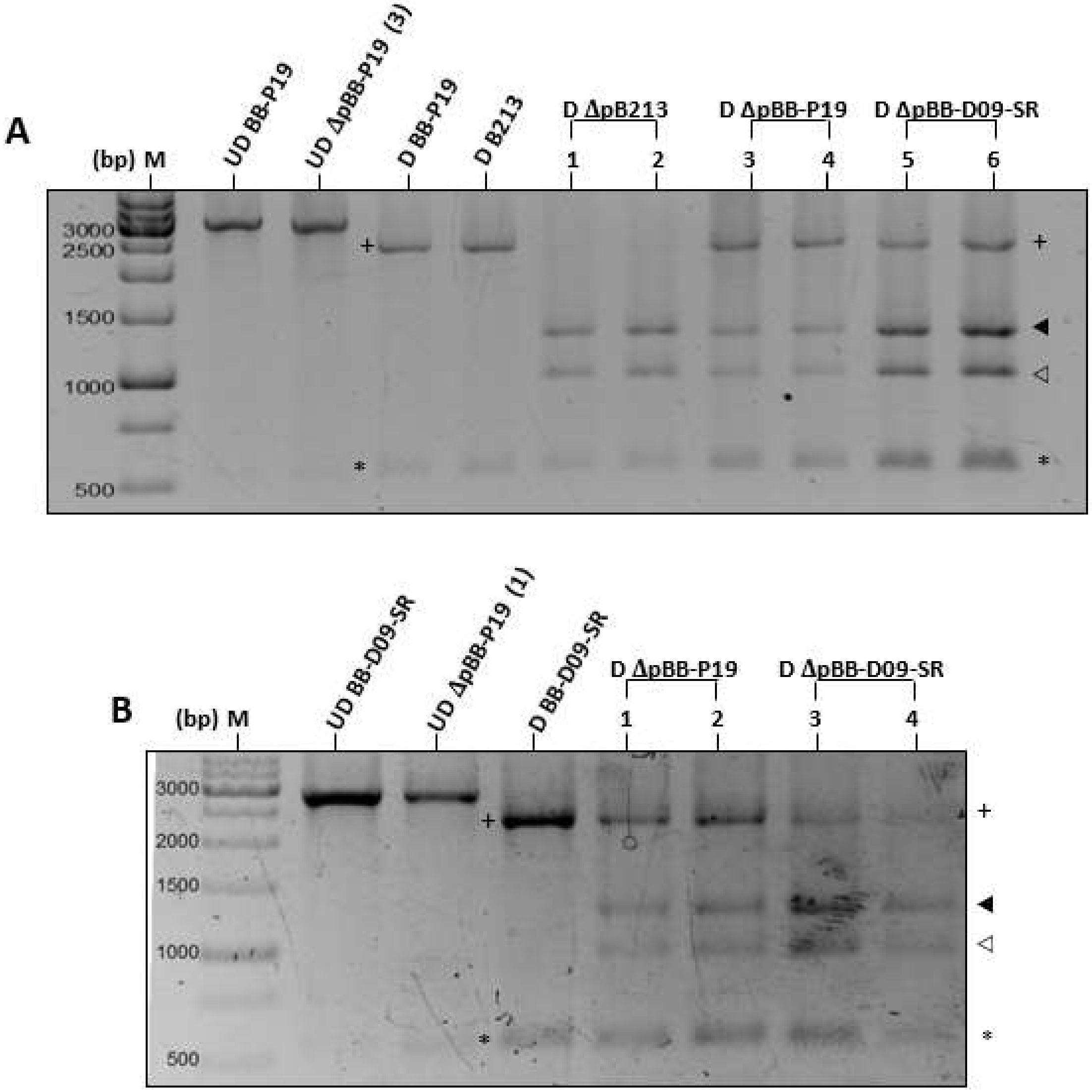


Figure 5.5.2. Digestion with restriction enzyme BglII of *lpxL1-2* obtained through PCR amplification with Fw-upLpxL1 and Rv-dwLpxL2. The PCR product of the positive strains for *lpxL1-2* knock-out harboring either the expression plasmid (A) pMMB67EH LpxL1-2 or (B) pMMB67EH LpxL2 (ΔpB213, ΔpBB-P19 and ΔpBB-D09-SR) was digested with BglII. Undigested (UD) *lpxL1-2* from BB-D09-SR, BB-P19 and *ΔlpxL1-2* from a clone harboring each plasmid were used as controls for the digestion. Digestion (D) of *lpxL1-2* from B213, BB-P19 or BB-D09-SR were used as negative controls. The digestion of the WT genes produces two fragments of 547 bp (*) and 2322 bp (+) in size. The digestion of the knock-out *lpxL1-2* produces three fragments of 547 bp and 971 bp (◁) and 1273 bp (◀) in size.

5.6. B213 ΔLpxL1-*2* pMMB67EH LpxL1-2 Displayed LPS structural differences but no fitness alterations

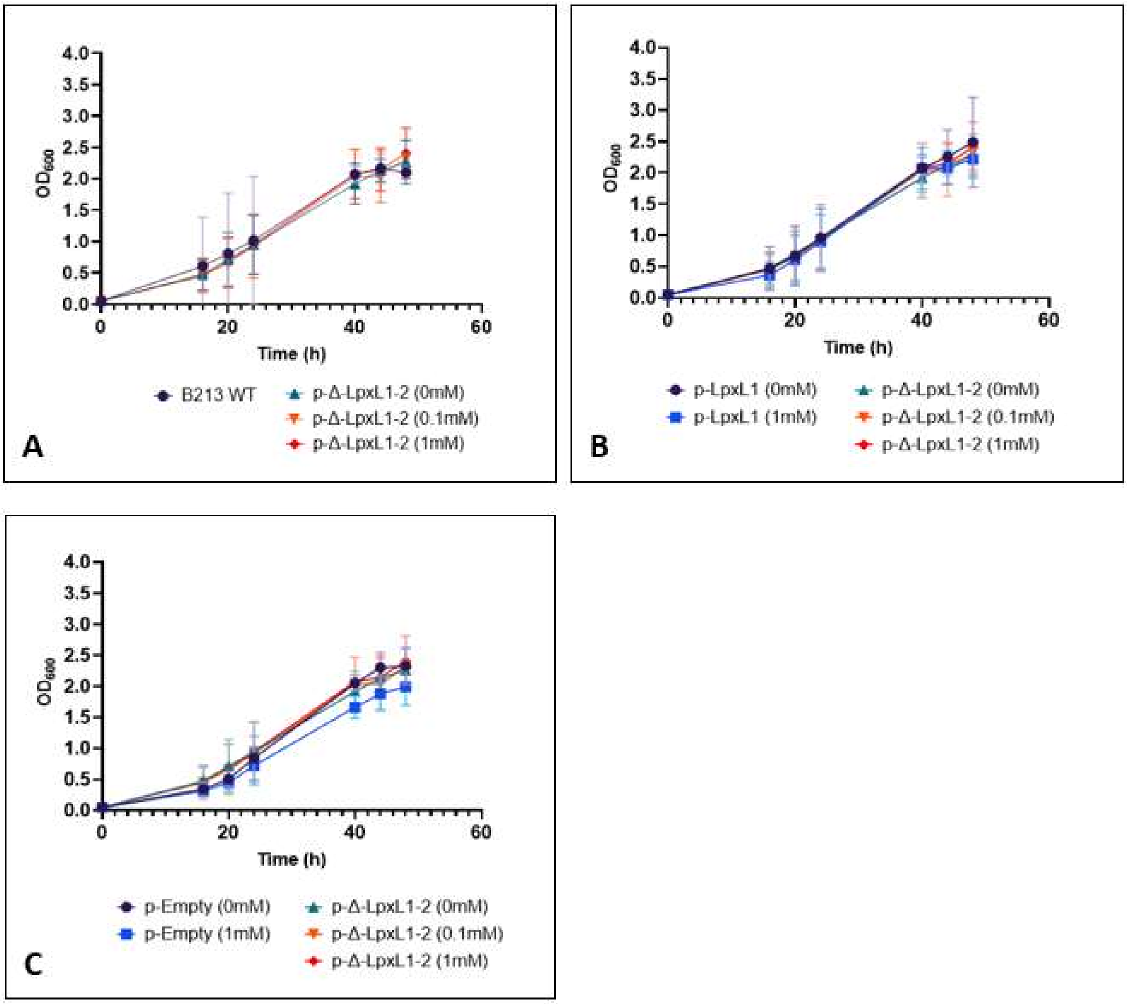
Following the knock-out of *lpxL1-2* in B213 pMMB67EH LpxL1-2, the strain was evaluated for effects of LpxL1-2 depletion in strain growth. The two independently obtained clones of strain B213 ΔLpxL1-2 pMMB67EH LpxL1-2 expressing *lpxL1-2* from plasmid (see Figure 5.5.2.A, ΔpB213 number 1 and 2) were exposed to a preliminary growth test with different IPTG concentrations (0, 0.1, 0.5 and 1 mM). Clone 2 grew roughly 3 times faster in a 3-day period than clone 1 for all IPTG concentrations (data not shown). Further studies were focused on clone 2, as time restrictions impeded further evaluation on clone 1, which was stored at -80°C. Clone 2 was grown for 48h and the OD_{600} was measured at several time points. Expression of the plasmid-encoded *lpxL1* and *lpxL2* genes was induced with 0.1 and 1 mM IPTG. For statistical analysis, the test was performed in duplicate and repeated 5 times in the same conditions. Contrary to what was expected, the absence of IPTG and, therefore, absence of *lpxL1-2* expression did not affect the growth of the mutant strain. Before inoculation in liquid medium, the strains were cultivated on BG plates with 1mM IPTG. To exclude the possibility that residual inducer from the plates affected the mutant's growth in liquid medium, the cultures were successively diluted to an OD_{600} of 0.05 in fresh medium every 24h. No significant IPTG-dependent differences in growth were observed (Figure 8.4).

Growth assessment revealed no statistically significant differences when compared to the controls displayed in Figure 5.6.1.A – C. Since *B. pertussis* B213 WT (panel A) could not be grown in amp as the mutant, *B. pertussis* harboring pMMB67EH LpxL1 (panel B) or the empty vector pMMB67EH (panel C) were also included as controls. The strain with the empty plasmid, although not carrying a gene, could be exposed to the same growth conditions, serving as a proximate substitute for the WT. Although not statistically significant, a slight difference in growth can be observed for B213 pMMB67EH. This strain showed slower growth when supplemented with 1mM IPTG. While *lpxL2* seems to be essential for bacterial survival, *lpxL1* is not normally expressed in *B. pertussis* [25], and its inactivation should, therefore, not impact bacterial growth. The growth of B213 ΔLpxL1-2 pMMB67EH LpxL1-2 was also compared with that of a strain expressing LpxL1 (B213 pMMB67EH LpxL1). Both strains behaved very similarly in the 48h growth period.

To determine whether LpxL1-2 depletion affects LPS structure, the strains were collected during the log phase and submitted to SDS-PAGE and silver-saining of the gel (Figure

5.6.1.D). Two bands were expected. The slower migrating band, designated band A, which represents a charged trisaccharide linked to the LPS core and lipid A and a band with lower molecular mass, band B. This band corresponds to the core region missing the trisaccharide [60]. The presence or absence of IPTG did not result in significant differences in band A in the mutant strain. Comparison with the WT strain showed similarities in band A intensity, possibly from the loading. The strain with the empty vector displayed the lower band ratio and the presence of a third band, band C.

The presence of inducer resulted in the appearance of a band above band B, marked with asterisk, in both the mutant strain and the *lpxL1*-expressing control. When expressed, LpxL1 mediates the transference of a secondary acyl chain. Previous studies demonstrated that extrachromosomal expression of *lpxL1* generated a hexa-acylated lipid A in *B. pertussis* [25]. Hence, the appearance of the band marked with asterisk can indicate hexa-acylated lipid A, deriving from the overexpressed *lpxL1,* with an additional secondary acyl chain substituted to the primary chain at the 2 position.



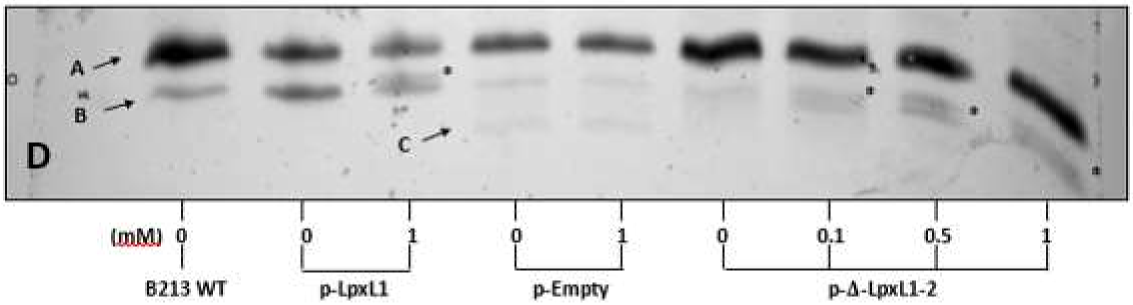


Figure 5.6.1. B213 ΔLpxL1-2 pMMB67EH LpxL1-2 growth and LPS structure analysis. Growth curve of B213 ΔLpxL1-2 pMMB67EH LpxL1-2 (p-Δ-LpxL1-2) measured at OD_{600}, in Verwey medium supplemented with ferrous sulfate heptahydrate (10 µg/ml). Expression was induced with 0, 0.1 and 1mM IPTG. Panels represent the growth curve of the mutant compared to (A) *B. pertussis* B213 WT (B213 WT), (B) B213 pMMB67EH LpxL1 (p-LpxL1) and (C) B213 pMMB67EH (p-Empy). Statistical analyses of five replicates (growth in different IPTG concentrations measured in double) were performed with Kruskal-Wallis test and Dunn's multiple comparation test ($p>0.999$). No statistically significative differences were detected between the growth of the mutant and the controls. (D) SDS-PAGE (16%) silver-stained gel of strain B213 ΔLpxL1-2 pMMB67EH LpxL1-2 in 0, 0.1, 0.5 and 1 mM IPTG. The LPS structure from the mutant strain was compared to the controls used in the growth assessment.

5.7. *lpxC* and *lpxL2* Expression could not be regulated

LpxC is responsible for the first committed step in lipid A biosynthesis. The cellular amount of LpxC correlates with the amount of LPS present in the outer membrane [27]. To regulate *lpxC* and *lpxL2* in the bacterial genome, the genes were placed under inducible promoter control (Figure 5.7.1.). To amplify a large part of *lpxC* and *lpxL2* genes, the forward primer was designed to introduce an NdeI restriction site while the reverse primer introduced AatII restriction site and a premature STOP codon, resulting in a shortened gene sequence. The amplicon was inserted under *lac/tac* promoter control, in a plasmid also carrying amp and ery (Ery^R) resistance sequences. The construction was inserted into the chromosome by homologous recombination events that placed the construction within the WT gene sequence. Translation of the transcript starting from the WT gene promoter should be interrupted by the premature STOP while the transcript of the gene under *lac*/*tac* promoter control uses the WT STOP resulting in the complete transcription and translation of the enzyme.

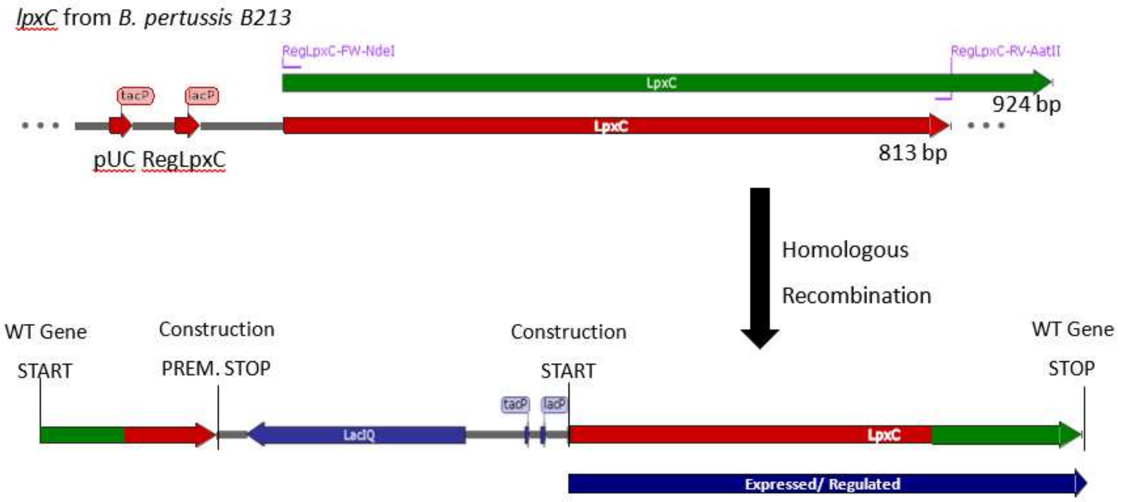


Figure 5.7.1. Diagram describing pUC RegLpxC integration into *B. pertussis* genome and *lpxC* regulation. A large fragment of *lpxC* was PCR amplified from *B. pertussis* B213 with primers intended to insert a premature STOP codon and 2 restriction enzyme sites (NdeI and AatII). After digestion, the amplicon was inserted into pUC plasmid, providing amp and ery resistance, and placing the gene under *lac*/*tac* promoter control. B213 was electrotransformed with the construct, which ought to be inserted into the chromosome by homologous recombination with the chromosomal *lpxC* gene. Once recombined into the chromosome, the translation of the gene expressed from the WT promoter is interrupted by the premature START codon, whilst an intact copy of the gene can be regulated using the *lac/ tac* promoter.

Construction of pUC RegLpxC and pUC RegLpxL2 involved amplification of fragments of the genes from the B213, BB-D09-SR and BB-P19 genomes by PCR. The PCRs resulted in amplicons of 819 bp and 861 bp in length for all *lpxC* (Figure 5.7.2.A) and all *lpxL2* (Figure 5.7.2.B) genes, respectively. The different amplicons and pUC18-ybcG-tolQ plasmid were then double digested with AatII and NdeI and ligated together. The ligation mixture was used to transform *E. coli* DH5α. The plasmids pUC RegLpxLC and pUC RegLpxL2 were chosen after gene sequencing that met all criteria with respect to length, no mutations or silent mutations and existence of START and STOP codon. Both constructs derived from BB-P19. *lpxC* from pUC RegLpxC presented 100% sequence homology with *B. pertussis* Tohama I and *B. bronchiseptica* KM22. *lpxL2* from pUC RegLpxL2 presented 98.82% homology with *B. pertussis* Tohama I and 100% with *B. bronchiseptica* KM22. Two conservative substitutions (Ile>Val and Thr>Ala) occur in the sequenced *lpxL2* when compared to the same gene from *B. pertussis* Tohama I.

After electrotransformation of B213, BB-D09-SR and BB-P19 the strains were selected with amp and ery. Screening for the gene under *lac/tac* promoter control was done with primers PeNP-sh-Fw-XbaI and either Rv-dw-LpxC for the *lpxC* construct or 3'LpxL2-R for the *lpxL2* construct. Since no PCR product were obtained, the downstream primers were also paired with primer Amp for RT-PCR, which aligns in the amp-resistance gene present on the plasmid (Figure 8.5.). However, also in this case none of the electrotransformants tested gave a positive result, suggesting the absence of the vector in the chromosome.

Compared to BB-P19 and B213, BB-D09-SR strain presented the higher number of colonies after electroporation with the pUC Reg plasmids. However, when screened in PCR for the presence of the Ery^R cassette (Figure 5.7.2.C), there was no amplification product, suggesting that the plasmid was not present in the bacteria. After re-plating the clones on thrice the original ery concentration (150 µg/ml), growth was still observed.

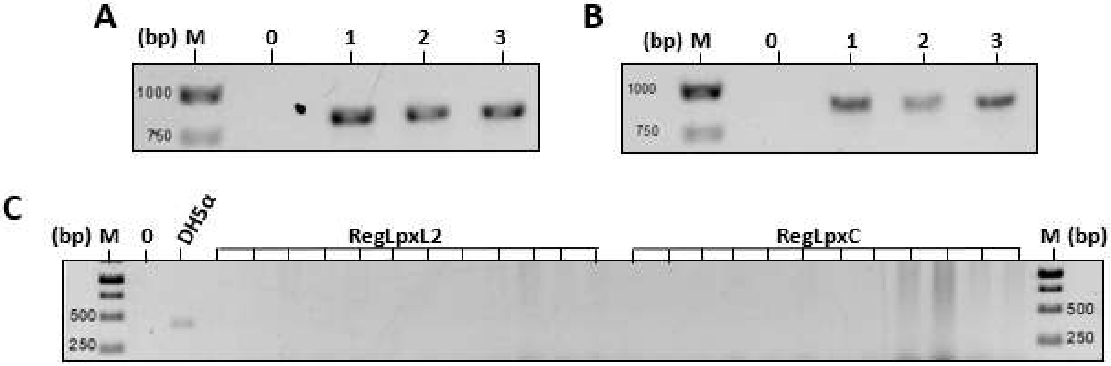


Figure 5.7.2. PCR amplification of shortened *lpxC* and *lpxL2* from *Bordetella* spp. Screening for pUC RegLpxC and pUC RegLpxL2 presence in putative electrotransformants. PCR amplification of a fragment of (A) *lpxC* and (B) *lpxL2* from (1) BB-P19, (2) BB-D09-SR and (3) B213 using RegLpxC-FW-NdeI / RegLpxC-RV-AatII or RegLpxL2-FW-NdeI / RegLpxL2-RV-AatII. (C) PCR amplification of Ery^R cassette in BB-D09-SR electrotransformed with pUC RegLpxC (RegLpxC) and pUC RegLpxL2 (RegLpxL2) using primers ERY-FOR/ ERY-REV. Ery^R cassette from *E. coli* DH5α pUC RegLpxL2 was used as positive control (DH5α). Five colonies per pool were tested. Lane 0 – Non-template control. Lane M – DNA Ladder. PCR products were separated by electrophoresis on 0.8% agarose gel.

6. Discussion

Whole-cell and acellular vaccines have been developed for *B. pertussis* and *B. bronchiseptica*. *B. pertussis* vaccines have shown to either generate excessive reactogenicity [11] or a declining of the acquired immunity [14]. Vaccination against *B. bronchiseptica* was regarded as not fully effective or safe for the animal [18, 19]. Variation of reactogenicity of the two vaccines was caused, among others, by the presence and quantity of LPS [43]. Lipid A is responsible for LPS endotoxicity. Lipid A biosynthesis involves nine conserved enzyme-catalyzed steps regulated by nine genes. It begins with the unfavorable UDP-GlcNAc acylation by LpxA, followed by the irreversible deacetylation by LpxC [20, 30]. After completion of *Bordetella* lipid A structure, LpxL1 and LpxL2 mediate the transfer of secondary acyl chains [61]. The first enzymatic step mediated by LpxA is responsible for the acyl chain present at the 3 and 3' positions [62]. To reduce LPS content in the cell, *lpxA* inactivation was targeted. The production of a construct carrying *lpxA* interrupted by Cam^R failed because it was not possible to introduce the antibiotic-resistance cassette in pCRII LpxA. The main challenge was to get transformants resistant to cam. The Cam^R gene was obtained by PCR, originally from pEN11-Imp and later from pEN11-GFP. DNA sequencing results for the PCR product derived from pEN11-lmp displayed a silent mutation that did not affect the gene transcription. The sequenced Cam^R was lacking the AscI restriction site, meant to introduced by the reverse primer. Initially this result did not seem to be the reason for the absence of cam resistant transformants since the length of the Cam^R fragment was above the precision limit of the sequencing technique. Upon further research, this assessment was revised. Sites for restriction enzymes AscI and SacII were expected to be present within the *lpxA* sequence on pCRII and were confirmed by single digestions of the plasmid with these enzymes. Thus, presumably, the absence of an AscI restriction site in Cam^R PCR fragment hindered the enzyme cleavage, which prevented the insertion of the antibiotic cassette into the plasmid. This could not be confirmed by analysis of the digestion product on agarose gel due to the small size of the released fragments (11 bp).

Despite the many similarities between *B. pertussis* and *B. bronchiseptica*, lipid A acylation patterns are species specific and can even differ between strains of the same species [62]. The acyltransferases LpxL1 and LpxL2 mediate the transfer of secondary acyl chains during lipid A biosynthesis. The latter mediates the addition of the secondary C14 acyl chain to the position 2', while LpxL1, which is active only in *B. bronchiseptica*, catalyzes the transference of a secondary 2-hydroxyl laurate at position 2 [61]. Studies suggest that the number of acyl chains is a determinant of endotoxic activity [25].

Subcloning of ΔLpxL2 BP into pKAS32 was not achieved and subcloning of ΔLpxL2 BB could not be confirmed. Transformants were selected with gen and amp, although the lack of a distinctive antibiotic resistance of the recipient plasmid impaired the subsequent results and testing. Growth on plate of amp- and gen-resistant mutants indicated that either pCRII ΔLpxL2 BB or pKAS32 ΔLpxL2 BB could be providing the resistance. The line of testing followed resulted from the lack of PCR amplification provided by the gentamycin-pKAS primer combinations. pKAS32 forward primer was not available. Instead, the presence of the donor plasmid and ΔLpxL2 BB insert were screened. Without confirming the presence of the recipient plasmid, it was not possible to ascertain the reliability of the negative results for pCRII presence. Assuming the absence of donor plasmids indicates the presence of recipient plasmid, the negative results for pCRII were screened for the insert. Regardless of the host plasmid, bacterial growth in the presence of gen should already indicate the presence of Gen^R cassette, but the 4 negative results in panel C are inconsistent with this assessment. These 4 clones, which were growing in the presence of the antibiotic, could represent mutants spontaneously resistant to gen, which is supported by the spontaneous resistance observed on control plates lacking the insert. In the absence of the donor plasmid, the gene should therefore be inserted in the recipient plasmid.

Several studies suggest that *lpxL2* is essential in *B. pertussis* [35]. Geurtsen *et al.* [25] reported the inability to create *lpxL1-2* and *lpxL2* mutants in *B. pertussis* through allelic exchange with an antibiotic resistance cassette. Several years later, the failed efforts to partially or completely delete chromosomal *lpxL2* persisted, as demonstrated by Arenas *et al.* [35]. To achieve the inactivation of *lpxL2*, allowing for bacterial survival despite the production of incomplete LPS molecules, two strategies were tried. First, the ΔLpxL1-2 construct on pKAS32 in SM10λpir was conjugated into B213 ΔPldA-MlaF. Then, the ΔLpxL1-2 construct was conjugated with *Bordetella* strains carrying pMMB67EH LpxL1-2 or pMMB67EH LpxL2. As mentioned, inactivation of PldA and Mla membrane maintenance systems has been shown to facilitate the cell's capability to reach homeostasis when LPS levels are interrupted in *A. baumanni* [21, 40].

Knock-out of *lpxL1-2* in B213 ΔPldA-MlaF was not successful. Str antibiotic selection to mediate the excision of the plasmid always resulted in reversion to WT, which does not explain the poor growth observed after the second crossover. In the second approach, inactivation of *lpxL1-2* was achieved in *B. pertussis* B213 after introduction of pMMB67EH LpxL1-2 into the bacteria. ΔLpxL1-2 could not be integrated into B213 mutants harboring pMMB67EH LpxL2 alone. In *B. bronchiseptica* strains overexpressing either *lpxL1-2* or *lpxL2*, *lpxL1-2* inactivation was not achieved since the WT genes were not excluded from the chromosome. Neither of these results was expected. Contrary to *lpxL2*, *lpxL1* is not crucial for the cell survival. Previous studies have successfully achieved *lpxL1* inactivation in *B. pertussis* [25] and in *B. bronchiseptica* [31].

The only two B213 ΔLpxL1-2 pMMB67EH LpxL1-2 mutant isolates obtained showed a remarkable difference in growth rate in a preliminary study where 0, 0.1, 0.5 and 1 mM IPTG were tested. Further analysis was done with the mutant with the best growth performance, clone 2. Growth curve of B213 ΔLpxL1-2 pMMB67EH LpxL1-2 was analogous to the WT, B213 pMMB67EH LpxL1 and B213 pMMB67EH strains. To reduce the number of proteins produced when the cells were pre-grown in the presence of inducer, the cells were diluted every 24h three times. The growth behavior of the strain was similar to the observed growth prior to the dilutions for all inducer concentrations. In every dilution, the growth was expected to decrease as the cells get depleted from proteins induced by the IPTG, particularly in the medium where no inducer was added and *lpxL2* expression would decline. Given similar growth of the mutant in the absence or presence of inducer, the results may imply that the pMMB67EH promoter is leaky and basal transcription is occurring. In the absence of inducer enough LpxL1-2 is produced to maintain normal growth.

Silver-stained gel of mutant LPS and controls revealed structural differences. The appearance of a band just above band B for the mutant strain and the B213 pMMB67EH LpxL1 control suggested that lipid A of *B. pertussis* is more acylated when expressing *lpxL1,* i.e. the enzyme adds a secondary acyl chain to the lipid A producing an hexa-acylated LPS with increased endotoxicity [25]. The different growth rate observed for clone 1 and clone 2 suggests a growth defect for the first. LpxL1-2 depletion should impose growth restrictions and the mutant was expected to grow slower than the WT. Hence, clone 1 growth behavior should be compared to the same controls and LPS structure should be assess.

Overexpression of the ABC transporter MsbA was attempted in the ΔPldA-MlaF mutant strain and the three WT strains. This methodology can be beneficial for cells with inactivated *lpxL1-2*, as it has been shown to compensate for *lpxL* loss in *E.coli* [32, 33].

Introduction of pMMB67EH MsbA was only possible in *B. bronchiseptica* BB-P19. Previous studies in this research group indicated that *msbA* overexpression in *B. pertussis* can be lethal in the WT strain. The extrachromosomal gene was continuously expressed, suggesting that unlike *B. pertussis*, *B. bronchiseptica* strains may be able to tolerate MsbA overproduction.

The attempt to regulate *lpxC* expression was supported by several studies that categorize LpxC as one of the key enzymes in lipid A biosynthesis, and consequently, LPS formation. It also controls the ratio between phospholipids and LPS in the membrane [63]. Controlling LpxC levels is a possible strategy when aiming to reduce the amount of LPS in the cells.

Attempts to place the chromosomal copies of either *lpxC* or *lpxL2* under a regulatable promoter failed. Two hypotheses can derive from the data collected: the construction failed at i) the recombination level or ii) the electroporation. Constructs were built using the same methods and vector, changing merely the gene sequence. Through homologous recombination, the vector would be incorporated into the chromosome producing a duplication of the target gene. Expression of the original chromosomal gene would be interrupted by the insertion of a premature STOP codon while an inducible promoter would allow the regulation of the expression of a complete gene. PCR screening for chromosomal introduction of either construct was negative. To assess the presence of the plasmid in the strain with the higher number of colonies (BB-D09-SR) PCR screening targeted ery, one of the two antibiotics present in the vector. The construct was absent from BB-D09-SR.

Zealey *et al.* (1988) [64] enumerated a few conditions that could affect electroporation rate in *Bordetella*, such as the shape and duration of the electric pulse, the field potency and the physiological conditions to which the cells were submitted. In this case however, it seems that the attempt failed at the recombination level. *Bordetella* transformation by electroporation has demonstrated high transformation efficiency and cell survival following electrotransformation at a field strength close to 25 kV/cm [64, 65].

This study aimed the reduction of LPS amount and endotoxicity in *B. pertussis* and *B. bronchiseptica*. While an expression plasmid harboring *lpxA*, *lpxL1-2* or *lpxL2* was introduced in the genome of all three WT strains, the constructs for inactivation of *lpxA* and *lpxL2* were not completed. Thus, it was not possible to inactivate *lpxA* or *lpxL2* alone. The Cam^R cassette was not introduced in the recipient plasmid due to the absence of AscI restriction site in the Cam^R sequence. The redesign of the reverse primer, which inserts the restriction site during PCR amplification should enable the Cam^R full digestion and ligation to pCRII plasmid, thus confirming the original primer incapability.

A construct carrying ΔLpxL1-2 was introduced in B213 pMMB67EH LpxL1-2 resulting in two clones. Clone 2 displayed similar fitness to the WT. The preliminary test, but not statistically significant, showed that clone 1 growth rate was slower than clone 2 for all IPTG concentrations. A *lpxL1-L2* deficient *B. pertussis* is expected to decrease the growth rate as the inducer concentration decreases because *lpxL2* is seemingly an essential gene [35]. Although at 1 mM IPTG the growth curves of both clones should not diverge, the growth behavior of clone 1 requires further study. Statistically significant growth curves using the previous controls should be performed and the LPS structure of clone 1 should be analyzed and compared to the same controls and to clone 2. LPS structure analysis of clone 2 displayed hexa-acylated LPS, similar to the *lpxL1*-expressing control. Following the growth and LPS structure evaluations of clone 1, the endotoxic levels of both strains should also be analyzed.

The attempt to place *lpxC* or *lpxL2* under inducible promoter control was unfeasible due to the inability to introduce the designed sequence into the chromosome in *B. pertussis* or *B. bronchiseptica* strains. The literature has reported the success of *Bordetella* electrotransformation with increased efficiency at the filed strength of 22.5 kV/cm used [65]. Thus, the required recombination into the chromosome after the plasmid was taken up by the cells was the challenging step. This approach should be repeated for all strains and a greater number of clones ought to be tested for the chromosomal incorporation of the construct.

Even though this study failed to achieve the outlined strategies, the need for less reactogenic, safe and efficient vaccine for both humans and animals remains. The studied genes represent an attractive target for decrease of endotoxicity and consequent reduction of host innate immune system stimulation in Gram-negative bacteria, and in particular the *Bordetella* genus.

7. Bibliography

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8. Supplementary Data

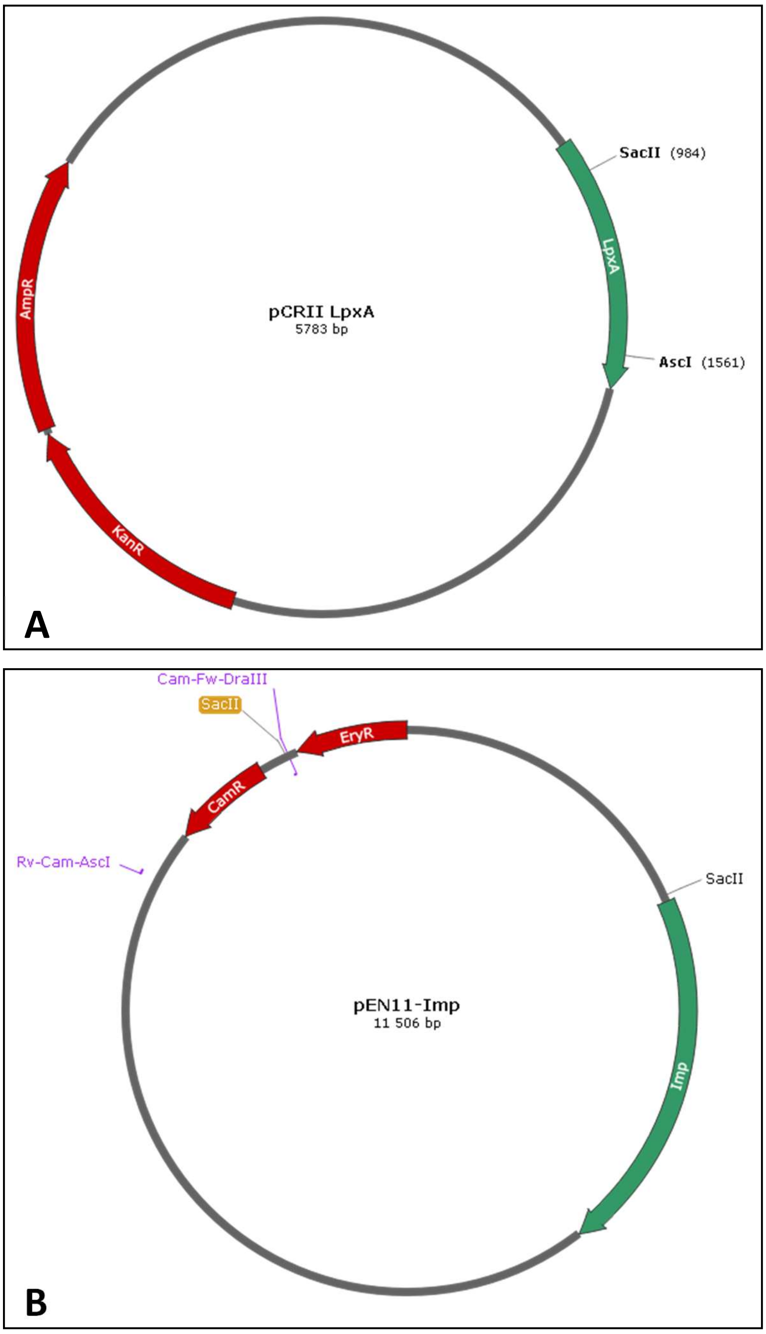
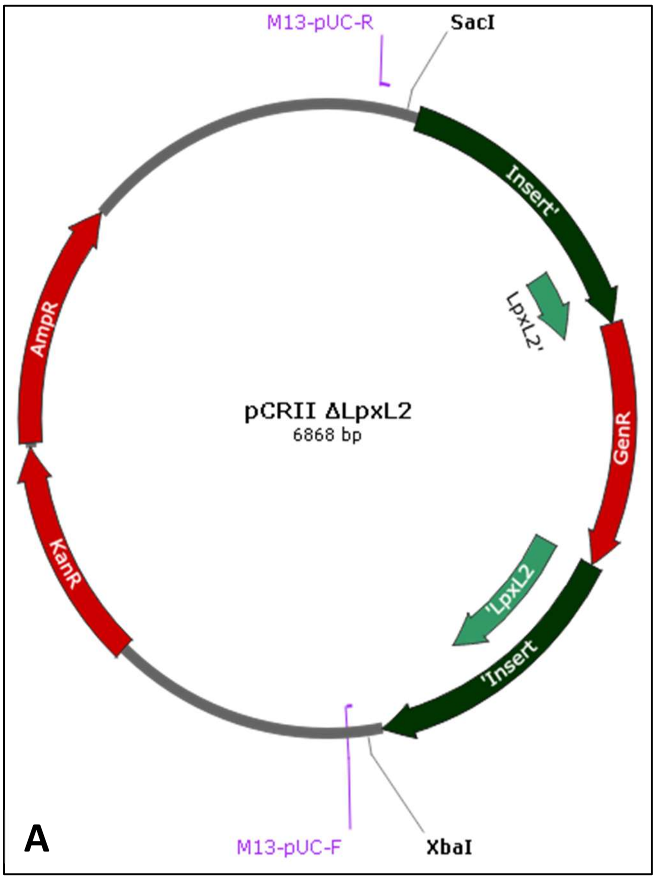


Figure 8.1. Plasmids pEN11-Imp and pCRII LpxA features. Antibiotic-resistance cassettes are presented in red, gene sequences in green and primers in purple. (A) Plasmid pCRII LpxA displays the restriction sites for AscI and SacII in *lpxA* gene and the antibiotic-resistance gene to amp and kan. (B) Plasmid pEN11-Imp displays the region for alignment of the primers Cam-Fw-DraIII / Rv-Cam-AscI for PCR amplification of Cam^{R}. SacII restriction site on Cam^{R} is highlighted in yellow.



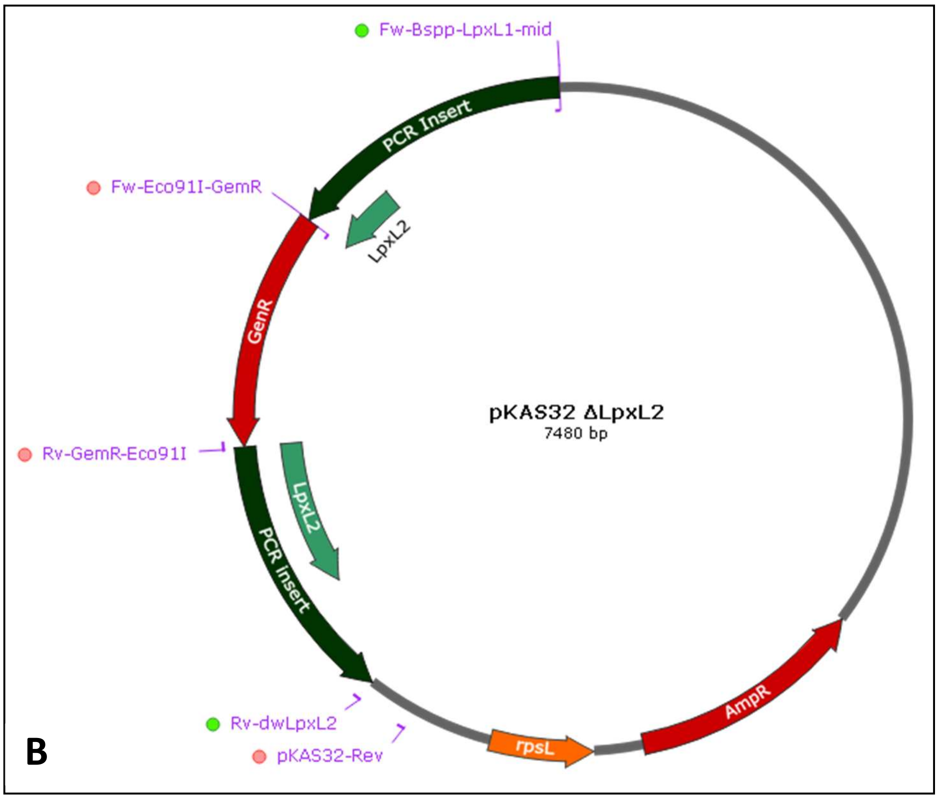


Figure 8.2. Plasmids pCRII ΔLpxL2 and pKAS32 ΔLpxL2 features. Antibiotic-resistance cassettes are presented in red, str sensitivity cassette in orange, gene sequences in green and primers in purple. Beads of the same color represent primer combinations. (A) Plasmid pCRII ΔLpxL2 displays the interrupted *lpxL2* gene by the Gen^R cassette and the restriction sites for SacI and XbaI. Backbone primer combination presented (M13-pUC-F/M13-pUC-R) was used to test

for the presence of pCRII after *E. coli* SM10λpir transformation. (B) Plasmid pKAS32 ΔLpxL2 obtained after *lpxL2* gene insertion by double digestion of both plasmids and subsequent ligation of the interrupted gene to pKAS32. Screening for the presence of the insert used primers aligning in the gene region (Fw-Bspp-LpxL1-mid/ Rv-dwLpxL2) and the antibiotic cassette with the vector (Fw-Eco91I-GemR/ pKAS32-Rev and Rv-GemR-Eco91I/ pKAS32-Rev).

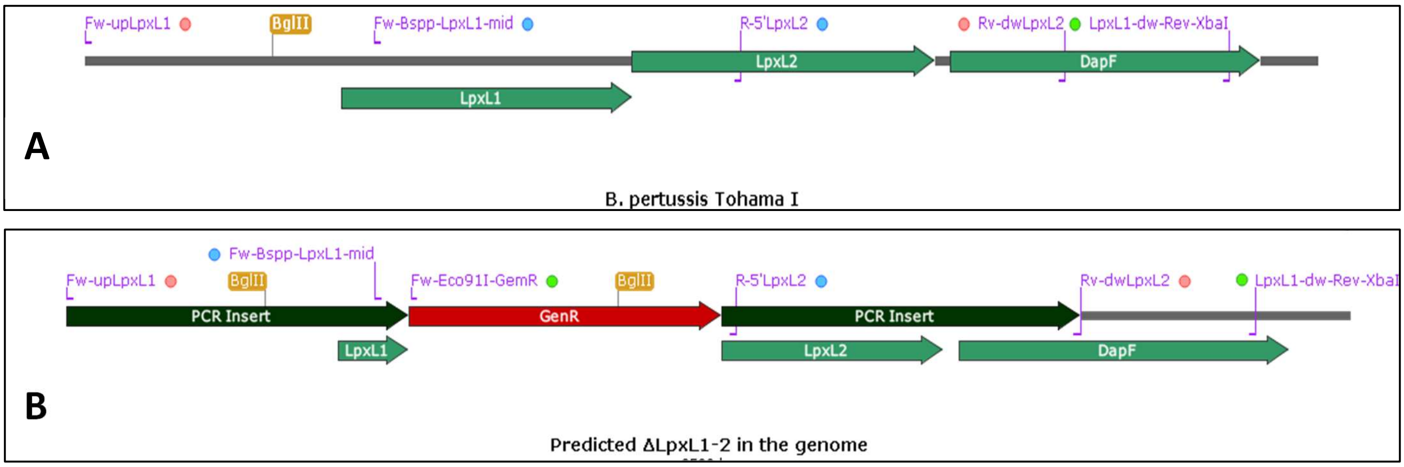


Figure 8.3. Features of the *lpxL1-2* genes interrupted by Gen^R cassette and predicted sequence after introduction of ΔLpxL1-2 in *Bordetella* genome. Plasmid pKAS32 harboring ΔLpxL1-2 was used for plate-mating conjugation with *Bordetella* spp. Antibiotic resistance cassette is marked in red, gene sequences in green, BglII restriction site in yellow and primers in purple. Beads of the same color represent primer combinations. The primers marked with red and blue beads were used for complete and partial PCR amplification of the insert after conjugation, respectively. To assess the incorporation of the insert in the genome Fw-EcoI91I-GemR and LpxL1-dw-Rev-XbaI were used. LpxL1-dw-Rev-XbaI does not align in the construct, but in the genome downstream from both genes. The region amplified by Fw-upLpxL1 / Rv-dwLpxL2 was used to test the excision of WT *lpxL1-2* from the genome. (A) Upstream and downstream region of *lpxL1-2* in *B. pertussis*. Restriction enzyme BglII produces two fragments of 547 bp and 2322 bp in size. (B) Predicted ΔLpxL1-2 introduction in *B. pertussis* genome. Restriction enzyme BglII produces three fragments of 547 bp and 971 bp and 1273 bp in size.

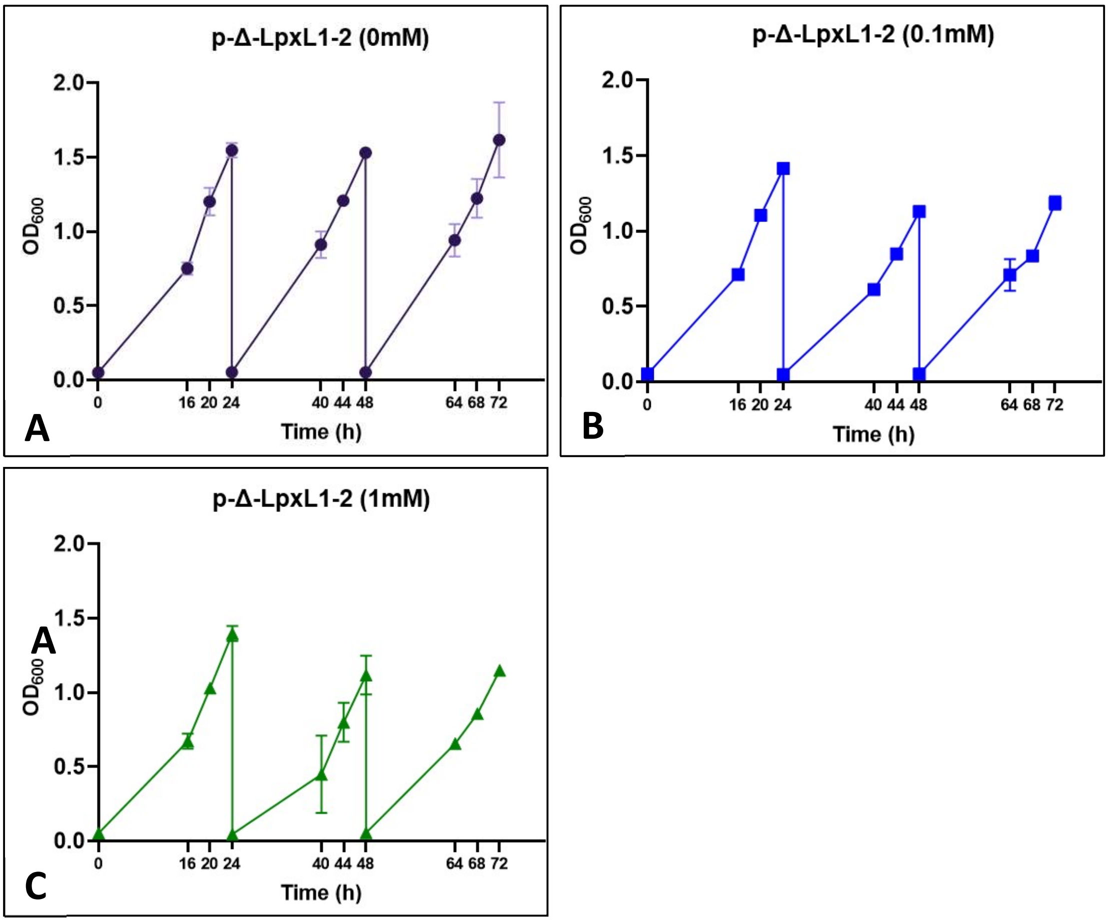
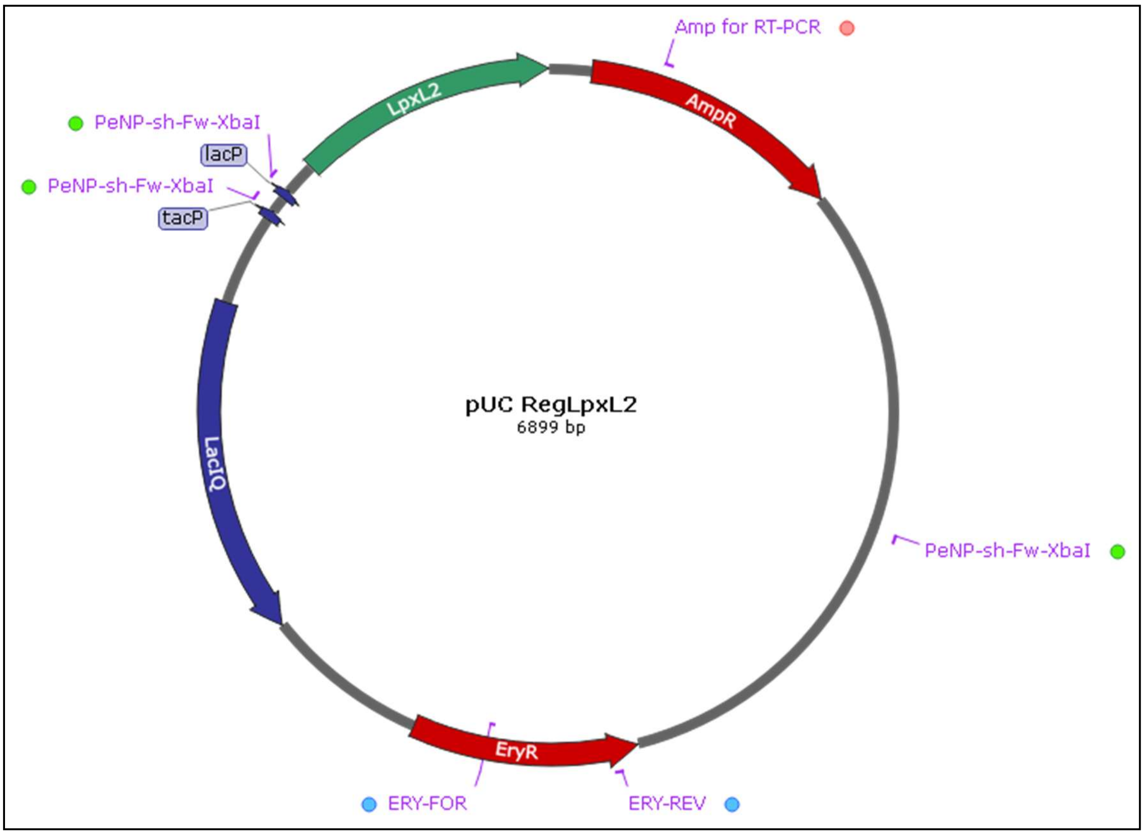


Figure 8.4. B213 ΔLpxL1-2 pMMB67EH LpxL1-2 during 72h after successive 24h dilutions to OD ±0.05. Growth curve of B213 ΔLpxL1-2 pMMB67EH LpxL1-2 (p-Δ-LpxL1-2) measured at OD_{600}, in Verwey medium supplemented with ferrous sulfate heptahydrate (10 µg/ml). Expression was induced with (A) 0mM, (B) 0.1 mM and (C) 1mM IPTG. Statistical analyses were performed with Kruskal-Wallis test and Dunn's multiple comparation test ($p>0.999$). No statistically significative differences were detected between 24h periods.



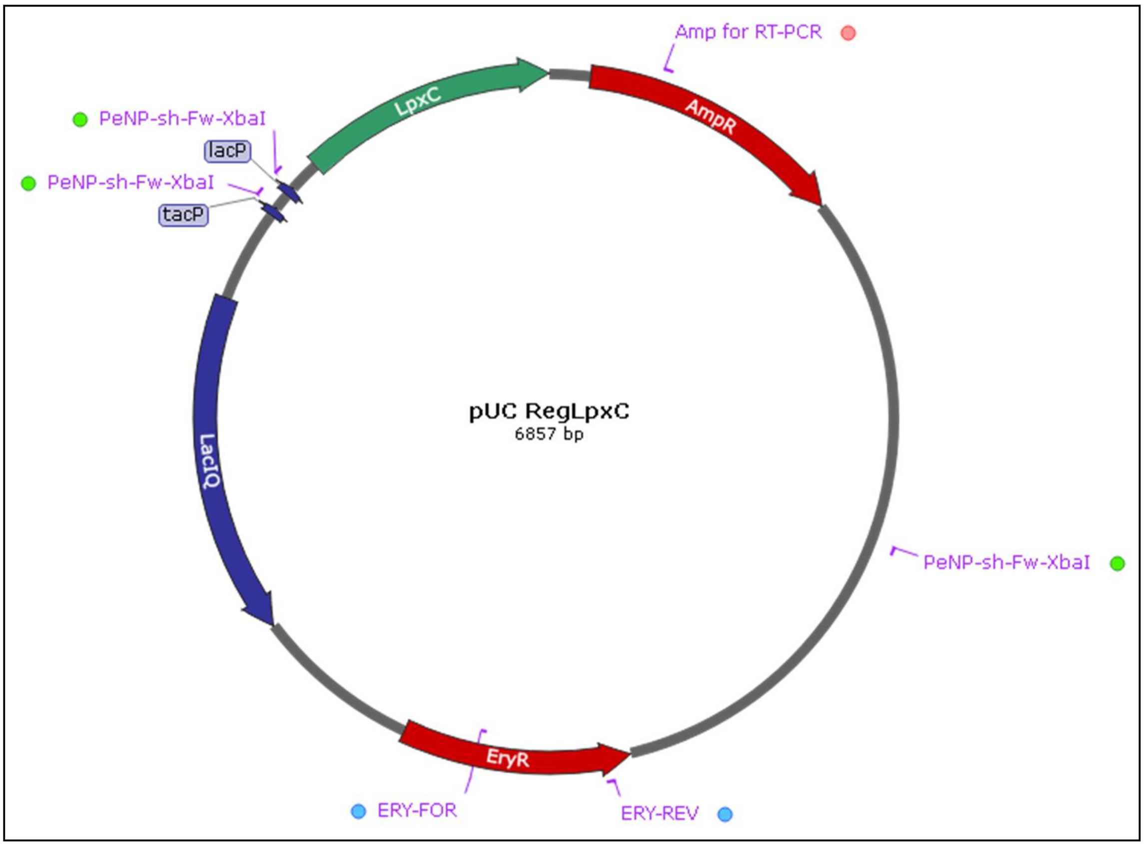


Figure 8.5. Plasmids pUC RegLpxL2 and pUC RegLpxC obtained after gene insertion. Antibiotic-resistance cassettes are presented in red, *lac* repressor (LacIQ) and promoters *lac/tac* sequences in blue, gene sequences in green and primers in purple. Beads of the same color represent primer combinations.

Both genes derived from BB-P19. *lpxC* presented 100% sequence homology with *B. pertussis* Tohama I and *B. bronchiseptica* KM22. *lpxL2* presented 98.82% homology with *B. pertussis* Tohama I and 100% with *B. bronchiseptica* KM22. The genes were introduced with the same orientation as the inducible promoters (*lac/tac*). After transference of the plasmid to *Bordetella* strains by electroporation the presence of the plasmid in the bacterial cell was assessed by PCR amplification of the Ery^R cassette with ERY-FOR/ERY-REV primer combination. Introduction of pUC RegLpxC into the bacteria chromosome was evaluated with 2 different primer combinations – PeNP-sh-FW-XbaI/Rv-dw-LpxC and Amp for RT-PCR/Rv-dw-LpxC. The primer Rv-dw-LpxC aligns downstream form *lpxC* in the genome. The same method was used to assess pUC RegLpxL2 introduction in the chromosome. Primer combinations (PeNP-sh-FW-XbaI/3'LpxL2-R and Amp for RT-PCR/3'LpxL2-R. The primer 3'LpxL2-R aligns downstream form *lpxL2* in the genome.